THE EFFECTS OF ALCOHOL ON SLEEP IN RATS

By

SANJIB MUKHERJEE

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

WASHINGTON STATE UNIVERSITY
Program in Neuroscience

DECEMBER 2008
To the Faculty of Washington State University

The members of the Committee appointed to examine the dissertation of SANJIB MUKHERJEE find it satisfactory and recommend that it be accepted.

________________________
Chair
ACKNOWLEDGMENTS

I am grateful to my advisor, Dr. Steve Simasko, for his excellent and astounding mentoring. I consider myself fortunate to work with Dr. Steve Simasko whose constant intellectual stimulation made this project so exciting to me. I would also like to thank my thesis committee members Drs. Barbara Sorg, Heiko Jansen and Levente Kapas for their thoughtful suggestions and comments. In addition, I would like to thank Dr. James Krueger, for his support and for drawing my interest to sleep research. Moreover, I also like to thank my current and former laboratory colleagues including Morvarid Kazerooni, Dr. Alok De, Richard Brown, Dallas Kinch and Adrienne Karpel for helping me to carry on my project in an efficient manner. Furthermore, I would like to thank my wonderful wife, Rashmita, for her love, dedication, support and motivation; and my beautiful son, Shoumodeep, for providing me with boundless happiness. Additionally, I am thankful to my father and mother-in-law Satyaranjan and Bithika, brother and sister-in-law, Amit and Susmita, and my nephews, Shohom and aditya for their inspiration, love and support. Also I would like to thank my only and amazing brother, Joydeep, and his wife, Piu for their love and support. Finally, I would like to convey my inexpressible gratitude to my parents, Tilottama and Chiranjib, for everything they have done for me. This project was made possible through funding provided by the National Institute on Alcohol abuse and Alcoholism.
THE EFFECTS OF ALCOHOL ON SLEEP IN RATS

Abstract

By Sanjib Mukherjee, Ph.D.
Washington State University
December 2008

Chair: Steven M. Simasko

Sleep pathology is a frequent complication in human alcoholics and is associated with relapse drinking. Progress in understanding this pathology requires an animal model but an optimal alcohol treatment paradigm that produces robust sleep pathologies in the rat has not been defined. Alcohol was delivered either in water (6% and 12%) or in liquid diet (3% and 6%). Result shows that 6% alcohol in liquid diet for 6 weeks produces the greatest alteration of sleep. This includes flattening of the distribution of wake, slow-wave sleep (SWS) and rapid-eye-movement sleep (REMS) across dark and light period, without any alteration of daily time spent in each vigilance state. In addition slow wave amplitude across dark and light period is also flattened.

In order to describe better the effects of alcohol on sleep in rats a more precise description of sleep in rats is required. Rats sleep in episodic manner, however a formal definition of sleep unit is lacking. Analysis of wake periods in rat suggested that rats have at
least two modes of wake, the long duration wake (LDW) and the brief wake (BW). When rats are not in LDW, they cycle relatively rapidly through SWS, REMS and BW, a period we call vigilance cycling (VC). In the light period VC episodes are extended, more intense, but less in number. In contrast, the number of VC is greater in the dark period but they are less intense. We propose that VC represents a sleep unit in rats.

The usefulness of VC and LDW becomes apparent when we analyze the effects of chronic alcohol treatment on sleep. The extended VC episodes in the light period were fragmented after chronic alcohol treatment in addition to a suppression of REMS. The effect of chronic alcohol treatment on sleep is found to be different from the effect of acute alcohol treatment on sleep. These results demonstrate that the effects of chronic alcohol in humans (REMS suppression and inability to remain asleep at night) are also produced in the rat. Additionally, it also demonstrates the usefulness of the analysis of rat’s vigilance states into LDW and VC.
<table>
<thead>
<tr>
<th>CHAPTERS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>I. Problems of alcoholism</td>
<td>1</td>
</tr>
<tr>
<td>Pathologies caused by alcohol</td>
<td>1</td>
</tr>
<tr>
<td>a. Gastrointestinal system</td>
<td>1</td>
</tr>
<tr>
<td>b. Cardiovascular</td>
<td>3</td>
</tr>
<tr>
<td>c. Immune system</td>
<td>3</td>
</tr>
<tr>
<td>d. Central Nervous System</td>
<td>4</td>
</tr>
<tr>
<td>Fetal alcohol syndrome</td>
<td>5</td>
</tr>
<tr>
<td>Alcohol addiction</td>
<td>6</td>
</tr>
<tr>
<td>II. Sleep</td>
<td>10</td>
</tr>
<tr>
<td>III. Neuronal regulation of SWS</td>
<td>12</td>
</tr>
<tr>
<td>a. Cholinergic neuronal groups</td>
<td>13</td>
</tr>
</tbody>
</table>
b. Norepinephrinergic neuronal groups.......................13

c. Histaminergic neuronal groups..............................14

d. Orexinergic neuronal groups.................................14

e. Other neurotransmitters.....................................15

Humoral Regulation of SWS......................................16

a. Somatotropic axis..............................................16

b. Tumour Necrosis Factor and Interleukin.................17

c. Prostaglandin..................................................17

Neuronal regulation of REMS....................................18

Humoral regulation of REMS....................................18

Circadian regulation...............................................19

Theories of sleep...................................................20

a. Thalamocortical synchronization and SWS..............20

b. Flip-flop switch theory....................................21

IV. Alcohol and sleep..............................................23

a. Alcohol and somatotropic axis............................25

b. Alcohol and HPA axis.......................................25

c. Alcohol and circadian rhythm............................26

V. References.....................................................27

B. Dose response study of chronic alcohol induced sleep changes in rats.........45

I. Abstract.......................................................46

II. Introduction.................................................47

III. Methods....................................................49
IV. Results......................................................................................52
V. Discussion.................................................................................57
VI. Acknowledgements...............................................................68
VII. References..............................................................................69

C. Novel analysis of sleep patterns in rats separates VC from LDW...........73
 I. Abstract..................................................................................74
 II. Introduction...........................................................................75
 III. Methods...............................................................................76
 IV. Results..................................................................................79
 V. Discussion..............................................................................90
 VI. Acknowledgements...............................................................124
 VII. References............................................................................125

D. Chronic alcohol treatment fragments VC in the light period..............128
 I. Abstract..................................................................................129
 II. Introduction...........................................................................130
 III. Methods...............................................................................131
 IV. Results..................................................................................134
 V. Discussion..............................................................................142
 VI. Acknowledgements...............................................................168
 VII. References............................................................................169
E. Discussion/Conclusion .................................................................173

References ......................................................................................175
LIST OF TABLES

Table

2.1 SWS and REMS episode durations and number of episodes when alcohol was administered in water.

2.2 SWS and REMS episode durations and number of episode when alcohol was administered in liquid diet.

3.1 Summary of the characteristics of VC and LDW.

3.2 Summary of the characteristics of VC and LDW when the 3-epoch rule applies.

4.1 Summary of the effect of 6 week treatment with alcohol on sleep parameters.

4.2 Summary of sleep parameters on day 16 following after cessation of alcohol.

4.3 Summary of sleep parameters day 2 after cessation of alcohol treatment.

4.4 Summary of sleep parameters acute dark onset administration of alcohol.

4.5 Summary of sleep parameters acute light onset administration of alcohol.
LIST OF FIGURES

Figure

2.1 Average weight gain (in g) per day from rats in the various treatment groups.

2.2 Total time spent in different vigilance states (wake, SWS, REMS) after different treatment paradigms.

2.3 Variation of SWA after different treatment paradigms.

3.1 Representative hypnograms from two control animals.

3.2 Cumulative probability density and probability density plots of the distribution of wake event durations.

3.3 Spectral power of the component frequencies in the EEG for wake and REMS.

3.4 Comparisons of episode durations and percent SWS and REMS for the first quarter of VCs and fourth quarter of VCs.

3.5 Spectral power of the component frequencies of the EEG from SWS epochs in the first quarter of VCs and last quarter of VCs.

3.6 Distributions of the indicated vigilance states with time.

3.7 Distribution of the indicated vigilance states across time (in 2 hour blocks) in the dark period when different epoch durations were used in the primary analysis.

3.8 Cumulative probability density and probability density of the distribution of wake events when different epoch durations were used in the primary analysis.

3.9 Same as 3.8 with except 3 epoch rule was used.
3.10 Power of the EEG in BW as BW progresses. Spectral power of the first 3 epochs of BW were averaged and compared to the averaged value in the subsequent BW epochs in groups comprising epochs 4-6, epochs 7-12, epochs 13-18, epochs 19-24 and epochs 25-30.

3.11 Power of the EEG in LDW as LDW progresses. Spectral power of the first 3 epochs of LDW were averaged and compared to the averaged value in the subsequent LDW epochs in groups comprising epochs 4-6, epochs 7-12, epochs 13-18, epochs 19-24 and epochs 25-30.

3.12 Power of the EEG in BW as BW progresses. Spectral power of the epochs 4, 5, and 6 of BW were averaged and compared to the averaged value in the subsequent BW epochs in groups comprising epochs 7-12, epochs 13-18, epochs 19-24 and epochs 25-30.

3.13 Power of the EEG in LDW as LDW progresses. Spectral power of the epochs 4, 5, and 6 of BW were averaged and compared to the averaged value in the subsequent BW epochs in groups comprising epochs 7-12, epochs 13-18, epochs 19-24 and epochs 25-30.

3.14 Comparison of the power in the frequency bands of BW vs LDW over the first 300 sec of each period.

3.15 Spectral power of wake EEG for BW and LDW in the frequency band 26-50 Hz.

3.16 Spectral power of the component frequencies of the EEG between BW in light and dark period.

3.17 Spectral power of the component frequencies of the EEG from SWS epochs in the first quarter of VC episodes as it progresses to the final quarter.

4.1 Vigilance state diagram for alcohol treated and pair-fed rats after 6 weeks of alcohol treatment.
4.2 Representative hypnograms from two pair-fed and two alcohol treated rats after 6 weeks of alcohol treatment.

4.3 Slow-wave amplitude after 6 weeks of treatment for pair-fed and alcohol treated animals.

4.4 Vigilance state diagram for alcohol treated and pair-fed rats 16 days after complete cessation of alcohol exposure.

4.5 Slow-wave amplitude on 16 day after cessation of alcohol treatment for pair-fed and alcohol treated animals.

4.6 Vigilance state diagram for alcohol treated and pair-fed rats on day 2 after complete cessation of alcohol exposure.

4.7. Vigilance state diagram for dark onset administration of and light onset administration of alcohol after water gavage control and alcohol gavage.

4.8 Representative hypnograms from two rats from dark onset and light onset recordings.

4.9. Slow-wave amplitude after water gavage and alcohol gavage after dark onset administration of alcohol and after light onset administration of alcohol.
LIST OF FREQUENTLY USED ABBREVIATIONS

ADH (Alcohol dehydrogenase)
ALDH (Aldehyde dehydrogenase)
BW (Brief wake)
DAT (Dopamine transporter)
EEG (electroencephalogram)
FFT (fast fourier transformation)
fMRI (functional magnetic resonance imaging)
GABA (gamma-aminobutyric acid)
GHRH (growth hormone releasing hormone)
I.C.V (Intracerebroventricular)
IL (Interleukin)
LDW (long duration wake)
LDT (laterodorsal tegmental nuclei)
NE (norepinephrine, noradrenaline)
NO (nitric oxide)
NOS (nitric oxide synthase)
PET (positron emission tomography)
PGD2 (prostaglandin D2 )
POA (preoptic area)
PPT (pedunculopontine tegmental nuclei)
REMS (rapid eye movement sleep)
SWS (slow wave sleep)
SWA (slow wave activity)

TNFα (tumor necrosis factor-alpha)

VC (vigilance cycling)

VIP (vasoactive intestinal peptide)
DEDICATION

To my family and friends for their constant inspiration, support and caring
Chapter 1 Introduction

ALCOHOL

Problem of alcoholism

Alcohol has been present in the human society from antiquity. The existence of the practice of making alcoholic beverages from fermented rice, honey and fruits has been documented as early as 7000 B.C [McGovern, 2004]. Although it is not known how big of a problem alcohol addiction was in ancient times, currently alcoholism inflicts a huge problem worldwide. For example, in the United States alcoholism imposes an estimated economic burden of $ 184.6 billion per year [NIAAA, 2000]. This includes both direct and indirect damage to body, property, and work; such as road traffic accidents, alcohol related absence from work, as well as medical expenditure due to alcohol induced pathologies. Alcohol can potentially affect all the organs of human body. So morbidity and mortality are usually high after an extended period of alcohol use. It has been estimated that each year around 85,000 people die of alcohol related causes in the US [Mokdad, 2004]. Worldwide, alcohol abuse and dependence is estimated to account for 3.2% of global mortality [Rehm, 2003].

Pathologies caused by alcohol

Gastrointestinal Drinking is the only mode of alcohol intake in humans and alcohol is predominantly metabolized in the liver. Thus the gastrointestinal system, mainly liver and pancreas are prime targets for damage from long term alcohol use. Alcohol increases secretion of gastrin and HCl. In addition, alcohol use may reduce the tone of lower esophageal sphincter and esophageal motility. All these factors may give rise to gastroesophageal reflux disease.
Although alcohol has not been shown to cause peptic ulcer, it may facilitate the development of superficial gastritis and chronic atrophic gastritis [Bujanda, 2000]. Alcohol use may also result in the development of pancreatitis and pancreatic insufficiency [Migliori, 2004]. Alcohol is calorically dense and with chronic alcohol use the calories in alcohol can become a major caloric source displacing other nutrients, leading to malnutrition and vitamin deficiencies (e.g., B1 and B12). In addition, alcohol exerts toxic effects on both liver and gut, so malnutrition may also result from alteration of GI function and associated maldigestion and malabsorption of essential nutrients [Leiber, 1975].

Alcohol use may give rise to acute alcoholic hepatitis, fatty liver, and cirrhosis of liver. Several factors are thought to participate in the pathogenesis fatty liver but the important one is the interaction of alcohol and its oxidation product, acetaldehyde, with lipid metabolism in the liver. Acetaldehyde and reactive oxygen species have been implicated in the mitochondrial injury hampering fatty acid oxidation. Alcohol ingestion results in enhancement of lipid biosynthesis as a result of excessive NADPH formation due to oxidation of ethanol in hepatocytes [Yu, 1997]. The enhanced production and lack of metabolism of fatty acid results in harmful accumulation of fatty acids in hepatocytes, which is initially diluted by converting fatty acids to triglycerides and by partially releasing it in the plasma. But this buffering action soon comes to an end, leading to the development of liver injury [Baraona, 1998]. The advanced progression of liver damage, characterized by inflammation and fibrosis of liver, is known as cirrhosis of liver, an irreversible disease condition with grim prognosis. At the beginning of the development of cirrhosis of liver, fibrous septa appears in the liver and together with the regeneration of entrapped hepatocytes give rise to the characteristic nodule formation and shrinkage of liver.
**Cardiovascular** Acute alcohol consumption has arrhythmogenic actions on the heart, causing mainly, paroxysmal atrial arrhythmias [Ettinger, 1978], the so called, holiday heart syndrome. The reason for the development of this arrhythmia is still obscure. However, various alcohol induced alteration in the body, such as, potassium ion depletion has been implicated, in the development of paroxysmal atrial arrhythmias [Fauchier, 2003]. Chronic heavy alcohol consumption may raise blood pressure and may cause dilated cardiomyopathy. Females are more prone to the alcohol induced damage to the heart [Urbano-Marquez, 1995; Fernandez-Sola, 2002]. The mechanism of the pathogenesis of cardiomyopathy is not clear but the implication is the development from apoptosis and altered function of myocytes [Piano, 2002]. Alcohol induced cardiomyopathy manifest as heart failure. In contrast to the damaging action of heavy drinking on heart, several studies have suggested beneficial effect moderate alcohol consumption on cardiovascular system for example, decrease in the risk for heart failure in older people with moderate consumption of alcohol [Jerome, 2001]. A possible reason for this beneficial effect of alcohol could mediate through increase in high density lipoprotein (HDL) or good cholesterol, which reduces the risk of the development of atherosclerosis by light or moderate alcohol drinking [Gaziano, 1993].

**Immune system** Prolong alcohol use may result in harmful effect on immunity and alcoholics are more prone to infections. Studies have suggested that alcoholics also have decreased cellular immunity which may be due to disturbed sleep pattern [Redwine, 2003]. In the above mentioned study, it has been observed that compared to control subjects, alcoholic patients exhibit a reduction of NK cell activity, a decrease in levels of IL-6 formation and IL-6/IL-10 ratio together with loss of delta sleep and augmentation of REMS. Altered sleep and immunity can be associated with increase levels of cortisol found in alcoholics [Adinoff, 2003].
**Central Nervous System:** Alcohol is classified as a CNS depressant drug but studies have also documented that alcohol can elicits both stimulatory and depressant properties depending on the dose of alcohol consumed and the phase of the blood alcohol concentration (BAC) [Pohorecky, 1977]. The stimulatory effect is prominent at moderate dose of alcohol and when BACs ascend to peak, whereas the sedative effect is prominent at higher dose of alcohol and when BACs decline. Alcohol suppresses the CNS in a dose dependent manner resulting in sleep, unconsciousness and death with increasing dose. Death occurs mainly due to respiratory failure.

Moderate amount of alcohol has variable effect of respiration but ventilatory response to CO$_2$ is always depressed. Very high level of blood alcohol concentration (400mg/dl or more) may cause lethal suppression of respiration.

Prolong alcohol use is directly associated with serious neurological and mental disorder such as neural damage, memory loss, sleep disturbances, unprovoked seizures and psychosis. Alcoholics may suffer from impaired memory function associated with Korsakoff’s syndrome. The development of Korsakoff’s syndrome results from the nutritional deficits rather than the direct effect of alcohol. Alcohol abuse in human is associated with attenuation of learning and memory [Fadda and Rosetti 1998]. The effect of alcohol on learning and memory is also observed in animal studies. Long Term Potentiation (LTP) in the hippocampus is considered to be a key component for the development of learning and memory, and, a reversible decrease in LTP in the CA1 region of the hippocampus in response to tetanic stimulation of the Schaffer collaterals is observed by intermittent (daily 14 h) exposure of ethanol vapor for 2 weeks in rats [Roberto, 2001]. Besides affecting neurons in the brain, alcohol may also affect cerebral vascular smooth muscles [Li, 2004] and heavy alcohol drinking increases the risk of having stroke [Reynolds, 2003].
Binge drinking can cause neurodegeneration in a very short time. In a study done on rats, researcher observed significant damage, as assessed by apoptosis and necrosis, in the agranular insular cortex, anterior piriform cortex, perirhinal cortex, lateral entorhinal cortex, and the temporal dentate gyrus, after 4 days of binge drinking [Obernier, 2002]. In this study alcohol was administered through surgically implanted intragastric catheter in a dose of 5g / Kg of body weight in liquid diet. Alcohol was administered every 8h for 4 consecutive days.

Fetal Alcohol Syndrome (FAS): Drinking during the gestational period is associated with many detrimental effects on developing fetus. Although suspected long ago, the deleterious effect of prenatal exposure of alcohol was first documented by researchers in France in 1968 [Lemoine, 1968]. Babies with FAS exhibit a wide spectrum of symptoms, ranging from severe physical and mental problems to near normalcy. Babies affected with the severe form exhibit particular craniofacial features like small head circumference, small eye opening, thin upper lip, skin fold at the corner of the eye, low nasal bridge, short nose, indistinct philtrum. They also exhibit prenatal and/or postnatal growth deficiencies, CNS dysfunction, psychiatric disturbances and impairment in psychosocial skills. Anatomically, the brains of babies with FAS show small or even absent corpus callosum, smaller cerebellum, diminished size of the vermis, significant reduction in the volume of basal ganglia, and damaged prefrontal cortex and hippocampus. This may also compromise the function of the affected brain areas and result in a number of behavioral and cognitive deficits, such as, diminished or loss of motor control, mental retardation, problems with learning and memory, lack of executive function, attention deficits, and difficulty to recognize and utilize visual / spatial relationships [Mattson & Riley, 1998].
Alcohol addiction

Serving alcoholic beverage is considered typical and is an expected practice at weekend and holiday parties. For the vast majority of individuals, this social drinking causes no undue problems as light alcohol use has actually been associated with some health benefits. However, this is not true for a few individuals, who are prone to the addictive property of alcohol use. They rapidly transit from occasional, experimental and social use of alcohol, to a regular heavy drinking pattern despite negative consequences. After becoming addicted, alcoholics become preoccupied with the compulsion of alcohol consumption and pathologically narrow their goals to focus on continual alcohol use. Many of them become oblivious to other crucial aspects of personal and family life and thus creating a whirl of misfortune that brings havoc in their lives, in the lives of their families and in the society at large. Given the magnitude of the harmful effect of alcohol addiction, an understanding of the basis of the addictive nature of alcohol, like many other drugs of abuse, is critical to helping these afflicted individuals regain control of their lives. The current thinking about alcohol addiction is that it is multifactorial and complex. More than 90% of Americans use drugs of abuse, including alcohol, at least once in their lifetime but the actual number of addicts is very low [Robinson and Berridge, 2003]. It has been reported that an estimated 12-13% of people become alcoholics within 10 years of their first use. The corresponding figure for cocaine is only 15-16% [Wagner and Anthony, 2002]. These reports demonstrate that use in and of itself are not the cause of addiction and suggest that other factors, which may be biological, psychological, or sociological in nature heavily contribute to alcohol addiction.

Some individual are genetically prone to alcohol addiction. Part of the reason may lie on the variant of enzymes, responsible for the metabolism of alcohol. Alcohol is oxidized first to
acetaldehyde by alcohol dehydrogenase (ADH) which is subsequently oxidized to acetate by mitochondrial aldehyde dehydrogenase (ALDH). Both ADH and ALDH show genetic polymorphism. This affects their catalytic property and the rate of metabolism of alcohol and acetaldehyde in the body and hence the duration of their presence in the body. Unlike the euphoric effect of alcohol, acetaldehyde exerts many untoward feeling (such as flushing in the face, increase in pulse rate) and increase in acetaldehyde level in the blood is associated with decrease in alcohol intake [Graver, 2001]. Aldehyde dehydrogenase (ALDH) in human has two main isozymes (ALDH-1) and (ALDH-2), which have low and high Km for acetaldehyde respectively. Thus ALDH-1 oxidizes acetaldehyde faster than ALDH-2. Asians, who have the atypical ALDH-2 gene are more sensitive to alcohol as a result of slow metabolism of acetaldehyde and hence they have elevated levels of acetaldehyde in their blood, which is associated with unpleasant feelings [Agarwal 1987; Mizoi, 1983].

Among the factors playing important role in the development and maintenance of addiction, the reward and motivational system of the brain is currently thought to be one of the key players. This system ensures the reward feeling from natural incentives such as food, sex etc for immediate survival of the organism and propagation of the species. All drugs of abuse exploit this natural reward system and elicit pleasure in the user and a development of compulsory seeking to satisfy the desire for the drug. This is the primary reason for why people go after drugs of abuse in the first place. Areas of the brain currently thought to play critical role in motivational behaviors include, midbrain dopamine neurons in the ventral tegmental area (VTA), nucleus accumbens, amygdala and prefrontal cortex. Repeated use of drugs of abuse causes tolerance by downregulating this dopaminergic reward system so that it escalates amount of the drug use among the addicts to achieve the same amount of high or euphoria [Koob and
Kreek 2007]. It has been reported that human alcoholics with greater severity of alcohol cravings have significantly lower D2 receptor availability in the nucleus accumbens [Heinz A, 2004]. Beside dopamine, GABA, glutamate, serotonin and opioids are also involved in alcohol reinforcement [Koob, 1994]. In addition, opioid receptors are also linked to the alcohol induced dopamine release in nucleus accumbens. Naltrexone, an opioid receptor antagonist has been reported to block the alcohol induced dopamine release in nucleus accumbens [Benjamin, 1993]. Naltrexone is also reported to decrease alcohol consumption in rats with prior exposure to alcohol [Kiefer, 2005].

Addiction is also thought to be an experience dependent learned behavior which involves the activation of nucleus accumbens-related circuitry in reward learning. Like natural rewards (e.g. food drugs etc), animals and humans consciously expect the reward of drug [Robinson and Berridge 2003]. Prolonged use of drugs of abuse is also thought to result in plastic changes in the brain. For example long-term recurrent use of drugs, such as amphetamine, cocaine, nicotine and morphine, in the addicts have been reported to change the physical connectivity of the neurons by altering the dendritic spines in the parts of the brain that play pivotal role in maintaining and perpetuating addiction, such as nucleus accumbens (motivation and reward) and prefrontal cortex (higher thought process, judgment and inhibitory control of behavior). This structural reorganization may be responsible for increasing the sensitivity of the reward system [Robinson, 2004].

According to Koob [2007], before the development of addiction, impulsive drug seeking behavior prevails, which is characterize by increasing sense of arousal before act of drug taking; experiencing pleasure, gratification and relief at the time of the commission of the drug taking act and feeling guilt, regret and self-reappraise following commission of the drug taking act.
However a full fledged addiction has the feature of compulsive behavior, which is characterized by anxiety and stress before committing a repetitive behavior (drug seeking) and relief from stress following compulsive behavior (after taking drug). Drug seeking behavior thus progresses from initial impulsivity to compulsivity during full blown addiction. The stages of drugs of addiction comprise preoccupation/anticipation stage, binge and intoxication stage and withdrawal and negative affect stage [Koob and Kreek 2007]. It is the negative affect, which motivates the addicts to engage in drug seeking behavior after getting addicted. There is an implication that the negative affect is mediated by the activation of the hypothalamo-pituitary-adrenal (HPA) axis by prolonged use of drugs. Activation of HPA axis increases the release of corticotrophin releasing factor (CRF), mediates anxiety and dysphoria. Relapse into drug seeking behavior may occur in order to avoid dysphoria and associated negative effects during withdrawal [Koob, 2008].

The fact that an addict often continues to carry out his addictive behavior despite negative consequence to his personal, professional and social life also point to the fact that addicts are unable to inhibit their intense drug seeking urge. Frontal cortex is thought to execute higher order thought processes including decision making, inhibition of inappropriate desires including drug seeking behavior, etc. Chronic exposure to drugs of abuse is reported to depress the neural processing of frontal region [Jentsch and Taylor, 1999]. Alcoholics with Korsakoff’s syndrome also show abnormality in frontal system functioning [Oscar- Berman, 2004]. In addition chronic alcohol use is reported to reduce the size of brain in human [Kril, 1999]; particularly frontal lobe [Harper, 1987], which indirectly implies malfunctioning of frontal cortex and hence putting a brake to the intense urge for drinking among alcoholics. Dysregulation of the areas thought to
help higher order thinking and planning is thought to orchestrate the vicious cycle of drug seeking behavior including alcohol and its associated pathologies.

**SLEEP**

We spend approximately one third of our life in sleep, a state characterized by reversible and diminished behavioral responsiveness to external stimuli. However, variation exists across different animals regarding the time of sleep, as well as behavioral and electrophysiological characteristics of sleep [Siegel JM, 2008]. For example, total sleep time may vary from 8 h in human to 17 h in owl monkey to 5.3 h in goat. Also unlike high amplitude EEG observed in human and rats and other mammals during SWS, amphibians have low amplitude EEG when they are in sleep and high amplitude EEG when they are awake [Allada and Siegel 2008]. The fact that sleep increases the vulnerability to predation and other injuries indirectly implies the importance of sleep from survival standpoint. However the precise need for sleep remains elusive. Sleep is thought to play a role in several physiological processes. Extended total sleep deprivation or selective SWS and REMS deprivation are reported to be lethal in rats [Rechtschaffen, 1989]. Before death, rats show skin lesions, food intake increases despite decrease in body weight and body temperature decreases before death ensues. Sleep has a suggestive role in memory consolidation [Stickgold, 2008]. It has been reported that sleep augments the plasticity in developing visual cortex [Frank, 2001]. Restricted and poor quality of sleep is reported to decrease performance and cognitive function [Belenky, 2003]. Disturbed sleep is associated with many health problems in humans. For example, both short daily sleep time (less than 6h), as well as extended daily sleep time (more than 8 h a day) are associated with increased mortality risk [Kripke 2002]. It has been documented by a recent study that hypertension and obesity are more prevalent among subjects with less than 5 h of sleep, whereas
hyperglycemia and triglyceridemia are more prevalent among subjects with sleep time more than 9 h [Choi 2008]. It is not clear whether disturbed sleep is associated with the development of various health issues or has a causal relationship. Despite the magnitude of importance of sleep, we still lack a complete understanding of sleep.

**Measurement of sleep:**

The gold standard technique for characterizing vigilance states is by analyzing the brain waves obtained from electroencephalogram (EEG) along with electromyogram (EMG), beside behavioral observations. The importance of electrophysiological measurement of sleep can be assessed by the use of some common terms routinely used in sleep research, such as, slow wave sleep (SWS), slow wave activity (SWA) etc. Sometime other electrophysiologic measurements, such as, electro-oculogram (EOG) and electrocardiogram (ECG) are also used in sleep study to get additional insight into eye movement and autonomic activation during sleep, especially in human. Animals exhibit three states of vigilance. These are SWS, characterize by high amplitude slow waves due to synchronized firing of cortical neurons, rapid eye movement sleep (REMS) characterized by fast cortical desynchronized cortical wave, hippocampal theta wave along with muscle atonia, and wake characterize by high frequency low amplitude desynchronized EEG and muscle activities.

The privilege of having modern day functional imaging technologies, such as positron emission tomography (PET) scan, functional Magnetic Resonance Imaging (fMRI), greatly enhanced our understanding of regional brain activities during sleep in human subjects [Nofizinger 2002]. Increase in activity of a localized brain area is associated with corresponding increase in glucose and oxygen utilization. Since neurons cannot store glucose and there is limited supply of glycogen in the glial cells, increase in activity of a focal brain area results in
corresponding increase in regional blood circulation of that particular area to adequately supply the glucose and oxygen demand of that active brain region. This idea is utilized by many functional imaging techniques. For example, PET scan utilizes the positron emission from radiotracer uptake in the localized brain tissue depending on the neural activity. The commonly used radiotracer for PET scan is $^{18}\text{F}$- radio labeled 2-deoxy-2-fluoroD-glucose ($^{18}\text{F}$-FDG). The uptake of radio labeled FDG in the particular area of brain increases with the activity and so is the positron emission from that particular brain region. Functional magnetic resonance imaging (fMRI) also utilizes this alteration of regional blood flow with neural activity. But it is the iron of the hemoglobin that is detected in fMRI. Deoxygenated hemoglobin has greater magnetic susceptibility than oxygenated hemoglobin. Image of a particular area of the brain is dependent on the ratio of oxygenated and deoxygenated hemoglobin, depending on regional blood flow [Otte and Halsband 2006]. All these imaging techniques provide us with greater insight into the areas of brain those are activated and deactivated during sleep. For example, the majority of the brain regions are deactivated during SWS, compared to wake and REMS. Brain metabolism decreases during SWS, especially in association cortices (frontal, parietal, temporal and occipital), primary visual cortex and thalamus (anterior and dorsomedial). But increase in activity is observed in basal forebrain, hypothalamus, ventral striatum, amygdala, hippocampus and pontine reticular formation, suggestive of their critical role in sleep [Nofzinger, 2002].

**Regulation of sleep:** The precise regulation of sleep wake cycle is not clear. However, the current thinking prevailing in the field suggests that sleep wake cycle is regulated at multiple levels. The major categories are neuronal, homeostatic and circadian regulation of sleep.

**Neuronal regulation of SWS:** Various studies including, stimulation of specific brain region, firing characteristics of group of neurons during a particular state of vigilance of sleep
wake cycle and transection of brain in animals established the concept of neuronal control of sleep wake cycles. In search of sleep regulating region of brain, earlier researchers studied the EEG of the brain in response to transection performed at various anatomical region of the brain. For example, transection of the brain in the pretrigeminal root region of the pons results in continuous wake in the part of the brain rostral to the cut. This observation suggested the probable existence of an activating system rostral to the cut and an inhibiting system caudal to the cut [Batani 1958]. The growing body of evidence in the past several decades suggests the presence of various sleep and wake promoting areas of the brain. These areas contain neurons with specific neurotransmitters and are involves in the sleep regulation as discussed below.

The brain stem and basal forebrain cholinergic neuronal groups: Cholinergic neurons are important in modulating wake by activating the cortex. Cholinergic neurons are found in the mesopontine area of the brain stem. These are laterodorsal tegmental nuclei (LDT) and pedunculopontine tegmental nuclei (PPT). Beside brain stem mesopontine region, cholinergic neurons in the basal forebrain constitute another major group. The cholinergic neurons in LTD and PPT project to thalamus, lateral hypothalamus and to the basal forebrain and from there to whole cerebral cortex [Jones 1991; Gottesmann 1999]. The excitatory cholinergic projection to thalamus is thought to gate the transmission of sensory information through the thalamus by preventing hyperpolarization of thalamic relay neurons and thus preventing them from firing in burst mode causing thalamo-cortical desynchronization [Fuller, 2006].

Locus coeruleus and Norepinephrinergic neurons: NE-ergic neurons in the locus coeruleus are thought to play a significant role in the control of alertness and wakefulness. The neurons in the LC are most active in the wakefulness and less active in SWS but silent in REMS [Hobson 1975; Aston-Jones 1981]. Moreover suprachiasmatic nucleus talks with the LC
through dorsomedial hypothalamus (DMH) and provides the circadian input to the firing patterns of NE neurons in the LC [Aston-Jones 2001].

**Posterior hypothalamic histaminergic neurons:** The common experience of drowsy feeling after taking H1 antihistaminic drug, such as Dyphenhydramine (Benadryl) suggests the role of Histaminergic neurons in the brain in sleep. Located in the tuberomammillary nucleus of the posterior hypothalamus, Histaminergic neurons have been implicated in promoting wakefulness. Histaminergic neurons project to preoptic anterior hypothalamus and modulate this sleep promoting regions of the brain and histamine when administered in the preoptic anterior hypothalamus increases wakefulness in cats. Administration of Antihistaminic drug, such as, Mepyramine, or histidine decarboxylase blocker, increases SWS in cat with corresponding decrease in wakefulness in cat [Lin, 1988; 1994]. Mice lacking histamine, histidine decarboxylase knockout mice, fall asleep more quickly and show decrease ability to maintain wakefulness in response to interaction with a novel environment [Parmentier 2002].

**Lateral hypothalamic orexinergic neurons:** These neurons are located in the posterior lateral hypothalamus and are currently thought to play critical in maintaining wakefulness. Orexin neurons have wide spread projection in the cortex and other areas in the brain, such as hippocampus, amygdala, diagonal band of Broca, olfactory bulb, bed nucleus of stria terminalis, thalamus, anterior and posterior hypothalamus, midbrain, brainstem, and spinal cord [Willie 2001]. Orexin neurons fire most during wake (Lee, 2005). They are thought to be responsible for stabilizing SWS and wake state as mice lacking orexin show less long wake episode as well as SWS episodes [Mochizuki, 2004]. Diminished orexin level or complete absence of orexin results in narcolepsy, a condition with intrusion of REMS in the middle of wake period. At present two types of orexin receptors (A and B) have been documented. Both are G-protein
coupled receptors. Non selective orexin antagonist, 078573, when administered in rats and dogs in active period promotes sleep without any signs of narcolepsy or cataplexy [Brisbare-Roch, 2007]. Beside sleep, the orexinergic neurons are also thought to play a role in pleasure seeking behavior [Harris, 2005].

Other neurotransmitter thought to play a role in the sleep wake cycles are:

**Adenosine:** The refreshing action of caffeine is well known to most of us. The wake promoting action of caffeine is mediated by blocking the action of Adenosine. A common idea prevailing in the sleep field is that the level of adenosine builds up with neuronal activity during wake period through the breakdown of Adenosine triphosphate (ATP) and mediates the homoeostatic sleep pressure mainly through activating A1 receptor. Adenosine may then inhibit the wake promoting cholinergic and non cholinergic neurons in the basal forebrain and may also disinhibit sleep promoting preoptic anterior hypothalamus [Strecker 2001]. It has also been suggested that adenosine through A1 receptor may modulate orexinergic neurons in the lateral hypothalamus [Thakkar 2008]. This group of researchers observed that microinjecting antagonist to A1 receptor in the perifornical lateral hypothalamus increases wakefulness in rats.

**Dopamine:** Dopamine is present in the ventral tegmental area of midbrain and basal ganglia specifically in substantia nigra. Although controversial, it has been suggested that wake promoting action of amphetamine and midofinil is mediated through dopamine [Wisor 2001]. The action of Dopamine is primarily terminated by reuptake of dopamine by dopamine transporter (DAT) from the synaptic cleft. DAT (KO) mice are reported to exhibit increase in wake with decrease in SWS [Wisor, 2001].
**Humoral regulation of sleep:**

Several lines of evidence have substantiated the importance of cytokines and other humoral substances in the homeostatic regulation of sleep [Steiger, 2003; Krueger 2003]. The well characterized humoral SWS regulating substances are growth hormone releasing hormone (GHRH), interleukin 1β, tumor necrosis factor-alpha (TNFα), adenosine and prostaglandin D2.

GHRH and somatostatin act on somatotropic axis and have been reported to play critical role in SWS regulation [van Cauter, 1998, Obál and Krueger, 2004]. For example, intrapreoptic microinjection of GHRH increases duration of SWS in rats, whereas microinjection of GHRH antagonist suppresses the augmentation of sleep after sleep deprivation in rats [Zhang, 1999]. SWS is also reported to increase in response to exogenous GHRH administered through icv injection in rats and rabbits [Nistico, 1987; Obál, 1988]. Systemic injection or nasal spray of GHRH also enhances SWS in humans [Kerkhofs, 1993; Perras, 1999]. Further support for the role of GHRH on sleep comes from the genetic alteration of GHRH system. For example, Obál et al (2001) reported that dwarf rat with a mutation in the GHRH receptor, show less spontaneous SWS and REMS. Mice overexpressing GH (which exerts feed back inhibition of GHRH release) show less SWS [Szabo, 1995]. Somatostatin, on the other hand, decreases SWS by suppressing the release of GHRH from the hypothalamus. Octreotide, an analogue of somatostatin, decreases stage 4 sleep (deep sleep) in male human subjects [Ziegenbein 2004]. In rats, microinjection of octreotide in the medial preoptic, arcuate nucleus and lateral hypothalamus suppresses SWS [Hajdu, 2003]
Tumor necrosis factor (TNF) and Interleukin –1 (IL-1):

TNF-α is reported to have somnogenic actions in animal and human. For example, intrapreoptic microinjection of TNF-α enhances SWS in rats [Kubota, 2002]. Increase in circulating of TNF-α is observed in human with sleep apnea associated with excessive subjective feeling of sleepiness in day time [Vgontzas, 2000]. TNF-α is suggested to have a role in alteration of sleep in chronic fatigue syndrome [Moss, 1999] HIV, [Darko, 1995] and other disease conditions.

Like TNF-α, IL –1 is also reported to have somnogenic effects [Obal, 1990] and an IL 1-receptor antagonist blocks IL 1-induced sleep and fever [Opp, 1991]. IL1β message is reported to increase after sleep deprivation [Mackeiwicz, 1996]. The variation of message and protein of TNF/IL1β in the brain match with the sleep propensity [Cearley, 2003].

Prostaglandin D 2

Prostaglandin D2 has been implicated in the modulation of sleep. Microinjection of PGD2 into the POA of hypothalamus promotes sleep in rats [Ueno, 1985]. Mizoguchi et al (2001) reported that infusion of prostaglandin (PGD2) into the lateral ventricle of the brain induced an increases in the SWS in wild-type (WT) mice but not in mice deficient in the PGD receptor. PGD synthase immunoreactivity is observed to be widely distributed in the leptomeninges of the entire brain. However, the region with the highest PGD receptor immunoreactivity was clearly defined as bilateral wings in the leptomeninges of the BF located lateral to the optic chiasma, and the tuberomammillary nucleus, the two regions with playing important role in regulation of sleep.
Neuronal regulation of REMS:

The pioneer transection studies by Jouvet had implicated pons to be critical for REMS generation. Jouvet introduced a series of brain transections in the cat and looked for signs of REM sleep throughout the brain. He found that the forebrain did not show typical signs of REM sleep when isolated from the rest of the brain. More specifically, he demonstrated that lesions in the dorsal pontine tegmentum disrupted normal REM sleep. If transection is made at the level of pontomedullary junctions, REMS is observed only rostral to the Pons [Siegel 1984]. But REMS is abolished in both cranial and caudal part of transection, when transection is made through pons [Siegel 1986]. Carli et al (1965) showed that lesions in the ventral portion of the oral pontine reticular nucleus (RPO) were the most effective at suppressing REM sleep.

The McCarley Hobson model of REMS suggests that ultradian rhythm of REMS is generated by the reciprocal interaction of presumptive cholinergic neurons (REMS on cells) in the mesopontine nucleus and brain stem monoaminergic neurons (REMS off cells) [Pace-Schott and Hobson 2002]. Cholinergic LTD-PPT neurons are most active during wake and REMS and project to thalamus and prevent generation of thalamocortical synchronization [krout 2002]. In support to this model it has been observed that cholinergic activation, such as administration of cholinergic agonist (Carbachol), anticholinesterase (e.g. Neostigmine) increases REMS. Fos activation, which is a marker of neuronal activity is reported to increase in the dorsolateral pontine after carbachol induced REMS in rats [Shiromani. 1996].

Humoral regulation of REMS:

Among the various humoral regulators of REMS, prolactin, vasoactive intestinal peptide (VIP) and Nitric Oxide (NO) are most prominent in the literature. Systemic and central administration of prolactin increases REMS. For example, administration of prolactin and VIP is reported to
enhance REMS [Obal, 1989]. On the other hand, blocking the action of prolactin by administering antiserum to prolactin or blocking action of VIP by administration of VIP antagonist decreases REMS in rats [Obal, 1992, Mirmiran, 1988]. Simiarly, microinjection of SNAP, a donor of NO, in the PPT enhances REMS, where as microinjection of L-NAME, a NOS inhibitor, in the same area, decrease REMS [Datta, 1997]

Circadian Regulation: Like activity, food intake, temperature and release of many hormones, sleep wake cycle is under a strong circadian regulation and maintains a phase with other biological rhythms. For example, in normal adult human (male) the initiation of sleep occurs approximately 6 h before temperature trough and four hours before melatonin peak release and sleep ends about 2 h after temperature nadir [Duffy, 1998]. Suprachiasmatic nucleus of the hypothalamus is implicated to be the master biological clock and modulate the activity of LC and many other arousal systems. The interplay between four genes and their products is currently thought to have a role in the regulation of periodicity of circadian rhythm in mammals [Pace-Schott and Hobson, 2002]. These are Clock, Bmal and Per and Cry. The heterodimer products of Clock and Bmal binds to the regulatory DNA sequences of Per and Cry genes and promotes the their transcription. mRNA of Per and Cry exit out of the nucleus and get translated in the cytoplasm. Per and Cry protein form complex which then reenter the nucleus and modulate the transcription of Clock and Bmal genes. Cry containing protein complex provides a negative feed back control on the Clock-Bmal heterodimer, whereas Per2 protein augments the transcription of Bmal. During the day the increased level of Clock-Bmal heterodimer (accumulate over the subjective night) increases the Per and Cry products which peaks at the end of subjective day. At this point feedback inhibition of Clock and Bmal reverses this trend.
Theories of generation of sleep: How sleep is generated is not clearly understood. However, several theories are available in the field, each having its own pros and cons. Dominant theories are discussed below.

**Thalamocortical synchronization and SWS:** It is one of the widely accepted models of generation of SWS and has the ability to explain the reversible loss of consciousness during sleep. Thalamus is the portal of entry for all sensory information going to the cortex. The axons carrying information synapse with the thalamic relay neurons; which then carry the information to the cortex. Thalamus also has (GABA-ergic) reticular thalamic neurons, which envelopes the thalamus. Both types of neurons have reciprocal connection with the cortical neurons. Thalamic relay neurons exhibit two distinct firing modes, a single action potential firing mode and a burst firing mode. Near the firing threshold of the resting membrane potential, the thalamic relay neuron is in transmission mode and it fires a single action potential in response to the stimulus it receives and transmits the excitatory impulse to the cortex. This happens during wake and makes the animal consciously aware of its environment. However, during transition from wakefulness to SWS, there is a period of hyperpolarization of thalamocortical relay neurons, which results in inhibition of incoming information from reaching the cerebral cortex. The hyperpolarization of thalamic relay neurons causes them to fire in a burst mode and cause synchronous firing of cortical neurons because of their reciprocal connection with thalamocortical relay neurons. This synchronous firing of thalamocortical neuronal network is thought to generate SWS [Steriade, 2003]. Thalamic relay neurons have special types of voltage gated calcium channels that remain inactivated near the resting membrane potential. However when the membrane gets hyperpolarized, these calcium channels open transiently and draw Ca- current that elevates the membrane above threshold causing the relay neurons to fire a burst of action potentials that
causes further opening of Ca-channels. This triggers calcium activated potassium current that hyperpolarizes the neurons, resetting it for another set of burst. The thalamic relay neurons become hyperpolarized in the first place by GABAergic neurons of the reticular thalamic nucleus, which envelopes the thalamus and have reciprocal interaction with the relay neurons. Dendrites of these reticular neurons receive collaterals from both thalamocortical and corticothalamic axons. The neurons of reticular thalamic nucleus also enter in a burst mode when hyperpolarized. When the reticular neurons fires they hyperpolarize the relay neurons, which then fires in burst mode in response to incoming signals. The thalamus gets inputs from various activational systems, such as cholinergic neurons from LTD and PPT [Fuller, 2006]. Chronic alcohol administration is suggested to affect thalamic relay neurons. For example chronic alcohol self administration in Macaque monkeys show attenuation of burst response in the LGN and reduces the Ca current in thalamic relay cells [Carden 2006].

Hypothalamus and Saper’s theory Flip-Flop switch of sleep: Historically hypothalamus was implicated to play a key role in the regulation of sleep. Von economo first reported the association of sleepiness among patients during the pandemic, Encephalitis Lethargica, with the lesion of posterior hypothalamus and rostral midbrain [Von Economo, 1930]. He also reported another group of patients with the same disease but suffered from prolong wake, with lesion in the anterior hypothalamus and basal forebrain. After around eight decades of sleep research his idea still holds true but now we developed a better understanding of specific nuclei in the hypothalamus and their role in sleep and wake. For example, sleep promoting regions of anterior hypothalamus, such as VLPO, mPOA have GABAergic sleep active neurons which fire at a high frequency during sleep. Increase in SWS by increasing the local temperature of hypothalamus as well as the study of the early immediate genes C-fos implicate anterior hypothalamus as a key
player for initiation and maintenance of SWS [McGinthy 2001; Saper 2001]. Microinjection of sleep regulating substance, such as GHRH, TNF α, in the anterior hypothalamus, increases SWS [Zhang, 1999; Kubota 2002b]. The posterior hypothalamus, on the other hand, has wake promoting histaminergic neurons and orexinergic neurons. Saper and his colleagues suggested that VLPO has two groups of neurons playing critical role in the regulation of sleep. The core of VLPO projects heavily to the histaminergic neurons in the tuberomammillary nucleus and has suggestive role in the sleep-wake regulation. The neurons surrounding the core of VLPO, project to locus coeruleus and dorsal and median raphe nuclei and thought to play a role in REMS regulation. Saper’s flip-flop theory is based on the mutually inhibitory interaction of sleep promoting vLPO neurons with the arousal systems. The important arousal systems are histaminergic neurons in the tubero mammillary nucleus, NE ergic neurons in the LC, cholinergic neurons in the PPT and LTD in the Pons and basal forebrain. This is analogous to flip-flop switch, which makes sure sharp transitions between states (Saper, 2001). In addition, dorsomedial hypothalamus integrates the circadian, emotional and other cues and modulates this Flip-Flop switch [Fuller, 2006; Saper 2005]. The transition to a sleep or a wake state is further thought to be stabilized by the orexinergic neurons in the lateral hypothalamus. This is supported by the observation that a decrement in the duration of SWS and wake are observed in orexin knockout mice [Mochizuki, 2004]. Saper’s Flip-Flop switch model better explains the sleep-wake transition but it does not provide any insight about the kinetic behavior of the sleep wake transition. This inability to provide insight about the dynamics of the sleep-wake transition is deficient in most of the models to my knowledge.
ALCOHOL AND SLEEP:

Chronic and recovering alcoholics suffer from disturbed sleep [Brower, 2000; Drummond, 1998]. The alcohol related sleep disturbance may account for 10% of all the cost imposed by alcoholism [Stoller, 1994]. If we look at the real number, it is mind-boggling. For example, in the year 1998 with total alcohol abuse related cost being around $184.6 billion [NIAAA, 2000], sleep disturbance alone accounted for nearly $18.46 billion. Acutely alcohol is somnogenic and increases SWS duration in human [Lands, 1999] and animals [Kubota, 2002, Mendelson, 2001]. The somnogenic action of alcohol has been demonstrated in other studies, for example, low dose of alcohol administration in nonalcoholic humans has been shown to induce sleep but at higher doses, alteration of sleep patterns is observed in second half of the sleep period [Roehrs, 2001; Landlot and Borbley, 2000]. However, with the recurrent use of alcohol, the somnogenic action of alcohol dwindles away with the development of various sleep pathologies. These are prolonged sleep latency, inhibition of REMS, reduced SWS duration and frequent awakening at night resulting in fragmentation of sleep [Brower, 2001; Johnson, 1970; Mello and Mendelson, 1970: Snyder, 1985; William and Rundell, 1981]. Disturbed and non-recuperative sleep at night may give rise to daytime sleepiness and compromise with daytime performance [Roth and Ancoli-Israel, 1999]. Other consequences of sleep disturbances associated or exacerbated by alcohol use are sleep apnea [Aldrich, 1999] and compromised memory function [Roehrs and Roth, 1995]. Alcohol is thought to exacerbate sleep apnea by relaxing the upper airway dilator muscles and by increasing the resistance to air inflow [Landolt and Borbely 2000]. Interestingly, disturbed sleep pattern, such as, longer sleep latency, shorter REMS latency and less deep sleep (stage 4 SWS), persist during recovery from alcohol addiction.
in many recovering alcoholics and is predictive of relapse into drinking behavior [Brower, 1998; Clark, 1998; Drummond, 1998; Gillin 1994; Gillin, 1990].

Dysregulation of sleep by chronic alcohol abuse is also documented by the fact that the normal increment of the power in the slow wave sleep (SWS) following sleep deprivation [Borbely, 1982] is not observed in alcoholics [Irwin, 2002]. Increase in sympathetic activation is also observed in recovering alcoholics in response to mild stress. For example, recovering alcoholics show increased heart rate and circulating NE level in response to sleep deprivation compare to non alcoholic normal subjects [Irwin and Zeigler 2005]. Given the profound disrupting effect of alcohol on sleep, the basis of this alteration of sleep by alcohol is not clear.

Several lines of evidence implicate alcohol induced alteration of major inhibitory neurotransmitter GABA [Weiner and Valenzuela, 2006; Lobo and Harris 2008] and major excitatory neurotransmitter glutamate in the brain. That alcohol mediates at least part of its action though GABA is apparent from the similarity of many of the behavioral action of alcohol consistent with increased GABAergic function [Criswell, 2008]. For example, ethanol shared many of the sedative, anxiolytic and anticonvulsant action of drugs known to enhance GABA-A receptor function, such as Benzodiazepine and Barbiturate [Deitrich, 1889]. Microinjection of alcohol in the medial preoptic hypothalamus, which is important for SWS, tends to increase total SWS with no effect on REMS. Flumazenil, a benzodiazepine antagonist, has been shown to block this SWS promoting action of ethanol microinjection in the anterior hypothalamus. This observation further, suggests that the acute sleep promoting action of alcohol in MPOA hypothalamus is, at least partly, mediated through GABA A benzodiazepine receptor [Mendelson 2001]. On the other hand long term alcohol exposure inhibits glutamergic neurotransmission and upregulate glutamate system [Fadda and Rossetti, 1998].
Alcohol and somatotropic axis

Alcohol is shown to have substantial effect on somatotropic axis [Land, 1999; van Cauter, 1998] and Somatotropic axis, mainly the growth hormone releasing hormone (GHRH) and somatostatin, is suggested to play role in the regulation of sleep [Krueger, 2003; Steiger, 2003]. Acutely alcohol is reported to decrease GH secretion in both rats and human [Ekman 1996; Emanuele 1992; Fernstrom, 1995; Valimaki 1990]. Alcohol also decreases basal and GHRH induced GH release in vitro [Emanuele, 1988]. Chronic alcohol exposure increases circulating GH in human [Redmond 1981; Zanoboni 1977]. However, in animals, the effect of chronic alcohol may vary. Decrease in GH is observed in rats [Badger, 1993], although no change [Mannisto, 1987] and increase increase in amplitude of GH pulse have also been reported [Sonntag, 1989].

Alcohol and HPA axis

Corticosterone releasing factor (CRF) promotes wakefulness in human and animals [Steiger, 2003; Krueger 2003]. When administered i.c.v., CRF decreases SWS in rats [Ehlers, 1986]. It has also been shown that urocortin, a CRF receptor agonist, increases wake and decreases SWS in the light period. This increase in wake is blocked by a CRF antagonist [Uchida, 2007]. Moreover, CRF is also reported to suppress the IL-1 induced increase in SWS and fever in rabbits [Opp, 1989]. Alcohol use is associated with increase in activation of HPA axis. Cortisol level increases in saliva after chronic alcohol use in human [Adinoff, 2003]. It has been documented that alcohol increases CRF in long sleep and short sleep line of mice in a dose dependent manner [Zgombick, 1988].

Alcohol and circadian rhythm:
Alcohol has profound effect on circadian rhythm including sleep [Lavie 1997]. Alcohol consumption delays the nocturnal peak of melatonin release. The melatonin peak has been shown to be associated with circadian sleep pressure, is thought to implicate the altered circadian rhythmicity and prolong latency to sleep in alcoholics [Kuhlwein, 2003]. Observing body temperature is another way of studying circadian rhythm. Alcohol initially decreases the core body temperature but later on it increases body temperature as evident by blunting of circadian peak rectal temperature, observed in human in response to alcohol [Dane, 2001].
REFERENCES:


Allada R and Siegel JM. Unearthing the phylogenetic roots of sleep. Current Biology, 2008; 18: 670-679


Baraona E and Lieber CS. Alcohol and lipids. Recent Dev Alcohol., 1998; 14: 97-134. Review


Bujanda L. The effects of alcohol consumption upon the gastrointestinal tract. Am. J. Gastroenterol., 2000; 95; 3374-3382. Review.


Cearley C, Churchill L, Krueger JM. Time of day differences in IL1 beta and TNF alpha mRNA levels in specific regions of the rat brain Neurosci Lett., 2003; 352: 61-63


Criswell HE, Ming Z, Kelm MK, Breese GR. Brain regional differences in the effect of ethanol on GABA release from the presynaptic terminals. JPharmacol Exp Ther, 2008; 326: 596-603


Datta S, Patterson EH and Siwek DF. Endogenous and exogenous nitric oxide in the pedunculopontine tegmentum induces sleep. Synapse, 1997; 27: 69-78


Fadda F, Rossetti ZL. Chronic ethanol consumption: from neuroadaptation to neurodegeneration. Prog. Neurobiol., 1998; 56: 385-431
Fauchier L. Alcoholic cardiomyopathy and ventricular arrhythmia. Chest., 2003; 123:1320


Frank MG, Issa NP and Stryker MP. Sleep enhances plasticity in the developing visual cortex. Neuron, 2001; 30: 275-287

Fuller PM, Gooley JJ and Saper CB. Neurobiology of sleep-wake cycle: Sleep architecture, circadian regulation and regulatory feedback. Journal of Biological Rhythm, 2006; 21: 482-493


Hajdu I, Szentirmai E, Obal F, Krueger JM. Different brain structures mediate drinking and sleep suppression elicited by the somatostatin analog, octreotide in rats. Brain Res., 2003; 994: 115-123


Jentsch JD and Taylor JR. Impulsivity resulting from frontostriatal dysfunction in drug abuse: implications for control of behavior by reward related stimuli Psychopharmacology (Berl.), 1999; 146: 373-90


Koob GF, Rassnick S, et al. Alcohol, the reward system and dependence. Exs., 1994; 71: 103-14

Krueger JM and Majde JA. Humoral links between sleep and immune system: research review Ann. NY. Acad. Sci., 2003; 992: 9-20


Kubota T, Li N, Guan Z, Brown RA, Krueger JM. Intrapreoptic microinjection of TNF α enhances non-REM sleep in rats Brain Res. 2002; 932: 37-44


Lee MG, Hassani OK, Jones BE. Discharge of identified orexin/hypocretin neurons across sleep-wake cycle. J. Neurosci., 2005; 25: 6716-6720
Leiber CS. Alcohol and malnutrition in the pathogenesis of liver disease. JAMA, 1975; 233 No.10.


Mannisto PT, Vedernikova NN, Borisenko SA, Tuominen RK, Kiianmaa K, Burov YV. Effect of chronic ethanol administration and abstinence on serum thyroid-stimulating hormone, prolactin and growth hormone concentrations in rats with high and low ethanol intake. Alcohol, 1987; 22: 251-256


Mendelson WB. The sleep-inducing effect of ethanol microinjection into preoptic area is blocked by flumazenil. Brain Research, 2001; 892: 118-121.


Mokdad AH, Marks JS, Stroup DF, Gerberding JL. Actual Causes of Death in the United States. JAMA, 2000; 291:1238-1245


Obál F Jr and Krueger JM. GHRH and Sleep. Sleep Medicine Reviews, 2004; 8: 367-377


Reynolds K, Lewis LB, Nolen JDL, Kinney GL, Sathya B, He J. Alcohol Consumption and Risk of Stroke. JAMA, 2003; 289: No. 5, Feb5,

39
Roberto M, Nelson TE, Ur CL, Gruol DL. Long-term potentiation in the rat hippocampus is reversibly depressed by chronic intermittent ethanol exposure. J. Neurophysiol., 2001; 87: 2385-2397


Saper CB, Chou TC, and Scammell TE. The sleep switch: hypothalamic control of sleep and wakefulness, Trends Neurosci, 2001; 24: 726-732

Saper CB, Cano G and Scammell TE. Homeostatic, circadian and emotional regulation of sleep The Journal of Comparative Neurology, 2005; 493: 92-98

Shiromani PJ, Wintson S, McCarley RW. Pontine cholinergic neurons show Fos-like immunoreactivity associated with cholinergically induced REM sleep Brain Res Mol Brain Res., 1996; 38: 77-84

Siegel JM. Do all animals sleep? Trends Neurosci, 2008; 208: 208-223.
Siegel JM, Nienhuis R and Tomaszewski KS. REMS signs rostral to chronic transections at the pontomedullary junction, Neurosci. Lett., 1984; 45: 241-246

Siegel JM, Tomaszewski KS, and Nienhouis R. Behavioral states in the chronic medullary and midpontine cat, Neurosci. Lett, 1991; 130: 41-45


Steiger A. Sleep and endocrinology. Journal of Internal Medicine, 2003; 254: 13-22


Szabo M, Butz MR, Banerjee SA, Chikaraishi DM, and Frohman LA. Autofeedback suppression of growth hormone (GH) secretion in transgenic mice expressing a human GH reporter targeted by tyrosine hydroxylase 5‘ flanking sequences to the hypothalamus. Endocrinology, 1995; 136: 4044-4048


van Cauter E, and L Plat Interrelations between sleep and the somatotropic axis Sleep, 1998; 21: 553-565


Wagner FA and Anthony JC. From first drug use to drug dependence: Developmental periods of risk for dependence upon marijuana, cocaine, and Alcohol Neuropsychopharmacology, 2002; 26: 479-488

Weiner JL, Valenzuela CF. Ethanol modulation of GABAergic transmission: the view from the slice. Pharmacology and therapeutics, 2006; 111: 533-554


Zgombick JM, Erwin VG. Ethanol differentially enhances adrenocortical response in LS and SS mice Alcohol, 1988; 5: 287-294

Ziegenbein M, Held K, Kuenzel HE, Murck H, Antonijevic IA and Steiger A. The somatostatin analogue octreotide impairs sleep and decreases EEG sigma power in young male subjects Psychopharmacology, 2004; 29: 146-161
Chapter 2 Dose-Response Study of Chronic Alcohol Induced Changes in Sleep Patterns in Rats.

Sanjib Mukherjee, Morvarid Kazerooni, Steven M. Simasko

Program in Neuroscience, Department of VCAPP, Washington State University, Pullman, WA 99164-6520

Published in Brain Research 1208 (2008) 120-127
ANALYZE

The goal of the present study was to determine an optimum exposure regimen for alterations in sleep induced by chronic alcohol treatments in rats. We used two different exposure routes (alcohol in water and alcohol in liquid diet at two different doses in each regimen (6% and 12% alcohol in water and 3% and 6% alcohol in liquid diet). All treatments were for 6 weeks. We found that the effects of the 6% and 12% in water and 3% in liquid diet to be very similar, all three produced increases in slow-wave sleep (SWS) only in the dark period with no changes in rapid-eye-movement sleep (REMS). On the other hand 6% alcohol in liquid diet caused much more dramatic changes, with alterations in both SWS and REMS in both the dark and light period. These animals spent less time in SWS and REMS during the light period but more time in SWS and REMS in the dark period. Additionally, the variation of slow wave amplitude (SWA) across day and night in this group of alcoholic animals is blunted with the loss of the peak of SWA at the beginning of light onset compared to the other groups. We conclude that future alcohol treatment regimens used to investigate the effects of alcohol on sleep in adult rats should use an exposure protocol of at least 6 weeks with 6% alcohol in liquid diet.
INRODUCTION:

Disrupted sleep is a frequent complaint in human alcoholics. During periods of alcohol consumption sleep is fragmented with frequent wakenings and suppression of rapid-eye-movement sleep (REMS) [5, 21]. During early abstinence there is a rebound in REMS but sleep onset remains delayed and sleep efficiency (percent time in bed asleep) continues to be reduced [4, 7, 9, 14, 15, 16, 24, 27]. In addition, alcohol can also intensify other sleep disorders such as sleep apnea and periodic limb movements [4, 7, 13, 20]. Many of these altered sleep patterns persist even during prolonged recovery from drinking [4, 5, 9, 20, 27]. Disrupted sleep patterns, especially increased REMS pressure during early abstinence and continued insomnia, is predictive to relapse into drinking behavior [6, 8, 9, 14, 15]. Despite the well documented fact of disturbed sleep in alcoholics, and the strong relationship between poor sleep and relapse drinking, the basis for chronic alcohol induced alteration of sleep is poorly understood.

An animal model would be a powerful tool that could help us understand the cause of alcohol induced alterations in sleep. However, only a few studies have directly assessed this issue. Some studies have used indirect measures of sleep, such as patterns of running wheel activity or loss of righting reflex, that fail to reveal changes in sleep architecture. In studies that use EEG recordings to assess sleep, alcohol induced alterations in sleep architecture have been observed. For example, Ehler’s group has reported that rats exposed to 6 weeks of ethanol vapor had a reduction in the spectral power of the EEG in both low frequency and high frequency bands, but no changes in slow wave sleep (SWS) latency or duration [11]. They also found a suppression of the evoked potential and rebound enhancement of the EEG power during withdrawal [10]. However, one issue in these studies was that EEG recordings were performed for only short periods of time (4 hours), thus changes in daily sleep patterns could not be
observed. Veatch [26] and Mendelson et al. [22] both found loss of total sleep time with ethanol exposure, and a rebound in REMS during withdrawal, but their exposure protocols where relatively brief (4 days or less) and they focused mostly on the withdrawal period rather than on the pathologies associated with chronic alcohol exposure. Two prior studies have focused specifically on the alcohol induced pathologies that follow chronic alcohol exposure for 2-3 weeks. In Rouhani et al. [23] alcohol was given by scheduled infusions via an indwelling gastric catheter. After 13 days they observed decreases in percent SWS and REMS and increase in wake only in light period. In Kubota et al. [19] alcohol was given in a liquid diet (6% alcohol) for 3 weeks. They observed a suppression of SWS in the dark period and a blunting of the normal circadian variation of REMS, that is, the amount of REMS decreased in the light period but increased in the dark period. Finally, like the study by Ehlers and Slawecki [11], they observed a suppression of the power spectra of the EEG in the high frequency bands (>5.0 Hz). While these studies document that alcohol can induce changes in sleep architecture in rodents, they represent only the beginning in understanding the relationship between alcohol consumption and altered sleep.

In the present study we modeled our alcohol exposure paradigm on the one used by Kubota et al. [19], 6% alcohol in liquid diet. We decided to use this paradigm as opposed to alcohol vapor or scheduled alcohol infusions because if alcohol was inducing alterations in sleep patterns, a behavior with a very strong circadian rhythm, allowing the animal a relatively free choice as to when to or not to consume alcohol may have a significant impact on the alterations of sleep patterns observed. We addressed three questions. The first was whether a more prolonged alcohol exposure (6 weeks vs. 3 weeks) would reveal any additional pathologies in the sleep patterns. The second was whether a lower dose of alcohol (3% in liquid diet for 6 weeks)
would be effective in inducing alcohol induced changes in sleep patterns. And the third was whether alcohol delivered in water rather than food would produce sleep pathologies that differed from those when the alcohol was given in food. We found that both the lower dose (3% in liquid diet) and alcohol delivered in water were capable of inducing sleep pathologies similar to those observed in Kubota et al. [19]. However, we also found that the 6% alcohol in liquid diet for 6 weeks produced even more profound alterations in sleep than any of the other exposure regimens. This suggests that the 6% alcohol in liquid diet for 6 weeks should be at least the minimal exposure regimen in future studies in this area of research.

METHODS

Alcohol treatments. Forty-eight young adult (2-5 months of age) male Sprague-Dawley rats (Taconic, Germantown, NY) were used. The experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at Washington State University. Animals were housed individually throughout the experiment. Alcohol treated rats were given grain alcohol (Everclear) v/v in liquid diet (Bio-Serv, Frenchtown, NJ) or in tap water. For the alcohol in liquid diet experiments each pair-fed rat was weight matched to a treated rat and given alcohol-free liquid diet of equal caloric content (calories from sucrose) as that consumed by the weight-matched alcoholic rat. All rats in the protocol where alcohol was given in liquid diet had ad lib access to water. Animals treated with alcohol in water had forced drinking but had ad lib access to pelleted food throughout the treatments. The control animals for the alcohol in water animals had ad lib access to pelleted food and water. The duration of alcohol exposure in all the protocols was for six weeks. Body weight of the rats was noted every three to four days, until the end of the experiment. Consumption of liquid diet or water consumption in the alcohol in water groups was noted daily during the last hour of light period.
**Instrumentation for sleep recordings.** All rats were instrumented in the fifth week of alcohol treatment. We followed standard instrumentation procedures as previously reported [19]. Briefly, under ketamine (87 mg/kg) and xylazine (13 mg/kg) anesthesia, 3 EEG electrodes (Plastics One, Roanoke, VA) were implanted, one in the left frontal (5 mm A and 2 mm L, from bregma), one over the right parietal (-5 mm A and 6 mm L, from bregma) and the ground electrode over the left occipital cortex (-11 mm and 4 mm L, from bregma). One EMG electrode was implanted in dorsal neck muscle. The rats were allowed one week to recover from the instrumentation before sleep recordings commenced. The rats were maintained on the alcohol treatment during this period until the end of the recording.

**Sleep recordings.** To minimize any disturbances to the rats during the period of data collection, sleep recordings were made in enclosed environmental chambers (4 rats per chamber, each chamber ~6 ft X 2 ft x 6 ft; width x depth x height) with constant room air flow through the chamber. The interior of the chamber was illuminated by a 15 watt light (12 h: 12 h light: dark cycle) and maintained at 22 ± 2 °C. We began to collect EEG and EMG for analysis of sleep patterns after three days of acclimatization to the chambers. We recorded for two consecutive days. Recordings were interrupted for one hour each day (last hour of light period) for animal care. In the present study we report findings from recordings on the last recording day.

**Collection and analysis of EEG and EMG signals.** The EEG and EMG signals were collected through a wire harness tethered to an overhead multi-channel commutator (Plastics One, Roanoke, VA). The signals were amplified by polygraph amplifiers (Grass-Telefactor, Inc., West Warwick, RI) and digitized at 128 Hz through a computer software program (SleepSign for Animals, Kissei America Inc.). The EEG/EMG recordings were analyzed in 8-sec epochs and each epoch was assigned to a particular vigilance state: wake, SWS, or REMS by
standard criteria embedded in the software program. Each continuous period in the same vigilance state is referred to as an episode, and we placed a minimum requirement of three consecutive epochs before a change in vigilance state of episodes was noted. Thus minimum episode durations were 24 s in length. All scoring was visually inspected to ensure correct assignment of vigilance states. We scored recordings for 22 hours of each 24 hour period because of the daily disruption for animal care (12 hours in the dark period and 10 hours in the light period). In addition to assigning vigilance state, the software program enabled use to analyze the EEG waveforms by fast Fourier transform within each epoch so that we could examine the power of the component frequencies within the EEG and determine slow-wave amplitude (SWA) from the power in the delta band (0.5 – 4 Hz) during SWS.

Statistical Analysis. In the course of the experiment the instrumentation on four animals failed before the first sleep recordings, two pair fed in 6% alcohol in the liquid diet and one pair fed in 3% alcohol in liquid diet and one animal in 6% alcohol in water group. All the animals in the remaining protocols were well and the instrumentation remained intact to the end of the measurements. Sleep was analyzed for 12 h in the dark period and the following 10 h in the light period. In our analysis for total time spent in light period in each vigilance state, we correct for the last 2 h, by multiplying the total time by a factor of 1.2. We used SigmaStat version 3.5 for the statistical analysis. For alcohol in liquid diets, we used a regular, two-tailed, t-test to compare results from alcohol treated animals to the pair-fed controls. For other comparisons with more than two groups (alcohol in water) we used analysis of variance (ANOVA) with the Holm-Sidak post hoc multiple comparison test. In all cases p<0.05 was considered statistically significant.
RESULTS

Alcohol consumption. At the end of the treatment regimens the average daily alcohol consumption was calculated for each protocol. For the animals treated with alcohol in liquid diet the 6% alcohol animals consumed 11.1 ± 0.2 g alcohol/kg body weight/day whereas the 3% alcohol animals consumed 5.5 ± 0.1 g alcohol/kg body weight/day. We have previously reported that the 6% in liquid diet produces blood alcohol levels of 0.11 to 0.13% at the end of the dark period [19]. It was our intention that the amount of alcohol consumed in the alcohol in water diets would be similar to that consumed in the liquid diet, and we had noted that the rats drank about half the volume of water compared with the liquid diet (unpublished observation), so we selected 6% and 12% alcohol in water for these treatments. Unfortunately, when alcohol was added to the water, the amount of water consumed by the rats was less than we had anticipated and the average daily alcohol consumption for these rats were 3.9 ± 0.2 g alcohol/kg body weight/day for the 6% in water group and 7.3 ± 0.2 g alcohol/kg body weight/day for the 12% in water group. We did not want to push the concentration of alcohol in water any higher than 12% because in other experiments we have performed, we find that when rats consume 15% alcohol in water with ad lib access, they further reduce their water consumption, and start to show signs of dehydration as measured by rapid free water consumption when a source of tap water is placed into the cages (unpublished observation).

Weight gain during alcohol treatments. While all the rats used in the protocols were young adult males, because of the limited number of recording chambers, the start of the diets were staggered, and thus the average starting weights of the different groups tended to be slightly different. In addition, we have previously found that when rats are first started on alcohol in liquid diet, they eat less the first few days of the protocol until they acclimate to the presence of
alcohol in their diets. Finally, the rate of weight gain in rats is somewhat dependent on their starting weight in that as they get bigger, their weight gain tends to slow. Thus to make comparisons of weight gains for the different treatment groups we examined the rate of gain for each group when the animals had been into their individual protocols at least a week (range 7 to 25 days), and the group average weight was 350 ± 10 g. Weight gains were then calculated over a period of ~3 weeks (17 to 24 days). The one exception to this was the 6% alcohol in water group that was slightly larger than the other animals at the start of their regimen, so the starting point for their average daily gain was when the group average was 400 g. The values for the average daily gain are shown in Fig. 1. As can be seen, the group that had ab lib access to pelleted food and water group grew the fastest. While the other groups were not statistically different from each other, there was a trend for the 3% in liquid diet group (and their pair-fed controls) to gain at a slightly higher rate than the other groups. Finally, one would expect the 6% in water group to gain somewhat faster than what we observed since this was the group with the lowest alcohol intake, but they were virtually identical to the other treatment groups. This is perhaps due to their slightly larger size at the beginning of the measurement.
Comparisons of sleep among the control groups. We compared sleep parameters among the two pair-fed control groups and the control for the water treated groups (Fig. 2). While there were no differences found between the *ad lib* water and food control and the pair-fed to the 3% alcohol in liquid diet group in any parameter, the pair-fed to the 6% alcohol in liquid diet group had statistically significant more SWS and less wake than the other two control groups in the light period (compare solid bars in unshaded panels; p<0.007 for wake and p<0.008 for SWS). This group also had more SWS and less wake in the dark period that nearly reached statistical significance (compare solid bars in shaded panels; p = 0.12 for wake and p = 0.056 for SWS). There were no differences in amount of REMS between any of the control groups in either light or dark periods (Fig. 2).

Effects of alcohol treatment on sleep. Treatment with alcohol in water (12% or 6%) caused a decrease in time spent in wake and an increase in time spent in SWS only in the dark period (Fig. 2A and 2C). Time spent in REMS was not altered (Fig. 2E). When 3% alcohol in liquid diet was given to the rats, only time spent in SWS in the dark period was statistically significantly altered (Fig. 2D, left panel). There was a non-significant decrease in time spent in wake in the dark period that almost reached statistical significance (Fig. 2B, left panel, p = 0.097). As with the alcohol treatments in water, REMS was not affected (Fig. 2F, left panel).

While the altered patterns of sleep produced by the 6% and 12% alcohol in water, and 3% alcohol in liquid diet where very similar to one another, the effects of 6% alcohol in liquid diet was much more dramatic in altering sleep patterns. All three vigilance states were altered by the alcohol treatment (Fig. 2B, 2D, 2F, right hand panels). Interestingly, while wake was suppressed and SWS and REMS were increased in the dark period, the changes in the light period were in the opposite direction.
The duration and the number of episodes of SWS and REMS of animals treated with alcohol in water are reported in Table 1. While overall time spent in SWS is increased in the dark period for both these groups, at this sub-level of analysis the only parameter that was statistically significantly altered was an increase in episode duration in the 12% alcohol in water group, although the same parameter for the 6% alcohol in water almost reach statistical significance (p = 0.039 but critical level in the multiple comparison test was 0.025). The number of SWS episodes also tended longer in the alcohol treated groups but the changes were not statistically significant (p = 0.111 between groups). However, when these two parameters are multiplied together to produce total SWS time (Fig. 2C) the increase in SWS in the dark period is clearly significant.

As with the alcohol treatments in water, the sub-level analysis of episode duration and frequency for the 3% alcohol in liquid diet (Table 2) revealed no statistically significant alterations, although there were trends in both increase episode duration (p = 0.077) and increase episode frequency (p = 0.413), that while by themselves were not statistically significant, produced a statistically significant increase in total SWS in this group (Fig. 2D, left hand panel). On the other hand, animals treated with 6% alcohol in liquid diet had multiple alterations in the sub-level analysis (Table 2). These animals had a dramatic increase in SWS episode duration that mostly accounts for the prolonged SWS in the dark period. The loss of SWS in the light period was due primarily to a decrease in the frequency of SWS episodes, rather than a change in duration. The same is true of REMS episodes. The increase in total REMS in the dark period is due to a dramatic increase in REMS episode duration, whereas the loss of REMS in the light period is due to a dramatic decrease in the number of REMS episodes.
An additional parameter we examined was the slow wave amplitude (SWA) across the day. SWA represent the delta power (0.5 to 4 Hz) during SWS and is generally interpreted as an indicator of the depth of the SWS period. To examine SWA across the day we averaged the amplitude of all delta wave frequency components within SWS episodes within each 2-hr block of time across the day and then determined an average value for the entire day by averaging these 2-hr block values together. The SWA of each individual block is then expressed as a percent of this average value. In Fig 3 the SWA of the first 2 hours of dark, the first 2 hours of light, and the last 2 hours of light (hours 8-10 of the light period) are shown for each treatment group. As is normal, the SWA for almost all treatment groups is highest at the onset of sleep (i.e., the first two hours of the light period). The one exception was the animals treated with 6% alcohol in liquid diet. These animals had an abolition of the peak of SWA at sleep onset (Fig. 3C, middle panel). This loss of circadian variation in SWA is similar to the blunting of circadian variation of other sleep parameters produced by the 6% alcohol in liquid diet regimen.
DISCUSSION

In the present study we examined the effects of different chronic alcohol treatment regimens on sleep in rats. We found that alcohol given in water (6% or 12%) was capable of altering sleep parameters similar to that observed with 3% alcohol given in a liquid diet. Given that the measured amount of alcohol consumed in these various protocols were similar (3.9, 5.5, and 7.3 g alcohol/kg body weight/day), it would suggest that administration of alcohol in liquid diet or water makes no difference in the final effect of alcohol. However, the most significant finding was that the group treated with 6% alcohol in liquid diet had much more dramatic alterations in sleep patterns than any of the other groups. Further, the alterations seen in our group treated with 6% alcohol in liquid diet for 6 weeks were also much more dramatic than what our group has previously shown to occur with the 6% alcohol diet for 3 weeks (19). The major difference between the 6% alcohol in liquid diet and 3% alcohol in liquid diet is the exposure amount (11.1 g/kg/day vs. 5.5 g/kg/day). These findings demonstrate that exposures for periods shorter than 6 weeks, and at levels less than those achieved by 6% alcohol in liquid diet, do not fully elaborate all the changes that can occur with chronic alcohol exposure on sleep mechanisms.

The lesser alcohol exposure regimens did not necessarily produce alterations in sleep patterns that were fundamentally different than what we observed with 6% alcohol in liquid diet, rather the trends that were apparent in the lesser exposure regimens appear to not have fully elaborated. For example, all regimens increased SWS in the dark period, but only the 6% in liquid diet caused a reciprocal decrease in SWS in the light period. Likewise, all exposure regimens decreased wake in the dark period, but only the 6% in liquid diet increased wake in the light period. The nature of these changes agrees well with previous reports. Kubota et al. (19)
also observed an increase in SWS in the dark period after 3 weeks of 6% alcohol in liquid diet, similar to all protocols we used for 6 weeks. Interestingly, they observed the same suppression of REMS in the light period with a reciprocal increase of REMS in the dark period that we observed. This would suggest that perhaps REMS is more sensitive to the amount of alcohol (observed in both studies when 6% in liquid diet was used for either 3 weeks or 6 weeks) but that changes in SWS require longer treatments (decreased SWS in the light period was only observed after 6 weeks of 6% alcohol in liquid diet). In further agreement that REMS is more sensitive to the amount of alcohol given, Rouhani et al. (23) reported a decrease of REMS in the light period after 2 weeks when alcohol (10 g/kg/day) was given by gastric infusion. This is an exposure level very similar to our 6% in liquid diet. Rouhani et al. also observed increases in wake with loss of SWS, but only in the light period, again similar to our observations with 6% alcohol in liquid diet. On the other hand, Rouhani et al. did not observe any changes in sleep parameters in the dark period. Perhaps the failure of Rouhani et al. to observe changes in the dark is due to the nature of the schedule infusion of alcohol (equal volumes given at 4-5 hour intervals across the day to maintain a constant exposure level across the day) rather than allowing the rats to consume alcohol more in tune with their normal circadian patterns (the vast majority of drinking in our protocols (estimated at >80%) was during the dark period (unpublished observation).

These observations suggest that allowing the animals to consume alcohol on a more natural schedule may be important in observing changes in a behavior that has a strong circadian entrainment. While controlled gastric infusion and alcohol vapor may be very effective means to get rats dependent on alcohol in a relatively short period of time, these may not be ideal protocols for studies of alcohol on sleep. Indeed, our protocols may suffer from the mixing of alcohol with nutritive intake or water intake. Thus the motivation to consume alcohol is mixed
with other motivational drives, and may result in a somewhat unnatural pattern of alcohol consumption. This issue remains to be tested in future studies.

Another possible weakness in our study was that we did not get as close of a match as we had wanted between levels of alcohol consumption and the two delivery vehicles. This weakness will always be a potential confounding influence when the rats are allowed to self regulate their consumption levels. However, because the alcohol consumed in the two alcohol in water protocols bracketed the alcohol consumed in the 3% alcohol in liquid diet protocol, and for the most part the effects on the sleep patterns were similar, we are somewhat confident that we can conclude water or food delivery does not have a major impact on the nature of the changes, it is just easier to get more alcohol in the animals when the alcohol in mixed with food. On the other hand, perhaps if the exposure protocols where to run for longer than 6 weeks, we might be able to tease apart differences in the nature of the alcohol effect on sleep when given in water or food. This issue remains for future study.

A final issue with our study is that we did observe significant differences in rate of weight gain between our groups, and we did observe increased SWS in our pair-fed controls to the 6% alcohol in liquid diet as compared to the other two control groups (ad lib control and pair-fed to 3% in liquid diet). The fact that the ad lib control and pair-fed to 3% alcohol in liquid diet had no differences in their sleep while they gained weight at significantly different rates, suggests rate of weight gain in and of itself does not account for any of the effects we observed. A possible reason for the increased total SWS in the pair-fed controls to the 6% alcohol in liquid diet could be that others have found that food restriction, specifically when access to food is restricted to just the dark period, does increase SWS in the dark period (2). While the degree of decreased food consumption in our study is no where near that in the Alvarenga et al. study (2),
the changes we observed in this control group relative to the others was also more modest than that observed by Alvarenga et al. In spite of this change in the control group behavior, the alcohol treatment led to an even further increase in SWS in the light period. Because the 6% alcohol in liquid diet and their pair-fed controls consumed the same amount of food, we are confident that the change in SWS between these groups can be contributed to alcohol treatment and not a decrement in food consumption relative to the animals that had *ad lib* access to food and water.

The connection between disturbed sleep patterns in sober alcoholics and propensity to relapse drinking remains unknown. It could be related to the decrement in performance that accompanies poor sleep contributes to psychological and/or social pressures to begin drinking again, or that recovered alcoholics with insomnia actually return to drinking as a form of self-medication (6), but scant support exists for either of these hypotheses. A number of investigators have begun to incorporate strategies for improving sleep in abstinent alcoholics with the hope that this will decrease relapse drinking (1, 3, 12, 17, 18, 25, 28). Obviously, improvements in this potential therapeutic intervention could be made if a better understanding of alcohol induced sleep pathologies was available. If an animal model is to be useful in uncovering the mechanistic basis for sleep pathologies associated with alcohol consumption, then the animal model should demonstrate similar pathologies to that observed in humans. With this perspective it is interesting to note that in the animal studies of chronic alcohol consumption, especially at the higher exposure levels, there is a loss of REMS and SWS in the rest period. This is similar to the loss of REMS and insomnia complaints of chronic alcoholics (6). It is also worth noting that in the present study we found that an alcohol exposure of at least 6 weeks was required to observe a more fully elaborated spectrum of sleep alterations. Given that human alcoholics may
be abusing alcohol for decades prior to starting abstinence; it is possible that even 6 weeks of exposure in an animal model is too short to fully appreciate the effects that alcohol has to disrupt sleep. Thus we conclude that studies in this area should use a minimum exposure of at least 6 weeks in duration at a level of alcohol that is equivalent to 6% alcohol in liquid diet (or 11.5 g alcohol/kg body weight/day), and that future studies are warranted that further explore the relationship between amount of alcohol consumed and the length of time of exposure to the nature of the sleep pathologies produced.
Average weight gain (in g) per day from rats in the various treatment groups. Weight gains were monitored after at least one week in treatment over a period of 3 weeks. Values are averages ± SE (N = 6 to 8 rats in each group). Bar labeled Con is the ad lib food and water control, the bars labeled 12% and 6% are from the rats treated with 12% and 6% alcohol in water, and the bars labeled PF and ET are from the pair-fed and alcohol in liquid diet treated groups, respectively. The asterisk indicates significantly different from all other groups (p<0.01, ANOVA).
Fig. 2

A. SWS total min
B. Wake total min
C. SWS total min
D. SWS total min
E. REMS total min
F. REMS total min

Alcohol treatment in water
3% alcohol in liquid diet 6% alcohol in liquid diet

Con 12% 6% Con 12% 6%
Con 12% 6% Con 12% 6%
Con 12% 6% Con 12% 6%
Con 12% 6% Con 12% 6%
Con 12% 6% Con 12% 6%
Figure. 2  Total time spent in different vigilance states (wake, SWS – slow-wave sleep; REMS – rapid-eye-movement sleep). In this and other figures the shaded panel denotes the dark period and the unshaded panel denotes the light period. All values are expressed as total min within the period. Light period values, recorded for only 10 h, have been multiplied by 1.2 so they can be directly compared to dark period values (recorded for 12 h). The results from the animals treated with alcohol in water are shown in the left hand panels (A, C, and E). The solid black bars (labeled Con) are from the ad lib control animals, the light gray bars and dark gray bars are from the 12% alcohol and 6% alcohol in water groups, respectively. ET denotes alcohol treated animals and PF denotes pair-fed control animals in this and all other figures. Asterisks indicates that the value is significantly different from control (p<0.05 by ANOVA with Holm-Sidak post hoc comparisons). The results from the animals treated with alcohol in liquid diet are shown in the right hand panels (B, D, and F). Asterisks indicate significantly different from the adjacent PF value (p<0.05, non-paired t-test). Pound signs indicate significantly different from the control recordings from other groups (compare solid bars within shaded or within unshaded panels, p<0.05, ANOVA).
Variation of slow-wave amplitude (SWA) in alcohol in water treated animals (top panel), in 3 % alcohol in liquid diet group (middle panel), and 6% alcohol in liquid diet group (bottom panel). The shaded left most panels show SWA of 1st 2 h of the dark period. The unshaded middle panels show SWA in 1st 2 h of the light period, and the right most unshaded panels show SWA in the last 2 recorded hours of the light period (hours 8-10 of the light period). SWA amplitude is compared within a group across time. The peak of the SWA occurs during the first 2-h block after lights on (first long sleep period) in all animals except the animals treated with 6% alcohol in liquid diet. Comparisons within treatments made by one way ANOVA (asterisks indicate p<0.05 compared to other time periods).
Table 1. SWS and REMS episode durations and number of episodes when alcohol was administered in water.

<table>
<thead>
<tr>
<th>parameter</th>
<th>episode duration (sec)</th>
<th>number of episodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dark period</td>
<td>light period</td>
</tr>
<tr>
<td>SWS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control:</td>
<td>61.9 ± 3.9</td>
<td>95.3 ± 5.0</td>
</tr>
<tr>
<td>12% alcohol:</td>
<td>76.9 ± 2.5*</td>
<td>115.6 ± 6.0</td>
</tr>
<tr>
<td>6% alcohol:</td>
<td>73.8 ± 4.6</td>
<td>110.4 ± 10.4</td>
</tr>
<tr>
<td>REMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control:</td>
<td>67.8 ± 3.8</td>
<td>88.7 ± 3.9</td>
</tr>
<tr>
<td>12% alcohol:</td>
<td>58.0 ± 3.3</td>
<td>84.9 ± 3.2</td>
</tr>
<tr>
<td>6% alcohol:</td>
<td>58.7 ± 5.0</td>
<td>94.0 ± 5.7</td>
</tr>
</tbody>
</table>

Episode duration of SWS or REMS are the average duration of individual SWS or REMS episodes within dark or light periods, while number of episodes is the average of the total number of episodes for each individual animal in the respective periods. Dark period episode numbers are over 12 hours whereas light period episode numbers are for 10 hours. Values are averages ± SE; N = 6 for ad lib controls, N = 6 for animals treated with 12% alcohol, and N = 7 for animals treated with 6% alcohol. Asterisks: significantly different from the ad lib control (*p<0.05; one way ANOVA).
Table 2. SWS and REMS episode durations and number of episode when alcohol was administered in liquid diet.

<table>
<thead>
<tr>
<th>parameter</th>
<th>episode duration (sec)</th>
<th>number of episodes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dark period</td>
<td>light period</td>
</tr>
<tr>
<td>3% alcohol in liquid diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair-fed</td>
<td>63.7 ± 6.3</td>
<td>90.0 ± 4.4</td>
</tr>
<tr>
<td>Alcohol treated</td>
<td>83.5 ± 7.4</td>
<td>92.8 ± 14.7</td>
</tr>
<tr>
<td>REMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair-fed</td>
<td>68.9 ± 7.2</td>
<td>83.7 ± 4.8</td>
</tr>
<tr>
<td>Alcohol treated</td>
<td>67.8 ± 6.1</td>
<td>85.2 ± 4.7</td>
</tr>
<tr>
<td>6% alcohol in liquid diet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair-fed</td>
<td>70.1 ± 9.3</td>
<td>108.0 ± 10.5</td>
</tr>
<tr>
<td>Alcohol treated</td>
<td>99.8 ± 5.2*</td>
<td>113.3 ± 9.4</td>
</tr>
<tr>
<td>REMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pair-fed</td>
<td>57.6 ± 6.1</td>
<td>86.0 ± 4.5</td>
</tr>
<tr>
<td>Alcohol treated</td>
<td>85.7 ± 6.2*</td>
<td>81.2 ± 5.6</td>
</tr>
</tbody>
</table>

Episode duration of SWS or REMS are the average duration of individual SWS or REMS episodes within light or dark periods, while number of episodes is the average of the total number of episodes for each individual animal in the respective periods. Dark period episode numbers are over 12 hours whereas light period episode numbers are for 10 hours. Values are averages ± SE; N = 6 for 3% alcohol treated animals, N = 5 for pair-fed to 3% alcohol treated animals, N = 8 for 6% alcohol treated animals, and N = 6 for pair-fed to 6% alcohol treated animals. Asterisks: significantly different from the pair fed control (*p<0.05; non-paired t-test).
ACKNOWLEDGEMENTS

This work was supported by National Institute of Alcohol Abuse and Alcoholism grant No. 13248 awarded to S.M.S.
REFERENCES


Chapter 3 Novel Analysis of Sleep Patterns in Rats Separates Periods of Vigilance Cycling from Long Duration Wake Events

Steven M. Simasko and Sanjib Mukherjee

Program in Neuroscience, Department of VCAPP, Washington State University, Pullman, WA 99164-6520

Behav Brain Res. 2008 (in press).
ABSTRACT

Rats are polyphasic sleepers. However, a formal definition of when one sleep episode ends and another begins has not been put forth. In the present study we examine the distribution of wake episode durations and based on this distribution conclude there are multiple components of wake. If the wake episode exceeds 300 sec the wake episode is assigned to long duration wake (LDW), if the episode is less than 300 sec it is assigned to brief wake (BW). Further support for this separation was found in close analysis of the EEG power spectrum in BW versus LDW. We then used LDW episodes to separate one sleep episode from another. We term the sleep episodes vigilance cycling (VC) because the rat is cycling between the vigilance states of brief wake (BW), slow-wave sleep (SWS), and rapid-eye movement sleep (REMS). We find that the characteristics of VC are different in the light period versus the dark period. We further find that as VC episodes progress, SWS pressure lessens, but the amount of time spent in REMS increases. These findings suggest that VC episodes are regulated and meaningful to the sleep behavior of rats. The use of the concepts of LDW and VC provides additional insights into the description of sleep patterns in rats that may be important in the development of a complete description of sleep behavior in this animal.

Key words: power-law distribution, exponential distribution, brief wake, slow-wave sleep, rapid-eye movement sleep, arousal
INTRODUCTION

A common animal model in sleep research is the rat. An aspect to the rat as a model for sleep is that they are polyphasic sleepers. This creates several issues when studying sleep in rats. For example, neurobiological models of the control of sleep must account for the kinetic patterning of vigilance states and how they change at different times of the day. In addition, when comparing the sleep pattern in rats to humans, typically a monophasic sleeper with one ~8 h sleep session per day, the factors that control the length of sleep episodes are likely to be similar but executed in a different manner. Further, in humans a common unit that is measured is a sleep cycle which involves the time from one rapid-eye movement sleep (REMS) episode to another (REMS-REMS cycle). However, in rats, vigilance states frequently cycle through slow-wave sleep (SWS) to wake without REMS episodes. This limits the usefulness of REMS-REMS cycle as a parameter for sleep in a rat. An important parameter to help fully describe the polyphasic pattern in a rat that has not been defined is when a sleep episode begins and ends.

When rats sleep they cycle through vigilance states of wake, SWS, and REMS. However, close examination of the wake episodes in these animals reveals that many of these wake episodes are relatively brief and interspersed among SWS and REMS episodes, and some of these wake episodes are of a long duration without disturbances of SWS or REMS. Although these long wake periods are few in number, they can account for significant amounts of time spent in wake, even in the light period. Like rats, other animals, including humans, have brief wakenings during their sleep periods. Lo et al. [15] found that the distribution of wake episode durations of brief duration followed a power law with similar coefficients for humans, cats, rats, and mice. This is in contrast to sleep times (REMS plus SWS) uninterrupted by wake, which follow an exponential distribution which differ among animals [15]. However, to our knowledge
a definition of what separates brief wake (BW), that which occurs among SWS and REMS episodes, from long-duration wake (LDW), that would separates one sleep episode from another, has not been put forth. Defining this parameter would enable study of the factors that regulate this sleep period in rats.

In the present study we present an argument that durations of BW and LDW separate from each other at ~300 seconds. Using this definition we show that sleep patterns in rats can be separated into periods of LDW, and between LDW is a period we have termed vigilance cycling (VC) since the animal is rapidly cycling between SWS, REMS, and BW. We find that the pattern of VC is different in the light period and dark period, and further, that SWS pressure decreases, but REMS pressure increases, as the VC period continues.

METHODS

Subjects. To be able to have sufficient number of observations of any rare events (e.g., long wakes in the light period), we used recordings from a relatively large sample size (26 male Sprague-Dawley rats ~350 g at the start of the measurements). These recordings had been collected over a two years period of time as control values in various projects. The rats were housed individually at 22 ± 2 °C with a 12h:12h light:dark cycle. The treatment of the rats was done with the approval of the Institutional Animal Care and Use Committee of Washington State University.

Instrumentation for sleep recordings. At least one week prior to any sleep recordings the animals were instrumented by standard procedures as previously reported [10]. Briefly, under ketamine (87 mg/kg) and xylazine (13 mg/kg) anesthesia, 3 EEG electrodes (Plastics One, Roanoke, VA) were implanted, one in the left frontal (5 mm A and 2 mm L, from bregma), one
over the right parietal (-5 mm A and 6 mm L, from bregma), and a ground electrode over the left occipital cortex (-11 and 4 mm L, from bregma). One EMG electrode was implanted in dorsal neck muscle.

Sleep recordings. During the period of data collection sleep recordings were made in enclosed environmental chambers (4 rats in individual cages within a chamber, each chamber ~6 ft X 2 ft x 6 ft; width x depth x height) with constant room air flow through the chamber. The interior of the chamber was illuminated by a 15 watt light (12h: 12h light:dark cycle) and maintained at 22 ± 2 ºC. Rats were habituated to the chambers for at least 3 days before starting sleep recordings. Any animal care was performed during the last hour of the light period, and we routinely do not include any analysis from this period of time because of the interruption of the records and disturbances associated with animal husbandry.

Collection and analysis of EEG and EMG signals. The EEG (electroencephalogram) and EMG (electromyogram) signals were collected through a wire harness tethered to an overhead multi-channel commutator (Plastics One, Roanoke, VA). The signals were amplified by polygraph amplifiers (Grass-Telefactor, Inc., West Warwick, RI) and digitized at 128 Hz through a computer software program (SleepSign for Animals, Kissei America Inc.). The EEG/EMG recordings were analyzed with either 4-, 8-, or 12-sec epochs and each epoch was assigned to a particular vigilance state: wake, SWS, or REMS by standard criteria embedded in the software program. All scoring was visually inspected to ensure correct assignment of vigilance states and to eliminate any epochs with artifact noise. In addition to assigning vigilance state, the software program enabled use to analyze the EEG waveforms by fast Fourier Transform (FFT) within each epoch so that we could examine the power of the component frequencies within the EEG.
**Curve fitting.** Histograms of the probability density and cumulative probability density of wake episode durations for the light period and dark period were generated from the pooled results from all 26 animals. In the main analysis (8-sec epochs used for scoring sleep records) binning for the histogram was in 8 sec increments. When 4 or 12 sec epochs were used for scoring, histogram binning was in 4 or 12 sec increments, respectively. Probability density distributions were then determined by dividing the number of observed events in anyone bin by the total number of observed events. The cumulative probability density was determined by totaling all events equal to or greater than the bin duration, and then dividing that number by the total number of observed events (thus in the cumulative probability density the first bin in the distribution had a value of 1.0).

Long wake events in the cumulative probability density (cumPD) plots were fit to an exponential function:

\[ \text{cumPD} = \text{amplitude} \times \exp\left(-\frac{X}{\tau}\right) \]

where \( X \) is the event durations, amplitude is the amplitude of the cumPD at the y-axis, and \( \tau \) is the time constant of the fit. Both the amplitude term and \( \tau \) were allowed to vary during the fits. For brief wake episode durations a plot of the probability density (PD) of the wake episodes were fit to a power function:

\[ \text{PD} = \text{amplitude} \times X^\alpha \]

where \( X \) is the event durations, amplitude is the amplitude of the PD at the y-axis, and \( \alpha \) is the power coefficient. The amplitude term and the power coefficient \( \alpha \) were allowed to vary in the fits. Fits were evaluated by TableCurve 2D software (SYSTAT Software, Richmond, CA) which used a non-linear least squares procedure (Levenburg-Marquardt algorithm). Fits were evaluated
by visual inspection, and the statistical parameters returned by the fitting program (correlation coefficient, standard error, and F statistic).

**Statistical Analysis.** Paired t-tests were used to compare light period derived values to dark period derived values. However, because of the one hour period reserved for animal care, and because we express time series data in 2-h blocks, there was only 10 hours (five 2-h blocks) of light period recording vs 12 hours (six 2-h blocks) of dark period recording. Thus for some comparisons, normalized values (either expressed as a percent or the light period value normalized to 12 hours) were used. Paired t-tests were also used in the analysis of first quarter parameters versus fourth quarter parameters within vigilance cycling periods. To compare the distribution of vigilance states when different epoch durations were used in analysis of the sleep records, we used a one-way analysis of variance with repeated measures at each time point. To compare the power of frequency components in the EEG we used a two-way repeated measures analysis of variance (factors of frequency and period of data collection). All analysis of variance (ANOV) were performed with SigmaStat version 3.5 software (SYSTAT Software, Richmond, CA), and t-tests were performed with Microsoft Office Excel 2003.

**RESULTS**

**Sleep patterns in rats.** Hypnograms from two representative sleep recordings are shown in Fig. 1. In the dark period the rats have many brief periods of sleep activity (SWS with some REMS) separated by long periods of wake. Within the sleep periods are many brief wakenings. In contrast, in the light period, the number of periods in which the rat remains awake for extended periods of time is greatly reduced. In the periods between these few long wake periods the rats cycle relatively rapidly through SWS, REMS, and brief periods of wake. In subsequent
discussion of the results, the long periods of wake between periods of sleep activity will be referred to as long-duration wake (LDW), the brief periods of wake within sleep activity will be referred to as brief wake (BW), and the periods of sleep activity separated by LDW episodes will be referred to as vigilance cycling (VC).

**Separation of BW from LDW.** If one wants to analyze the characteristics of VC in the records, the critical issue is to determine a length of time when a wake episode shifts from BW to LDW. To accomplish this we examined the distribution of wake episodes when results were combined from all 26 rats (Fig. 2). (See supplementary Fig. S1 for a summary of vigilance state distributions with time of day for this group of rats.) We worked with a combined data set because some events (e.g., LDW in the light period) were relatively rare in any one rat (2-4 times during a light period) to make conclusions drawn from such few observations relatively unstable (e.g., curve fitting equations to two points). When the distribution of wake episode durations were expressed as a cumulative probability density, and the results plotted on a semi-logarithmic scale, the distribution clearly shows a biphasic nature (Fig. 2A). This was true in both the dark period and the light period. In order to separate the two distributions of wake durations, we fit the long events to an exponential starting at events 500 sec in duration or longer, thus concentrating on the distribution of long duration events at a time that the distribution of the brief events would be complete. The time constants ($\tau$) of the fits to the distribution of long wake events were similar in both the dark period and light period (~1000 sec). The fit to the distribution of the light period was not as good as that to the dark period. The deviation from the fit in the light period occurs mainly at extremely long periods of time were events are very rare. In addition, the logarithmic plot tends to exaggerate the magnitude of the deviation of the fit when the absolute y-value is very small.
To fit the distribution of the brief events we expressed the results as a probability density and plotted the results on a log-log scale (Fig. 2B). We did this in order to be able to directly compare our results with those of Lo et al. [15] who found a power function best fit the distribution of the probability density of brief wake episode durations in mice, rats, cats, and humans. We also restricted our fits to the data from 24 sec to 360 sec. The reasons for this restriction are that this is the range that Lo et al. fit their results, and the 24 sec time point is the first common time point in the analysis when we used different epoch durations for analysis of the sleep records (see below). As is seen (Fig. 2B) the distribution of brief wake episodes are linear on the log-log plot over this range and the coefficients of the fits (-2.29 in the dark period and -2.38 in the light period) are in excellent agreement with each other and with the results of Lo et al. [15] (their coefficients ranged from -2.0 to -2.3). Also, as in the results of Lo et al. [15], the episode durations less than 24 sec fall below the line of the fit, and the durations greater than 360 sec lie mostly above the line of the fit.

We used these results to select a time point that would serve to separate BW from LDW. An expansion of the fit to the cumulative probability density (Fig. 2C) reveals that the data begin to deviate from the fitted line starting around 300 sec, so in the subsequent analyses we selected 300 sec as the time point that separates BW from LDW. Using this separation time we have divided the state of the rat into periods of LDW and periods of VC (the cycling that occurs between LDW episodes). The resulting analysis is shown in Fig. 1 as the lines drawn above the hypnograms for the individual rats (lines indicate periods of VC). In some instances, two periods of LDW would be separated by a single SWS episode (e.g., Fig. 1, top hypnogram, period of time between 4 and 5 hours). We analyzed the results either by allowing such brief sleep episodes to separate LDW or not separate LDW, and the results of the fits to the wake
episode durations were barely affected (the distribution of the cumulative probability density was still biphasic on the semi-logarithmic plot with time constants of fits to the longer wake events shifted by 50 – 100 sec, with virtually no change in the power coefficients in the fits to the probability density; data not shown). Thus we decided to ignore these brief episodes of SWS with the rule that if no cycling occurred (only a single SWS event), then the LDW episode was considered as a single episode. The reason this rule had minimal effect is that on average, this rule caused us to eliminate only one event per rat in the dark period, and only one event in all 26 rats in the light period. However, eliminating these microsleep events provided more consistency to the average characteristics of VC episodes because the brief sleep episode was not included in the final averaged values for the characteristics of VC (i.e., these few brief episodes were outliers; data not shown).

**Characteristics of LDW and VC.** Summarized in Table 1 are the characteristics of LDW and VC when 300 sec is used as the cut-off between BW and LDW. In the light period there are fewer LDW episodes with a much smaller percent of time spent in LDW. Interestingly, the average duration of a LDW was independent of light or dark period. In contrast to the similar length of LDW episodes in the light and dark periods, VC episodes in the light period are extremely long compared to the dark period. There are also fewer number of VC episodes in the light period. In spite of the drastically reduced number of VC episodes in the light period, the extremely long nature of the VC episodes in the light period caused the percent time spent in VC to double in the light period compared to the dark period.

The characteristics of the vigilance states within periods of VC can also be analyzed (Table 1). In the light period we found that the average episode duration for both SWS and REMS within VC significantly increased relative to the dark period. Remarkably, the average duration
of BW episodes was independent of the light versus dark period. On the other hand, the percentage of time that the animal spent in BW during VC was significantly greater in the dark period. Also, the percentage of time spent in REMS was greater in the light period, but the percentage of time in SWS was only slightly different between the light and dark periods. Thus the characteristic of VC in the light period (the major rest time for rats) are that the VC episodes are relatively long, the average duration of individual SWS and REMS episodes are also relatively long, the percentage of BW is less, and the percentage of REMS is greater. These observations are all consistent with the VC events in the light period having a more intense nature of sleep than those that occur in the dark period.

We have also explored the data set using separation times briefer or longer than 300 sec. When a time less than 300 sec was used (100 sec) the periods of VC became highly fragmented (data not shown). The reason for this is that in any individual rat the number of wake episodes that occur that are less than 300 sec begins to increase at rapid rate as the time is shortened, thus producing frequent breaks in the VC periods even in the light period (data not shown). Thus we conclude that 300 sec is a minimal time for the separation. On the other hand, we have explored the results with a separation time as long as 500 sec and on the whole we obtain results very similar to those found with the 300 sec separation time (data not shown). The stability of the result when using the larger separation time results from the relatively few number of events that are actually observed between 300 and 500 sec in the record of any one rat, and thus the longer separation time removes at most two to four events from the record (e.g., in the upper hypnogram of Fig. 1 the relative short LDW events at 3-4 h period, around 6 h, and between 15-16 h would be eliminated and in the lower hypnogram the LDW events at around 4 h, 8 h, 10 h, and 20 h would be eliminated). Elimination of these few events has only minor effects on the
average characteristics of the VC (a slight reduction in number but a slight lengthening of
duration; data not shown).

**Alternative primary analysis parameters.** An issue that arises from this analysis is whether
the results we observe are dependent on the specific criteria by which we analyzed the raw sleep
records. One such issue is the duration of the epoch used in the analysis of the raw sleep records.
To investigate this issue we reanalyzed the records of the animals in the dark period with 4 and
12 sec epochs (see supplementary Fig. S2 for the effect of changing epoch duration on the
distribution of vigilance states with time), and then subjected the resulting distribution of wake
episodes to the same curve fitting routine as for the results with the 8 sec epoch (supplementary
Fig. S3). We found in each case that the distribution still had a biphasic nature in the semi-
logarithmic plot and that the time constant of the long duration events where very similar
(supplementary Fig. S3A; brief single SWS events surrounded by LDW ignored as described
above). The fit to the power function of the probability density was also similar (supplementary
Fig. S3B). Finally, as with the results with the 8 sec epoch, the cumulative probability density
began to deviate from the fit to the exponential equation at ~300 sec, thus reinforcing the
conclusion that a 300 sec cut-off was a good value to separate BW events from LDW
(supplementary Fig. S3C).

Another factor we examined was manipulation of short events that fell off the line of the
power fit (Fig. 2B). To eliminate these short events from the record we invoked a three-epoch
rule in which to register as a state transition, the new state had to be stable for at least 3
consecutive epochs (or 24 sec in the 8 sec epoch data set). If a new state did not last at least
three epochs, the epoch(s) in question was assigned the state of the preceding epoch. As with the
re-analysis with different epoch durations, the inclusion of the 3-epoch rule did not significantly
alter the results of the analysis (supplementary Fig. S4). The cumulative probability density still was biphasic with a time constant for the LDW events of ~1000 sec (supplementary Fig. S4A); the BW events were still fit by a power distribution with a power coefficient of approximately -2 (supplementary Fig. S4B); and the brief events began to deviate from the exponential fit at around 300 sec (supplementary Fig. S4C). Further, we analyzed the resulting pattern of VC and LDW using the records scored with the 3-epoch rule (see Supplementary Table S1) and the results of the analysis are virtually indistinguishable from the analysis of the data when a single 8 sec epoch was used except that the average durations of BW, SWS, and REMS episodes are longer, as would be expected if the numerous 8 and 16 sec events are ignored (37%, 43%, and 77% of all wake events were <24 sec for the 8-sec, 12-sec, and 4-sec epoch scoring, respectively). Thus the distribution of wake events into BW and LDW, with a break point of 300 sec (or 500 sec) is independent of a number of arbitrary parameters used to analyze the raw sleep records. This demonstrates that the division of BW and LDW is a biological phenomenon and not an artifact of the arbitrary selection of parameters used in the analysis.

**EEG of wake events versus REMS.** We next examine the EEG behavior of BW and LDW. We first wanted to establish that the EEG power spectrum of either BW or LDW was not similar to REMS. We compared the distribution of the power in the frequency bands 1-25 Hz in episodes assigned to BW and LDW compared to those from episodes assigned to REMS (Fig. 3A and 3B). As can be seen, epochs assigned to REMS had a relatively high power in the theta band (5-8 Hz) and relatively low power in the delta band (1-4 Hz) compared to either of the two components of wake. Thus both components of wake were clearly different from REMS.

**EEG of BW versus LDW.** Up to this point we have separated BW from LDW only based on the kinetic distribution of wake episode duration. While such a distinction suggests that the
neural networks that underlie the stability of BW and LDW are different, it is also possible that BW and LDW differ in other ways, such as the characteristics of the EEG. However, comparison of the EEG in between BW and LDW is complicated by the unknown relationship between BW and LDW. For example, it is not known if an animal always wakens into BW and then transitions into LDW, or whether the animal can enter directly into LDW from SWS or REMS. To address these issues we first compared the average EEG of all BW epochs to LDW epochs when LDW epochs less than 300 sec were excluded from the analysis. If BW precedes LDW, this would eliminate any contamination of the average EEG from LDW epochs by BW epochs that occur early in the LDW episode. By this approach we found that the EEG of BW had significantly greater power in the frequency bands from 1-16 Hz (Fig. 3A-D).

Since this analysis demonstrated subtle differences between the EEG of BW and the EEG of LDW, we could then address the issue as to whether a BW episode precedes a LDW episode by examining the EEG power in epochs at the beginning of LDW episodes. We collected the power of the frequency bands from each progressive epoch starting with the first epoch of a BW or LDW episode, and then combined the values into groups that included epochs 1-3, 4-6, 7-12, 13-18, 19-24, 25-30, and 31-36, thus covering the entire 300 sec period of BW episodes and the portion of the LDW episodes excluded in the prior analysis (results were also segregated into light period and dark period). To determine if the power spectrum of the EEG throughout the 300 sec period were similar within BW and LDW, we compared the power spectrum of the 1-3 epoch group to all successive groups within BW or LDW. For BW in both the light and dark period, we found that in epochs 1-3 the EEG power spectrum was elevated compared to subsequent groups primarily in the 1-13 Hz range (supplementary Fig. S5). For LDW we found that epochs 1-3 had higher power primarily in the 4 to 12 Hz bands (supplementary Fig. S6), but
only in the dark period. For LDW in the light period the 1-3 epoch group was not different from other groups, but because there are so few LDW episodes in the light period, the data are very noisy, and thus any conclusion from this observation must be interpreted with caution. These findings demonstrate that the EEG power spectrum in the 1-3 epoch group (the first 24 sec of the episodes) is different from all subsequent times within BW or LDW episodes.

We next compared epochs 4-6 with all subsequent groups within BW and LDW. In the dark period we found, with the exceptions of few frequencies here and there, that there were no differences between the power spectrums from the 4-6 epoch group and subsequent epoch groups (supplementary Figs. S7 and S8). In the light period the 4-6 epoch group did show some minor differences compared to subsequent groups in both LDW and BW (supplementary Figs. S7 and S8), but when the 7-12 epoch group was used as the bases for comparison, the differences in BW were no longer significant, and the differences in LDW were not different with the exception of a few individual frequencies in some groups (data not shown). These results demonstrate that once the 24 sec period at the beginning of an episode has been passed (perhaps a little longer than 24 sec in the light period), the EEG power spectrum was stable and constant within a BW or LDW episode.

We next compared the EEG power spectrum of all groups between BW and LDW (supplementary Fig. S9). We found that the BW groups had higher power components for every comparison similar to that found when we compared all BW episodes to all LDW episodes when LDW episodes that occurred less than 300 sec from the beginning were excluded (Fig. 3A-3D). These findings demonstrate that the animals did not pass through a BW episode at the beginning of a LDW episode, but rather entered directly into a LDW episode from the prior sleep period.
An additional parameter we examined was whether there are any differences between BW and LDW in bands >25 Hz. Up to 25 Hz the power was greater in BW compared to LDW, although this was not statistically significant above ~16 Hz (Fig. 3A-3D). This pattern (BW>LDW) continued into high frequency bands up to ~30 Hz where the power in LDW began to systematically exceed that found in BW, although the differences were not statistically significant (supplementary Fig. S10).

A final parameter we examined was whether the power spectrum of the EEG was different in BW between light and dark periods. We found that there were differences at a few frequencies, but that these did not appear systematic but rather seemed more likely to be due to random fluctuations (BW in light had more power at 1 and 6 Hz but BW in the dark had more power at 8 and 9 Hz; supplementary Fig. S11). This finding, along with the prior finding that BW episode durations are similar in the dark and light period (Table 1), suggests that BW is similar regardless of its occurrence in the dark period or light period.

Sleep pressure during VC episodes. Finally, we hypothesized that if VC events are a type of sleep unit for the rat, there is likely to be changes in the characteristics of the SWS or REMS events over the course of an individual VC event (similar to the decreased depth of SWS and increase REMS that occurs in humans as the sleep period progresses during the night). To examine this issue we analyzed the average duration of BW, SWS, and REMS events, and the percent time spent in each vigilance state, as well as the power in the EEG spectrum during SWS episodes over the course of individual VC events. For BW, SWS, and REMS event durations we restricted the analysis to the first quarter and fourth quarter of VC events. Further, we restricted our analysis to VC events of at least 2000 sec in duration for two reasons. First, for us to be able to evaluate individual episodes durations, we wanted each quarter we analyzed to contain at least
3 episodes for the averaged value, and with the 2000 sec event duration this held true for SWS and BW, but REMS episode numbers frequently were less than 3 (this also forced us to not analyze REMS-REMS cycle). Second, we reasoned that for there to be a significant shift in these characteristics enough time had to elapse so that sleep intensity could lessen over the VC, and this was unlikely to occur in brief VC events. The 2000 sec event duration limitation forced us to ignore almost all events in the dark period, so we only show analysis from events in the light period. Further, the 2000 sec event limitation forced us to drop, on average, about 1-2 events from the light period record of each rat, but these shorter events only represented 1.8 ± 0.5% of the total time spent in VC.

We found that the average duration of SWS episodes and the percent of time spent in SWS decreased in the fourth quarter of the VC events compared to the first quarter of the VC events (Fig. 4, top set of panels, black bars). We also found that the percent of time spent in REMS increased in the fourth quarter versus the first quarter of VC episodes (Fig. 4, middle set of panels, black bars). We also found that the duration of BW episodes decreased in the fourth quarter versus the first quarter, but that the percent of time spent in BW did not change (Fig. 4, bottom set of panels, black bars). Finally, we found that the power of the slow wave components during SWS episodes were smaller in the fourth quarter versus the first quarter, whereas the high frequency components were not different (Fig. 5). We extended the analysis of the power spectrum to include the second and third quarter periods and found that there was a smooth progression towards lower power in the 1-7 Hz bands as time in VC progressed (supplemental Fig. S12). These findings; shorter SWS episodes, less percent of time spent in SWS, and less power in the slow wave components of the EEG spectra during SWS, all support the idea that sleep pressure is lessened at the end of a VC compared to the beginning of a VC. Further, just as
in human sleep at the end of the night, the amount of REMS increased at the end of the VC compared to the start of the VC. A noticeable trend in all vigilance states was the tendency for episode durations to decrease as the VC continued. Thus at the end of the VC the cycling between states becomes progressively faster.

A final issue with regard to this analysis is that it is well known that in rats sleep pressure is greatest at the beginning of the light period. Thus it is possible that the trends we observed in our analysis of first quarter versus fourth quarter behavior within VC events is mostly due to the high sleep pressure that exists at the beginning of the light period (first VC event of the light period), and is not a change observed over individual VC events that occur later in the light period. To examine this issue we reanalyzed the results, only we dropped the first VC event of the light period from the analysis (Fig. 4, gray bars in all panels; Fig. 5, filled triangles in the lower panel). In all cases the trends we observed when all VC events were included remained significant when the first VC event of the light period was dropped from the analysis. In one case, REMS episode duration, the trend toward decreased durations became significant once the first VC event was dropped (Fig. 5, middle left panel). These findings provide further support to our contention that regardless of the time during the light period that a VC event occurs, as the VC event progresses SWS pressure is reduced, REMS becomes more prominent, and the rapidity of cycling increases.

DISCUSSION

Our findings indicate that BW and LDW are different components of wake and a time between 300 and 500 sec can be used to separate them. The distinction between these two components is based upon both the stability of the component in time and the slight differences
in the EEG power expressed within each component. Use of this break point enabled us to describe a sleep unit for rats, VC, that produces an additional descriptor for the polyphasic sleep patterns found in this animal. We also found that the characteristics of VC were different in the light period and dark period, and within individual VC episodes, especially long VC episodes in the light period, we found that SWS pressure waned but the percent of time in REMS increased.

**Multiple components of wake.** Our results also indicate that wake events occur in more modes than the two we have described above. In our analysis events less than 24 sec fell off the fitted power distribution, and these events account for a significant number of the total wake events. In addition, we also found that these brief periods had slight but significantly more power in their EEG compared to other components of wake, especially in the frequency bands between 5 and 15 Hz. Previous investigators have observed ultra-short wakenings (cut-off times from 2 to 16 sec) and they are known to occur in both humans [1, 11] and rats [6, 14]. Whereas some interpret these ultra-short events to be a sign of fragmentation of sleep [1], Halasz et al. [11] interpret these ultra-short events to be preparatory for future vigilance states. Halasz et al. [11] base their conclusion on the observation that synchronized EEG wake events occur in humans descending into deep slow-wave sleep, whereas desynchronized EEG wake events occur when ascending out of deep slow-wave sleep. However, the synchronized/deschronized EEG patterns in these brief wake events observed in humans are more complex than what we have observed in rats.

Multiple components of wake create a potential for confusion when discussing these components. For example, Lo et al. [15] refer to events that align exactly with our BW events as arousal, but Franken at al. [6] refer to ultra-short wake events (8-16 sec in duration) as brief wakes, with Halasz et al. [11] and Lena et al. [14] referring to the brief wake events they
investigate as arousal or micro-arousal, respectively. We selected to call the wake events that fall on the power distribution as brief wake events to avoid confusion with the arousal terminology of investigators that study shorter events; events that are likely to serve a different function than BW [11].

Additional findings that support the separation of arousal from other components of wake are the results of Horner et al. [12] who showed in rat immediately after wakening (3-10 sec) the animal has less pre-pulse inhibition to an audio signal compared to the animal in established wake (>30 sec). These authors concluded that this finding indicates the animal has less filtering of sensory inputs and increased responsiveness to external cues immediately after wakening, and further supports that the state immediately after wakening is neurophysiologically distinct from that which occurs in established wake.

It is of interest to note that when we eliminated these arousal events from the sleep record by use of the 3-epoch rule, there was little effect on the characteristics we determined for BW, LDW, or VC. This finding supports the conclusion that BW and LDW are the critical wake components in the determination of VC events.

**Power-law distribution of BW.** Our finding that brief wakes are well fit to a power-law in the dark period as well as the light period extends the analysis by Lo et al. [15]. Lo et al. concluded that finding a scale-invariant power-law behavior suggested that sleep-wake transitions involves fractal-like phenomena observed in systems undergoing phase transitions or self-organized criticality. However, the power-law distribution arises in rats and mice by P21 [3, 4]. These latter investigators concluded that the basis for the power-law distribution must require additional neuronal circuits that develop as the rat matures and is not present in all circumstances of sleep-wake transitions. Our finding of the power-law distribution in the
circadian phase outside the normal rest period indicates that the power-law behavior is
generalized to both light and dark periods in adult animals.

**BW versus LDW.** Just as with arousal versus other components of wake, the stability and
EEG power for BW and LDW are different from each other. Because the EEG and EMG
patterns exhibited in both BW and LDW are very similar, it seems logical that the same basic
neural activity supports both components of wake. However, the increased stability observed in
LDW implies that a separate neural component must be involved in stabilization of these wake
periods. In support of this idea Mochizuki et al. [17] found in orexin knockout mice that long
wake events were selectively reduced whereas shorter wake events became more plentiful,
indicating that orexinergic neurons are part of the network that supports LDW. On the other
hand, Blanco-Centurion et al. [2], using various saporin constructions to lesion cholinergic,
histaminergic, and noradrenergic neurons, found brief events (<1 min) were greatly reduced but
that long duration wake events were not altered. Furthermore, different lines of inbred mice
have significant differences in the distribution of wake episode durations [7]. These studies
support the idea that there are different underlying neuronal circuits that regulate wake event
durations of different lengths.

As previously mentioned, another distinction we observed between BW and LDW are the
subtle differences in EEG power. While the differences in the EEG are not distinct enough to
assign epochs to different components of wake, they are distinct enough for us to determine that
the animals do not need to transit through a BW episode to enter into a LDW event. We found
that there was higher power in low to mid-frequency bands in the EEG of BW episodes. It is
possible that the higher power in low frequency bands in BW implies that if the animal enters
into wake and SWS pressure has not been completely dissipated, the animal soon falls back into
SWS. However, if SWS pressure has dissipated in the prior sleep period, the animal wakes and enters into a LDW period. This suggestion does not preclude the possibility that an animal could enter into a LDW episode from a BW episode if, during the BW episode, the animal becomes aware of external cues necessitating attention of a long duration.

**Role of LDW and BW in sleep regulation models.** The flip-flop model put forward by Saper and colleagues [20] has been presented as an explanation for the control of sleep-wake transitions. In this model a positive feedback due to mutual inhibition between sleep promoting GABAergic neurons in the ventrolateral preoptic nucleus (VLPO) and several arousal systems produces a bistable situation in which only the states of wake and sleep are stable. However, the study of animals lesioned with saporin constructs to destroy the forebrain cholinergic neurons, midbrain histaminergic neurons, and brainstem noradrenergic neurons has been interpreted as evidence against the flip-flop hypothesis since no enduring changes in the percentage of wake was observed [2]. Our results suggest an alternative explanation for these findings. While these lesions did not produce much loss of overall wakefulness, they did produce a dramatic loss of brief wake events. As alluded to earlier, perhaps the wake components supported by the lesioned areas are either arousal or BW events rather than all wake events. Thus these short wake events were severely altered by loss of these activational systems, but since LDW may involve other neurocircuitry, as long as the animal can activate these additional circuits, LDW episodes will occur. Although LDW are few in number, they account for the majority of time spent in wake in the dark period and a significant portion in the light period. Thus something that eliminates the frequency of BW but not LDW might not be expected to dramatically reduce overall wakefulness in an animal.
Saper and colleagues [5,21] have extended their original flip-flop model to also include a circuit that involves the dorsal medial nucleus of the hypothalamus (DMH). The DMH receives a variety of inputs such as circadian signals from the suprachiasmatic nucleus and feeding cues from the arcuate nucleus, and it projects to the lateral hypothalamus where orexinergic neurons are found [21]. They suggest that the circuitry involved in the DMH enables the animal to coordinate wakefulness with circadian and environmental demands be they visceral sensory, cognitive, or emotional in nature. This would suggest that what we have labeled LDW is stabilized by the output of the DMH.

In support of this interpretation are the results of lesions of the orexin system. Whereas lesions of multiple activational systems resulted in loss of the number of BW episodes with long wake periods relatively intact [2], loss of orexinergic neurons or action results in fragmentation of long wake episodes [8, 17, 22]. Furthermore, orexin knockout mice continue to exhibit power-law behavior, supporting the idea that BW events are not influenced by orexinergic neurons [3]. However, there must be additional circuits beyond orexin that help maintain wakefulness during LDW because even with loss of orexin, animals still have periods of LDW which enable them to interact with their environment. The nature of these additional wake stabilizing mechanisms remain to be identified.

**Purpose of BW and LDW.** While it is clear that LDW episodes are needed to enable the animal to interact with its environment, the need for BW episodes is more opaque. It should be noted that although BW episodes can be as long as 300 sec, the vast majority of these events are less than 100 sec. Lo et al. [15] found that whereas the behavior of BW was invariant from different size mammals, the time constant of the exponential fits to sleep (SWS plus REMS) increased with size of the animal. They suggest that the greater frequency of wakenings
observed in small animals enable these animals to more frequently monitor their environment, which may provide survival advantage in a small prey species. Perhaps it is the arousal period that allows the animal to monitor its environment, consistent with heightened sensory awareness as previously described by Horner et al. [12]. Then depending on the mix of internal (e.g., SWS pressure) and external (e.g., danger) cues, the animal can fall directly back to SWS (wake events <24 sec), or enter into a BW episode in which undissipated SWS pressure eventually causes the animal to fall back into SWS, or, if cues are appropriate (no SWS pressure, hunger, demanding external events, etc.) the animal enters into a LDW episode.

**Vigilance cycling.** The separation of BW from LDW results in the identification of a period between LDW episodes we termed VC, which we suggest is a sleep unit in rat. Support of this distinction was found in the differences between behavior within VC in light and dark periods, and that sleep pressure during the VC episode waned. However, the analysis of sleep pressure within VC episodes was not performed on VC episodes in the dark period and in the light period brief VC episodes were ignored. While these decisions were made for practical analytic reasons, some additional issues are worth considering. The amount of total VC in the light period that occurred within these brief episodes was miniscule (<2%), and these episodes were only ignored in the analysis of sleep pressure within VC, but were included in the calculation of average values for VC in this period. These brief episodes typically occur near the beginning or end of other VC episodes and become separate because of a relatively short LDW event that separates them from the prolonged VC episode that follows or has just ended. Thus they may arise out of an occasional event that has been assigned to LDW but really is a BW event (note that the cut-off is not an absolute, and because of this there is undoubtedly some events that are misclassified).
In regards to VC in the dark period, whether sleep pressure dissipates during each event remains difficult to assess. However, this does not dismiss the idea that these events are without purpose to the animal. Humans show napping behavior in the light period and these sleep episodes, even if very brief, can be refreshing even though it might be difficult to document that sleep pressure waned over the episode [13].

Conclusions. The kinetic analysis of vigilance states presented in this manuscript separates behavioral wake in at least three components; arousal, BW, and LDW, and defines a sleep unit in rats labeled VC. We contend that this unit of sleep is akin to the 8-hr sleep period in humans, and provides another descriptor of sleep patterns in this animal. It should be noted that like rats, humans also have brief wake episodes during their sleep period [15]. Current neurobiological models of sleep and wake are very good at defining the neurocircuits that control sleep-wake transitions; however they have not yet provided details that have the power to explain the kinetic control of these transitions. It is likely that sleep patterns are not generated by mechanisms within the sleep-wake [20] and REMS flip-flop switches [16] but by the actions of influences that impinge on the flip-flop switches [21]. For example, the circadian variation in SWS episode duration is likely due to the balance between sleep homeostatic drive, perhaps mediated by the accumulation of adenosine in the forebrain [19] or cytokines [9], with circadian and environmental cues generated by the hypothalamic integrator in the DMH [21], both of which impinge on the VLPO, which in turn, controls the sleep-wake flip-flop switch [18]. Our analysis provides a step in defining the kinetic behavior that ultimately will need to be accounted for in these models.
Fig. 1

Representative hypnograms from two animals (B3 and G3). The shaded regions in this figure and subsequent figures indicates the dark period. W, S, and R indicate times in wake, slow-wave sleep and rapid-eye movement sleep, respectively. Bars over the state hypnogram at the level indicated by VC are periods that were classified as vigilance cycling (see text for further explanation).
Cumulative probability density and probability density plots of the distribution of wake event durations. Circles illustrate results from the dark period (5,070 total events) and downward triangles indicate events from the light period (4,871 total events). In all plots the light period data is offset (one log unit in A and B, half log-unit in C) on the vertical axis for clarity. In all graphs the filled symbols indicate the data used to fit equations. In A and C an exponential equation was used to fit events >500 sec (the small vertical lines in graph A indicate 500 sec mark), and the solid line through the data points illustrates the fit of the equation to the data. The time constants ($\tau$) from the fits (in sec) are indicated on the graphs. In B a power function was used to fit events from 24 - 360 sec and the line through the data points indicates the fit of the equation to the data. The coefficients of the power term ($\alpha$) are indicated on the graphs. In C (an expansion of the plot in A) the vertical line that intersects the two fits indicates the 300 sec time point.
Spectral power of the component frequencies in the EEG for wake (open symbols) and REMS (filled symbols). In A and B upward triangles are from wake epochs in LDW after 300 sec from the start of the episode, and open circles are from wake epochs in BW. In C and D the power is expressed as a ratio of power in BW over power in LDW (normalized for each individual animal). The dashed line in C and D is for reference indicating equal power. Data are expressed as average ± S.E.; N = 26. The lines and numbers over the plotted data points in C and D indicate the frequencies that are significantly different (p<0.05; pairwise multiple comparisons by Holm-Sidak method).
Comparisons of episode durations for the indicated states (left hand panels) and percent time in the indicated state (right hand panels) for the first quarter of VCs and fourth quarter of VCs. Only results from VC >2000 sec were used. Results are from the light period only. Solid bars include results from all VCs, whereas the gray bars indicates the results after the first VC of the light period was dropped from the analysis (see text for explanation). Results were calculated for each individual rat and then expressed as the average ± S.E.; N = 26. Asterisks indicate significant differences (*<0.05; **<0.005; paired t-tests).
Top panel: Spectral power of the component frequencies of the EEG from SWS epochs in the first quarter of VCs (filled circles) and last quarter of VCs (open circles). VCs were selected as described in Fig. 4. Asterisks indicate significant differences between filled and open symbols (p<0.05; pairwise multiple comparisons by Holm-Sidak method). Bottom panel: Normalization of the spectral power of the component frequencies of the EEG from SWS epochs (power of the first quarter expressed relative to the fourth quarter). Filled circles include results from all VCs, whereas filled upward triangles are results after the first VC of the light period was removed from the analysis. The dashed line is for reference indicating equal power. Asterisks indicate significant differences between first and fourth quarter values for filled circles and # indicates significant differences between first and fourth quarter values for filled triangles (p<0.05; pairwise multiple comparisons by Holm-Sidak method). Values were calculated for each rat and the values expressed as averages ± S.E.; N = 26.
Supplementary Figure S1.

Left hand panels: Distributions of the indicated vigilance states with time. Right hand panels: Time spent in each vigilance state per 2 hours within the dark period and light period. Results are averages S.E.; N = 26. Asterisks indicate significantly different from corresponding light period (** p< 0.0001; paired t-test).
Supplementary Figure S2.

![Graph showing % wake, % SWS, and % REMS over time (h).](image-url)
**Supplementary Figure S2.** Distribution of the indicated vigilance states across time (in 2 hour blocks) in the dark period when different epoch durations were used in the primary analysis. Solid bars indicate 4 sec epoch, grey bars indicates 8 sec epochs, and open bars indicates 12 sec epochs. Asterisks indicate significant differences between 4 and 8 sec epochs; # indicate significant differences between 4 and 12 sec epochs; and y indicates significant difference between 8 and 12 sec epochs (p<0.05; Friedman repeated measures analysis of variance on ranks; a ranked test was used because the normality test failed in the one-way repeated measures analysis of variance). The averaged values were marginally different when the different epoch durations used in the primary analysis, although the 4 sec epoch duration did produce a systematic elevation of wake and decrease of SWS. This significance is primarily due to the paired nature of the statistical test.
Supplementary Figure S3.

A

12 sec epochs
\( \tau = 980 \)

8 sec epochs
\( \tau = 1040 \)

4 sec epochs
\( \tau = 870 \)

cumulative probability density

time (sec)

B

12 sec epochs
\( \alpha = -1.88 \)

8 sec epochs
\( \alpha = -2.29 \)

4 sec epochs
\( \alpha = -2.09 \)

probability density

time (sec)

C

12 sec epochs
\( \tau = 980 \)

8 sec epochs
\( \tau = 1040 \)

4 sec epochs
\( \tau = 870 \)

cumulative probability density

time (sec)
**Supplementary Figure S3.** Cumulative probability density (A and C) and probability density (B) of the distribution of wake events when different epoch durations were used in the primary analysis. Squares – 12 sec epochs (6,043 total events); circles – 8 sec epochs (5,070 total events); upward triangles – 4 sec epochs (14,548 total events). In all plots the results from the 12 sec epoch analysis and 4 sec epoch analysis have been shifted up and down, respectively, for clarity (one log unit in A and B, half log unit in C). All results are from the dark period. In all graphs the filled symbols indicate the data used to fit equations. In A and C an exponential equation was used to fit events >500 sec (the small vertical lines in graph A indicate 500 sec mark), and the solid line through the data points illustrates the fit of the equation to the data. The time constants (t) from the fits (in sec) are indicated on the graphs. In B a power function was used to fit events from 24 - 360 sec and the line through the data points indicates the fit of the equation to the data. The coefficients of the power term (a) are indicated on the graphs. In C (an expansion of the plot in A) the vertical line that intersects the two fits indicates the 300 sec time point.
Supplementary Figure S4.

Same analysis as in supplementary Fig. S3 except the 3-epoch rule was used in the primary analysis of sleep records with an 8 sec epoch.
Supplementary Figure S5.
**Supplementary Figure S5.** Power of the EEG in BW as BW progresses. Spectral power of the first 3 epochs of BW were averaged and compared to the averaged value in the subsequent BW epochs in groups comprising epochs 4-6, epochs 7 -12, epochs 13 -18, epochs 19 - 24 and epochs 25 - 30. Left panels with white background illustrate BW in the light period and right panels with gray background illustrate BW in the dark period. The left half of each figure shows the absolute power (µV²) while the right half of each figure shows the ratio of a particular epoch group with respect to 1-3 epoch group. Groups were compared by a 2-way repeated measure ANOV (frequency and power). Frequency significantly different from the 1-3 epoch group are indicated by the line and numbers above the plotted data points (p<0.05; pairwise multiple comparisons by Bonferroni method). Group 31-36 was not analyzed because of the few BW that extended into this period.
Supplementary Figure S6.

LDW 1-3 vs 4-6

LDW 1-3 vs 7-12

LDW 1-3 vs 13-18

LDW 1-3 vs 19-24

LDW 1-3 vs 25-30
Supplementary Figure S6. Power of the EEG in LDW as LDW progresses. Spectral power of the first 3 epochs of LDW were averaged and compared to the averaged value in the subsequent LDW epochs in groups comprising epochs 4-6, epochs 7-12, epochs 13-18, epochs 19-24 and epochs 25-30. Left panels with white background illustrate LDW in the light period and right panels with gray background illustrate LDW in the dark period. The left half of each figure shows the absolute power (µv^2) while the right half of each figure shows the ratio of a particular epoch group with respect to 1-3 epoch group. Groups were compared by a 2-way repeated measure ANOV (frequency and power). Frequency significantly different from the 1-3 epoch group are indicated by the line and numbers above the plotted data points (p<0.05; pairwise multiple comparisons by Bonferroni method).
Supplementary Figure S7.
Supplementary Figure S7. Power of the EEG in BW as BW progresses. Spectral power of the epochs 4, 5, and 6 of BW were averaged and compared to the averaged value in the subsequent BW epochs in groups comprising epochs 7 -12, epochs 13 -18, epochs 19 - 24 and epochs 25 - 30. Left panels with white background illustrate BW in the light period and right panels with gray background illustrate BW in the dark period. The left half of each figure shows the absolute power (µv²) while the right half of each figure shows the ratio of a particular epoch group with respect to 1-3 epoch group. Groups were compared by a 2-way repeated measure ANOV (frequency and power). Frequency significantly different from the 1-3 epoch group are indicated by the line and numbers above the plotted data points (p<0.05; pairwise multiple comparisons by Bonferroni method). Group 31-36 was not analyzed because of the few BW that extended into this period.
Supplementary Figure S8.

LDW 4-6 vs 7-12

LDW 4-6 vs 13-18

LDW 4-6 vs 19-24

LDW 4-6 vs 25-30
Supplementary Figure S8. Power of the EEG in LDW as LDW progresses. Spectral power of the epochs 4, 5 and 6 of LDW were averaged and compared to the averaged value in the subsequent LDW epochs in groups comprising epochs 7 -12, epochs 13 -18, epochs 19 - 24 and epochs 25 - 30. Left panels with white background illustrate LDW in the light period and right panels with gray background illustrate LDW in the dark period. The left half of each figure shows the absolute power ($\mu V^2$) while the right half of each figure shows the ratio of a particular epoch group with respect to 1-3 epoch group. Groups were compared by a 2-way repeated measure ANOV (frequency and power). Frequency significantly different from the 1-3 epoch group are indicated by the line and numbers above the plotted data points (p<0.05; pairwise multiple comparisons by Bonferroni method).
Supplementary Figure S9.
**Supplementary Figure S9.** Comparison of the power in the frequency bands of BW vs LDW over the first 300 sec of each period. Spectral power of BW and LDW were averaged in groups of epochs 1 – 3, epochs 4 – 6, epochs 7 -12, epochs 13 – 18, epochs 19 – 24 and epochs 25 – 30. The results were expressed as absolute power (left half of each graph) and also as a ratio BW / LDW (right half of each graph). Left panels with white background illustrate difference between BW and LDW in the light period and right panels with gray background illustrate difference between BW and LDW in the dark period. Groups were compared by a 2-way repeated measure ANOVA (frequency and power). Frequency significantly different between BW and LDW are indicated by the line and numbers above the plotted data points (p<0.05; pairwise multiple comparisons by Bonferroni method). Group 31-36 was not analyzed because of the few BW that extended into this period.
Spectral power of wake EEG for BW (filled circles) and LDW (open circles) in the frequency band 26-50 Hz. In A and B absolute power is shown. In C and D the power is expressed as a ratio of power in BW over power in LDW (normalized for each individual animal). The dashed line in C and D is for reference indicating equal power. Data are expressed as average ± S.E.; N = 26. Frequency bands were compared by a 2-way repeated measure ANOV. No statistically significant difference were observed but the power in the LDW began to systematically exceed the power in the BW at frequencies approximately above 30 Hz.
Supplementary Figure S11.

Spectral power of the component frequencies of the EEG between BW in light and dark period. BW from epoch 4 and up were averaged together and compared. The asterisks show sporadic and nonsystematic differences in frequency points 1, 6, 8 and 9 Hz (p<0.05; pairwise multiple comparisons by Bonferroni method).
Spectral power of the component frequencies of the EEG from SWS epochs in the first quarter of VC episodes (filled circles), second quarter VC episodes (open circles), third quarter VC episodes (filled triangles) and last quarter of VC episodes (open triangles). VC episodes were selected as described in Figure 4. Plotted results are slightly offset from one another on the X-axis for clarity. The bar above the graph denotes significant difference between each quarters in the 1-6 Hz band (p<0.05; pairwise multiple comparisons by Bonferroni method). There was a smooth graded reduction in the EEG power in the 1-6 Hz frequency band as VC episode progressed with only a few exceptions (e.g. at 1 Hz frequency power of the 2\textsuperscript{nd} quarter VC was higher than power of the 1\textsuperscript{st} quarter VC; also no statistical difference was observed between 1\textsuperscript{st} and 2\textsuperscript{nd} quarter VC at 2Hz frequency.
Table 1. Summary of the characteristics of VC and LDW

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dark Period</th>
<th>Light Period</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Long Duration Wake</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td># of LDW episodes</td>
<td>18.0 ± 1.0</td>
<td>5.3 ± 0.3 **</td>
</tr>
<tr>
<td>Duration of LDW</td>
<td>1420 ± 90</td>
<td>1300 ± 80</td>
</tr>
<tr>
<td>% time in LDW</td>
<td>55.0 ± 1.6</td>
<td>15.2 ± 0.9 **</td>
</tr>
<tr>
<td><strong>Vigilance Cycling</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td># of VC episodes</td>
<td>18.3 ± 1.0</td>
<td>5.1 ± 0.3 **</td>
</tr>
<tr>
<td>Duration of VC</td>
<td>1130 ± 80</td>
<td>7600 ± 380 **</td>
</tr>
<tr>
<td>% time in VC</td>
<td>44.5 ± 1.6</td>
<td>84.8 ± 0.9 **</td>
</tr>
<tr>
<td><strong>Brief Wake</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW episode duration</td>
<td>39.8 ± 1.4</td>
<td>38.9 ± 1.3</td>
</tr>
<tr>
<td>BW (% of VC)</td>
<td>37.5 ± 1.9</td>
<td>22.7 ± 0.8 **</td>
</tr>
<tr>
<td><strong>Slow-Wave Sleep</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS episode duration</td>
<td>60.7 ± 2.8</td>
<td>103.6 ± 4.0 **</td>
</tr>
<tr>
<td>SWS (% of VC)</td>
<td>55.7 ± 1.5</td>
<td>60.4 ± 0.8 *</td>
</tr>
<tr>
<td><strong>REMS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REMS episode duration</td>
<td>60.8 ± 2.9</td>
<td>88.2 ± 3.1 **</td>
</tr>
<tr>
<td>REMS (% of VC)</td>
<td>6.8 ± 0.6</td>
<td>16.9 ± 0.4 **</td>
</tr>
</tbody>
</table>

Durations are reported in sec. # of LDW episodes is the number of episodes assigned to LDW within the light or dark period. Duration of LDW is the average duration of LDW episodes within the period. Percent time in LDW and VC are relative to entire dark or light period. Percent BW, SWS, and REMS within VC are the percent of time within VC that is occupied by the respective state. Duration of BW, SWS, and REMS episodes within VC are the average duration of episodes that occurred within VC. Values are averages ± SE; N = 8 for alcoholic animals and N = 6 for pair-fed animals. Asterisks: significantly different from dark period for same treatment (*p<0.05, **p<0.005, paired t-test
Supplementary Table S1. Summary of the characteristics of VC and LDW when the 3-epoch rule applies.

<table>
<thead>
<tr>
<th>parameter</th>
<th>dark period</th>
<th>light period</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Long Duration Wake</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td># of LDW episodes:</td>
<td>19.8 ± 1.0</td>
<td>5.4 ± 0.3 **</td>
</tr>
<tr>
<td>Duration of LDW:</td>
<td>1240 ± 60</td>
<td>1300 ± 100</td>
</tr>
<tr>
<td>% time in LDW:</td>
<td>54.7 ± 1.6</td>
<td>15.2 ± 0.8 **</td>
</tr>
<tr>
<td>LDW (% of total wake):</td>
<td>78.7 ± 1.2</td>
<td>47.8 ± 2.2 **</td>
</tr>
<tr>
<td><strong>Vigilance Cycling</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td># of VC episodes:</td>
<td>17.2 ± 0.1</td>
<td>4.6 ± 0.3 **</td>
</tr>
<tr>
<td>Duration of VC:</td>
<td>1150 ± 70</td>
<td>8590 ± 530**</td>
</tr>
<tr>
<td>% time in VC:</td>
<td>43.3 ± 1.6</td>
<td>84.7 ± 0.8 **</td>
</tr>
<tr>
<td><strong>Brief Wake</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW episode duration:</td>
<td>54.0 ± 1.9</td>
<td>49.4 ± 2.0</td>
</tr>
<tr>
<td>BW (% of VC):</td>
<td>34.7 ± 1.5</td>
<td>19.7 ± 0.8 **</td>
</tr>
<tr>
<td><strong>Slow-Wave Sleep</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS episode duration:</td>
<td>87.8 ± 4.3</td>
<td>152.1 ± 7.9 **</td>
</tr>
<tr>
<td>SWS (% of VC):</td>
<td>58.6 ± 1.3</td>
<td>63.5 ± 0.7 *</td>
</tr>
<tr>
<td><strong>REMS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REMS episode duration:</td>
<td>65.6 ± 3.4</td>
<td>97.6 ± 3.0 **</td>
</tr>
<tr>
<td>REMS (% of VC):</td>
<td>6.7 ± 0.5</td>
<td>16.9 ± 0.4 **</td>
</tr>
</tbody>
</table>

Durations are reported in sec. # of episodes (LDW and VC) in the light period have been normalized to 12 h. Percent time in LDW or VC is with respect to total time. LDW (% of total wake) is percent of total wake that is LDW. Episode durations and percent of VC for BW, SWS, and REMS are with respect to periods of VC only. Values are averages ± SE; N = 26. Asterisks: significantly different from dark period (*p<0.05, **p<0.005, paired t-test).
ACKNOWLEDGEMENTS

This work was supported by grant no. AA13248 from the NIAAA awarded to S.M.S.
REFERENCES


[3] Blumberg MS, Coleman CM, Johnson ED, Shaw C. Developmental divergence of sleep-wake patterns in orexin knockout and wild-type mice. European J Neuroscience, 2007; 25: 512-518.


Chapter 4 Chronic Alcohol Treatment in Rats Alters Sleep by Fragmenting Periods of Vigilance Cycling in the Light Period with Extended Wakenings.

Sanjib Mukherjee and Steven M. Simasko

Program in Neuroscience, Department of VCAPP, Washington State University, Pullman, WA 99164-6520

Behav Brain Res, 2008 (in press).
ABSTRACT

Studies have shown that disturbed sleep produced by chronic alcohol abuse in humans can predict relapse drinking after periods of abstinence. How alcohol produces disturbed sleep remains unknown. In this study we used a novel analysis of sleep to examine the effects of alcohol on sleep patterns in rats. This analysis separates waking into multiple components and defines a period labeled vigilance cycling (VC) in which the rat rapidly cycles through various vigilance states. These VC episodes are separated by long duration wake periods (LDW). We find that 6 weeks of alcohol (6% in a liquid diet) caused fragmentation of extended VC episodes that normally occur in the light period. However, total daily amounts of slow-wave sleep (SWS) and rapid-eye movement sleep (REMS) remained constant. The daily amount of wake, SWS, and REMS remained constant because the alcohol treated rats increased the amount of VC in the dark period, and the sleep nature of VC in the dark period became more intense. In addition, we observed more wake and less REMS early in the light period in alcohol treated rats. All effects completely reversed by day 16 of alcohol withdrawal. Comparison of the effects of chronic alcohol to acute alcohol exposure demonstrated the effects of chronic alcohol are due to adaptation and not the acute presence of alcohol. The effects of chronic alcohol treatment in rats mimic the effects reported in humans (REMS suppression, difficulty falling asleep, and difficulty remaining asleep).

Key words: long duration wake, brief wake, circadian rhythm, REMS suppression, sleep homeostasis, insomnia
INTRODUCTION

Although acute alcohol is a soporific agent, a frequent complaint of alcoholics is disturbed sleep. Specifically, alcoholics entering rehab programs have been found to have high pressure for rapid-eye-movement sleep (REMS), an increase in sleep onset latency, and inability to maintain sleep through the night [3, 19, 22]. Although REMS returns to normal after cessation of drinking, the insomniac effects can remain for years after recovery [1, 5, 8, 12, 13, 34, 39]. Furthermore, disturbances in sleep patterns in recovering alcoholics have been found to predict the probability of relapse drinking [4, 6, 8, 12, 13]. Why disturbed sleep should predict relapse to further alcohol consumption remains unknown, but at an even more fundamental level, the mechanistic basis for disturbed sleep patterns produced by alcohol exposure also remains unknown. An animal model of altered sleep induced by chronic alcohol would be helpful in addressing this issue.

A common animal model for both sleep and alcohol research is the rat. However, a difference between rat and human sleep is that rats are polyphasic sleepers whereas humans are monophasic sleepers. Thus when human alcoholics (or recovered alcoholics) complain of failure to maintain sleep throughout their monophasic sleep period, it becomes difficult to assess this parameter in the rat since rats normally have frequent wakenings throughout the rest period. Recently we developed an analysis of sleep patterns in rats that separates wake episodes into brief wake (BW) and long duration wake (LDW), and we used this division to identify a sleep unit that occurs between LDW events [33]. The term vigilance cycling (VC) was used as the label for this sleep period because of the rapid cycling between different vigilance states. In our prior study we suggested that periods of VC are analogous to the 8-h monophasic sleep period in
humans. In the present study we used this analysis to examine the effects of chronic and acute alcohol consumption on sleep in rat.

We found that the long periods of VC that normally occur in the light period in untreated rats are fragmented into shorter periods by the chronic alcohol treatment. Further, since slow wave sleep (SWS) and rapid-eye movement sleep (REMS) occur within VC periods, there is also a drop in the overall amount of SWS and REMS in the light period. On the other hand, during the dark period VC takes on the more sleep intensive appearance of VC in the light period such that daily amounts of SWS and REMS are not altered by the chronic alcohol treatment. These effects of chronic alcohol are not mimicked by acute exposures to alcohol. These results document that the effects of chronic alcohol in rat (fragmentation of prolonged sleep episodes and suppression of REMS in the light period) are a result of adaptations to the presence of alcohol and closely resemble the pathologies observed in human alcoholics.

**METHODS**

**Chronic alcohol treatment.** Sixteen male Sprague-Dawley rats (250-275 g at the beginning of the treatment) were used; eight of them were assigned to the alcohol treated group and eight to the pair-fed control group. Beginning body weights were matched between the two groups. Rats were housed individually at 22 ± 2 °C ambient temperature with a 12h:12h light:dark cycle. Alcohol treated rats were treated with 6% alcohol (grain alcohol, Everclear) in liquid diet (Bio-serv) for 6 weeks. The alcohol treated rats consumed on average 11 ± 0.2 g alcohol/kg body weight/day. In a prior study we found that this protocol produces a blood alcohol level of ~0.12% when blood samples are taken at the end of the dark period [18]. Each pair-fed rat was given alcohol-free liquid diet of equal caloric content (calories from alcohol replaced with
maltose dextrin) to that consumed by the weight-matched alcohol treated rat. All rats had ad lib access to water. These rats are the same rats for which previous results have been reported [23]. The focus of this previous study was to determine the optimum treatment protocol to produce alcohol-induced changes in sleep patterns. The results contained within the present report are a more detailed analysis from this group of animals and includes the data from the recovery period after cessation of alcohol treatment.

**Acute alcohol treatment.** Twenty male Sprague-Dawley rats (approx 350g) were used for dark onset alcohol administration study and nineteen rats of similar specifications for light onset alcohol administration. Housing, ambient temperature and light cycles used in these experiments were the same as described in the chronic alcohol treatment study. Alcohol (Everclear) was administered within 30 min prior to a scheduled light change in a 30% solution in tap water (1 ml / 100 g body weight) by gastric gavage (final dose 2.4 g/kg body weight). Control recordings were obtained after an equivalent water gavage (1 ml/ 100 g body weight). Rats had ad lib access to food and water at all times. Administration of alcohol at 3 g/kg produces a blood alcohol level around 0.153 % after 90 minutes and 0.1 % at three hour [37].

All experiments were done with the approval of the institutional animal care and use committee of Washington State University.

**Instrumentation for sleep recordings.** Surgical implantation of permanent EEG and EMG electrodes were as described previously [23]. A week of recovery from the instrumentation was allowed before sleep recordings commenced. In the chronic treated animals the instrumentation was done during the 5th week of the alcohol exposure. The alcohol diet was continued during the time of instrumentation.
Sleep recordings. During the period of data collection sleep recordings were made in enclosed environmental chambers as described previously [23]. Alcohol and pair-fed treatments continued in the environmental chambers through the first two days of sleep recordings after which the alcohol was removed from the diet of the alcohol treated animals over a two day period (6% to 3% to 0%). Recordings were interrupted for one hour each day (last hour of light period) for animal care. However since we analyze data in 2 h blocks we excluded data in the last 2 h block in the light period. After weaning from alcohol, the liquid diet was continued and additional sleep recordings were collected periodically throughout the recovery period. In the present study we report findings from recordings on the last 6% alcohol day and those obtained 2 days and 16 days after termination of the alcohol treatment. In the acute alcohol studies the animals were also gavaged daily during the 3 day habituation period.

Collection and analysis of EEG and EMG signals. We collected and analyzed the EEG and EMG signals as described previously [33] using an 8-sec epoch in the primary analysis of the sleep records. A 300 sec cutoff was used to separate BW from LDW. VC events were those events between LDW episodes. Further, as described previously [33] any isolated SWS events that were surrounded by LDW were not considered VC (because no cycling occurred), and such events were not used to divide periods of LDW. When examining the variation in slow-wave amplitude (SWA; average of amplitudes in the 0.5 to 4 Hz frequency bands only during SWS episodes) across the 22 h of recording, we averaged the SWA across the entire recording for each rat, and then the average SWA within each 2-h block was expressed as a percentage of the average across the entire recording.

Statistical Analysis. In the course of the experiment the instrumentation on two pair-fed animals failed before the first sleep recordings were made at the end of the alcohol treatment
period. Thus the results reported from this phase of the treatment are from 8 alcohol treated animals and 6 pair-fed animals. During the recovery period (after day 2), the instrumentation on two of the alcohol treated animals failed. Thus the results for day 2 of recovery contain 8 alcohol treated and 6 pair-fed animals and results from day 16 of recovery from 6 animals in each group.

A two-way (light period versus dark period and alcohol treated versus pair-fed) analysis of variance (ANOVA) was used when comparing most parameters. However, because there was only 10 hours of recording in the light period versus 12 hours in the dark period, results that scale with time of recording were either normalized to minutes of vigilance state per 2-h time period and the two-way ANOVA used or a t-test was used to compare alcohol treated versus pair-fed only within the dark or light period (number of LDW and VC episodes). When comparisons were made across 2-h time blocks, a two-way repeated measure ANOVA was used. Post-hoc pair-wise comparisons were done by the Holm-Sidak method and Bonferroni’s method. We used repeated measures two-way ANOVA (time versus treatment) in the acute alcohol study. In all cases a p value <0.05 was considered statistically significant. All results are presented as averages ± SEM. All ANOVA were performed with SigmaStat version 3.5 software (SYSTAT Software, Richmond, CA), and t-tests were performed with Microsoft Office Excel 2003.

RESULTS

Effects of chronic alcohol treatment on sleep patterns. After 6 weeks of 6% alcohol in liquid diet rats show multiple alterations in the percentage of time they spend in various vigilance states as compared to the pair-fed control rats (Figure 1). Chronic alcohol treated rats show a suppression of wake and an increase of SWS and REMS in the dark period, but opposite changes
(increased wake but decreases in SWS and REMS) in the light period (Figure 1B, 1D, and 1F). However, when one examines the total time spent in each state over the entire 22 hour recording period, there was no net change in the time spent in any one of the vigilance states (Figure 1G, 1H, and 1I). A more detailed analysis of the sleep pattern broken down into 2-h blocks reveals that significant differences are observed in wake, SWS and REMS at various times of the day even within the light and dark periods (Figure 1A, 1C, and 1E). Most notable are the increased amount of wake (Figure 1A) and suppression of REMS that occur during the first half of the light period (Figure 1E).

Shown in Figure 2 are representative hypnograms from two pair-fed animals (top two traces) and two alcohol treated animals (bottom two traces) with periods of VC indicated by the bars drawn above the hypnograms. The effects of the chronic alcohol treatment on sleep parameters are quantified and summarized in Table 1. In the light period, after chronic alcohol exposure the number of VC events was greater and there was a decrease in the average duration of individual VC episodes. The decrease duration of VC episodes resulted in a decrease in the percentage of time spent in VC. However, within VC in the light period, the average episode durations for BW, SWS, and REMS did not change significantly. The one change in these parameters was the percentage of REMS was reduced, which from the analysis presented in Figure 1 can be seen as due to a REMS suppression that occurred early in the light period (this is also apparent in the hypnograms in Figure 2). In addition to the lack of change in episode durations for BW, SWS, and REMS from the alcohol treatment, the duration of LDW episodes was also not significantly greater, although this parameter was on the borderline of significance (the p value for alcohol treatment in the two-way ANOVA was p = 0.053). The conclusion form these observations is the major effect of chronic alcohol treatment on the sleep patterns of rats in
the light period is the alcohol exposure fragments the long periods of VC that normally occur in
the light period, with little change in the characteristics of the events within the VC except for
REMS suppression early in the light period.

In the dark period the chronic alcohol treatment caused the rats to spend more time in VC
than the pair-fed controls. The average duration of the LDW episodes and the average duration
of VC episodes were increased (the VC episode duration doubled), but neither change was
statistically significant. Although the number of these events did decrease, the decreases were
not statistically significant (p = 0.106 for VC and p = 0.084 for LDW). However, when analyzed
by percentage of time spent in VC, the alcohol treated rats did spend significantly more time in
VC than the pair-fed rats, indicating that there was a change in the distribution of VC. This was
due to the longer event durations of VC (a doubling), even if the doubling failed to reach
statistical significance in the ANOVA of VC event duration.

Within the VC episodes of the dark period the average duration of REMS episodes was
increased, and in fact became similar to the duration of these episodes in the light period.
Furthermore, the percentage of REMS within VC also became similar to the percentage normally
found in the light period. The average duration of SWS episodes increased by about 20 sec, but
this increase was not found significant in the two-way ANOVA. However, this may be due to
the fact that SWS duration did not change in the light period after alcohol treatment (the
interaction term in the analysis almost reached significance; p = 0.126). By a straight t-test
(alcohol vs pair-fed in the dark), the increase in SWS duration is easily significant (p = 0.0206).
Finally, in the dark period the duration of BW events was not affected by the alcohol treatment,
but the percentage of time spent in BW was significantly reduced compared to the pair-fed rats,
and was approximately equal to that found in both the pair-fed and alcohol treated rats in the

136
light period. The conclusion from these observations is the major effect of chronic alcohol treatment on the sleep patterns of rats in the dark period is the alcohol treatment causes the rats to spend more time in VC in the dark period, and the nature of the VC has characteristics of VC in the light period (generally longer durations episodes of VC, and within VC, longer episode durations of SWS and REMS, with a relatively less amount of BW).

Effects of alcohol on the slow-wave amplitude in the EEG. The analysis of chronic alcohol effects to this point indicated that alcohol fragments VC in the light period and that in the dark period, when the rats do sleep, the sleep appears to be more intense (i.e., dark period VC takes on more of the characteristics of light period VC). We next sought to determine if additional support for the latter conclusion could be found from the analysis of the EEG amplitude in the SWS periods. Increases in slow-wave amplitude (SWA) are typically interpreted as a sign of increase sleep intensity [2]. The SWA of the EEG varies over the course of the day with a peak that normally occurs soon after the onset of sleep in the normal rest period. This can be seen to occur in the pair-fed rats (Figure 3A, solid circles). However, in the alcohol treated rats the normal circadian variation of SWA was obliterated (Figure 3A, open circles). Since the SWA was normalized across the day, the loss of variation in SWA could be due to a suppression of SWA across the entire day, or an elevation of SWA across the entire day. In Figure 3B and 3C are the average power spectrum for the chronic alcohol treated rats and pair-fed rats shown at the first 2 h of the dark period (normally a low point) and the first 2 h of light period (normally the high point). The pair-fed rats have low amplitude in the delta band in the first 2 h of the dark period and high amplitude in the delta band in the first 2 h of the light period; whereas the chronic alcohol treated animals have high amplitude in the delta band in both periods. Thus the loss of variation was due to an elevation of SWA across the entire day. This observation is
consistent with the conclusion that chronic alcohol treated rats are sleeping more intensely in the dark period compared to the pair-fed controls.

**Recovery from the effects of chronic alcohol.** Sleep patterns were collected over 20 days following termination of the alcohol treatment (results from day 16 and day 2 only are shown). The vigilance state diagrams for the animals 16 days after termination of the treatment is shown in Figure 4 and at this point there are absolutely no differences between the animals in the two groups. Further, analysis of LDW and VC, and analysis of behavior within VC, show complete recovery of every parameter that was altered at the end of the 6 week alcohol treatment (Table 2). Finally, analysis of SWA amplitude also showed a complete recovery of the variation of SWA across the day (Figure 5A). Also of note in Figure 5B and 5C is that the absolute power of the EEG in the alcohol treated animals also matched the control values. It is possible that absolute values could be different between the two groups because of differences in the fidelity of the recording electrodes in the individual animals. However, in this set of animals, after recovery, the power spectra were almost identical. The observation that the SWA matched in the two groups after recovery supports the conclusion that the chronic elevation of the EEG power across the day at the end of the 6 week alcohol treatment (Figure 3B and 3C) was in fact due to an elevated EEG power spectra in the alcohol treated animals and not due to technical differences in the recordings.

A final issue is whether the changes observed after the 6 week alcohol treatment are due to adaptations to chronic treatment with alcohol or whether the changes are simply due to the acute presence of alcohol. We addressed this issue in two ways. First, we examined the sleep patterns of the chronic alcohol treated rats on day 2 after cessation of alcohol treatment, and second, we examined the effects of acute administration of alcohol at light and dark onset in a separate group
of rats. On day 2 of recovery the animals should be virtually free of alcohol (taper to 3% alcohol in liquid diet on day 0 of recovery, alcohol completely removed at the end of the light period, day 1 is the first alcohol-free 24-h period, day 2 recordings thus started 24 hours after complete removal of alcohol). The vigilance state diagrams for the animals at this point are shown in Figure 6. It can be seen that the wake disturbance was still present (more wake in the light and less in the dark), and that some of the SWS and REMS disturbances were still present, but the alterations were not as great as those observed when the alcohol treatment was ongoing (Figure 1). It can also be seen in the more detailed analysis of LDW and VC, and behavior within VC, that some of the parameters were still altered similar to the last alcohol treatment day (Table 3). For example, the percentage of time in LDW and VC were still altered. In the light period the VC episode duration in the alcohol treated animals had begun to approach that of the pair-fed animals but was still shorter (this parameter was almost still significantly different than the pair-fed animals in that p = 0.078 for the interaction term in the two-way ANOVA), and in the dark period the characteristics within VC were still similar to the characteristics of VC in the light period (long durations of SWS and REMS episodes with a small percentage of BW and elevated percentage of REMS). Thus many facets of the sleep pattern disturbed by chronic alcohol were still present even after alcohol was no longer present, and thus the changes are likely due to adaptations to the chronic treatment and not the acute presence of alcohol.

**Acute administration of alcohol.** The second way we examined whether the effects of 6 weeks of alcohol treatment were due to adaptation to the presence of alcohol rather than the acute presence of alcohol was to examine sleep parameters in animals acutely exposed to alcohol. Acute dark onset administration of alcohol decreased wake and increased SWS throughout the entire dark period (Figures 7A – 7D). Acute alcohol initially suppressed REMS
which was followed by a rebound in REMS (Figure 7E, and top two hypnograms in Figure 8).

No effect on wake, SWS, REMS was observed in the following light period when we analyzed the data in this manner (Figure 7A – 7F). In regards to daily wake, SWS, and REMS, dark onset administration of alcohol caused a significant decrease in 22 h wake with a corresponding increase in SWS (wake: 729 ± 14 min for control versus 657 ± 13 min after alcohol, p<0.001; SWS: 486 ± 14 min for control versus 553 ± 13 min for alcohol, p<0.001) with no significant change in REMS (105 ± 3 min for control versus 110 ± 6 min for alcohol).

Examination of the VC behavior revealed additional alterations in sleep from the acute alcohol challenge. Dark onset administration of alcohol increased the percentage of time spent in VC in the dark period and within VC it decreased the percent and duration of BW events and increased the percent and duration of SWS events (Table 4). These effects within VC in the dark period are similar to the effects seen after the chronic alcohol treatment (Table 1). However, in regards to REMS, there were significant differences. Whereas with chronic alcohol REMS was elevated throughout the dark period (Figure 1), with acute dark onset alcohol, REMS was initially suppressed followed by a rebound (Figure 7). Although the analysis of vigilance states suggested no changes occurred in the light period following dark onset administration of alcohol, analysis of VC and LDW event durations revealed that dark onset alcohol caused VC and LDW event durations to shorten without an overall change in the distribution of VC and LDW in the light period (Table 4). Thus, there was fragmentation of VC and LDW in the light period following alcohol administration at the onset of the preceding dark period, but the fragmentation that occurred was not as great as that seen after the chronic alcohol administration (Table 1). Further, the behavior of SWS and BW within VC in the following light period also revealed hangover effects. The percentage of time in BW within VC increased but BW episode duration
decreased, whereas the percentage of time in SWS decreased and the SWS episode duration also
decreased (Table 4). This increase in cycling within VC supports the conclusion that sleep was
further compromised in the light period following dark onset administration of alcohol. A final
bit of evidence that sleep was compromised at the beginning of the light period after dark period
administration of alcohol was that SWA was suppressed during the first two hours of the light
period after dark onset administration of alcohol (Figure 9A). In this set of animals we also
observed an enhancement of SWA during the first two hours after alcohol (Figure 9A).

Acute alcohol administration at the beginning of the light period caused a paradoxical
increase in wake and decrease in SWS during the first 2-h time block (Figure 7G and 7I). In
addition, like dark onset acute administration of alcohol, REMS was initially suppressed
followed by a REMS rebound; that in this case extended into the following dark period (Figure
7K and bottom two hypnograms in Figure 8). Consistent with the increase in wake, VC
episodes, especially at the beginning of the period, were fragmented (Figure 8, bottom
hypnogram, Table 5). In the following dark period the percentage of time in LDW was
decreased with a corresponding increase in VC (Table 5). However, within VC there were no
changes with regard to BW and SWS in the light period, or in the following dark period (Table
5). Thus the changes in wake and SWS distribution seen in the shaded portions of Figure 7G and
7I were due to changes in the distribution between VC and LDW and not the behavior within
VC. The lack of change in BW and SWS behavior was further supported by the complete lack
of change in SWA after light period onset alcohol (Figure 9B). REMS rebound appears to be
due to increases in the percentage of time within VC spent in REMS and not changes in episode
duration (Table 5). This suggests an increase in the number of REMS episodes, which we
confirmed in a separate analysis (data not shown). The lack of effect on REMS episode
durations by both light and dark onset administration of alcohol is in contrast to the increase in REMS episode duration observed in the dark period after chronic alcohol administration.

Finally, light onset administration of alcohol caused no changes in daily wake or SWS (wake: 738 ± 2 min for control versus 717 ± 3 min for alcohol; SWS 470 ± 3 min for control versus 460 ± 3 min for alcohol) but did increase 22 h REMS (112 ± 2 min for control versus 144 ± 2 min for alcohol, p<0.001).

**DISCUSSION**

Chronic alcohol treatment in rats caused a shift of sleep (SWS and REMS) from the light period to the dark period, with opposite shifts in wake, such that daily amounts of wake, SWS, and REMS did not change. In the light period these changes were mediated primarily by fragmentation of periods of VC with LDW episodes. In addition, in the early light period there was an overall decrease in wake and REMS. In the dark period there was a corresponding increased intensity of sleep as indicated by both an increase in the amount of VC in the dark period, and the VC that occurs in the dark period has a more intense sleep nature. The sleep disturbances in the light period (suppression of REMS and increase wake early in the period, and fragmentation of VC) are very similar to the effects of chronic alcohol consumption in humans where difficulty falling asleep, REMS suppression, delayed REMS onset, and sleep fragmentation are frequent complaints [3].

*Effects of chronic alcohol treatment requires adaptation.* It is possible that the effects we observed in our chronic alcohol treatment are due to the acute consumption patterns of alcohol and not adaptations to the chronic presence of alcohol. For example, in a separate study we monitored light/dark differences in alcohol consumption during the 6% alcohol in liquid diet
treatment and found that ~80% of the alcohol consumption occurs during the dark period (unpublished observation). Thus the increase sleep intensity in the dark period after chronic alcohol could be caused by the greater acute consumption of alcohol in this period. This could in turn cause less sleep pressure in the light period and hence the fragmentation of VC that we observe. Several lines of evidence argue against this interpretation. Although acute alcohol in naïve animals does cause an increase in SWS episode duration and percent time spent in SWS [18, and this study], this effect does not appear to be maintained after 2-3 weeks of chronic alcohol [18]. This early tolerance to the sleep inducing effects of alcohol is well supported in the literature [24]. Further, after only 2-3 weeks of alcohol, the entire spectrum of changes caused by prolonged alcohol treatment, particularly the changes in distribution of SWS between light and dark period, does not fully manifest [18, 23].

In addition, although dark onset administration of alcohol did cause some fragmentation of VC in the following light period, the degree of fragmentation of VC in the light period is not anywhere near the degree seen with chronic alcohol treatment. The effects of chronic alcohol on VC in the light period are also not mimicked by light onset administration of alcohol. Although light onset did cause some fragmentation of VC early in the period, mainly due to the paradoxical increase in wake, after chronic alcohol the percentage time spent in LDW in the light period was doubled, an effect not seen with either acute dark onset or light onset alcohol administration. The reason for the paradoxical increase in wake after alcohol at light onset remains obscure. Alcohol-induced increases in locomotor activity following alcohol have been observed, but this phenomenon occurs robustly in mice, but not in rats [9, 11, 26].

Another argument that favors the interpretation that chronic effects of alcohol require adaptations is that although acute dark onset alcohol blunted the peak of the SWA in the early
light period (as well as elevating SWA in the first 2 hours of the dark period), chronic alcohol treatment flattened any variation in SWA across the day. In total, these numerous observations support the interpretation that increase sleep intensity in the dark period after chronic alcohol exposure is a result of adaptation to light period changes (i.e., fragmentation of long periods of VC), not a cause of light period changes.

**Recovery from chronic alcohol effects.** By day 16 following stoppage of alcohol treatment the chronic alcohol treated animals had completely returned to normal sleep patterns. At day 2 of recovery, a time when no alcohol should be present, many of the disturbed sleep parameters were still present. This observation further supports our contention that the effects of chronic alcohol are due to adaptation and not the acute presence of alcohol. However, we were surprised by the rapidity of the recovery process. In humans, the alcohol-induced sleep disturbances can last for years [1, 5, 8, 12, 13, 34, 39]. This suggests that our treatment did not fully manifest the adaptations that occur in humans when they adapt to chronic alcohol. This might be due to either the pattern by which the alcohol was consumed (for example, a more episodic alcohol exposure may produce more enduring changes [24]), or that the 6 week treatment was insufficient to produce the enduring changes that might occur after decades of alcohol abuse in humans. This issue requires additional studies to be resolved.

**Use of novel analysis to describe alcohol effects.** A significant aspect of the present study is the use of the novel analysis that includes dividing wake into LDW episodes and BW episodes, which permits us to define a sleep unit in rats labeled VC [33]. First, this analysis allows us to describe effects of alcohol on the sleep patterns in rats that are not apparent in the traditional analysis by vigilance state distributions and analysis of vigilance state episode duration and number. For example, by the traditional analysis acute dark onset administration of alcohol had
no hangover effects in the subsequent light period, but with the new analysis we could observe that sleep quality as measured by continuity of VC episodes and more rapid cycling between vigilance states within VC occurred. In the chronic exposure paradigm, the fragmentation of VC in the light period becomes obvious; an effect we conclude is similar to the inability of human alcoholics to maintain sleep throughout the night. Without some definition of an extended sleep unit in rats, such conclusions would not be possible.

Another aspect of the analysis is that the different components in the sleep patterns in rat are likely due to unique aspects of the neuronal circuits that control each component. Thus by identifying which components are altered by alcohol, it helps to identify where it is likely that chronic or acute alcohol is likely to be interacting with sleep regulatory mechanisms. In this novel analysis we find there are four components of sleep patterns that can be identified in such a manner. These include daily sleep homeostasis, the distribution between LDW and VC, the behavior of BW and SWS within VC episodes, and the behavior of REMS.

**Total daily sleep in alcohol treated rats.** We observed that after 6 weeks of alcohol that the homeostatic regulation of the amount of daily sleep (SWS and REMS) is intact. It has been proposed that the sleep homeostat may be mediated by accumulation of extracellular adenosine in the basal forebrain [27] or possibly cytokines [17], and that these substances eventually influence the flip-flop circuitry of sleep-wake transitions [31] at the level of the ventral lateral preoptic area (VLPO) [25]. The observation that after chronic alcohol the animals still had normal total daily sleep suggests that alcohol does not disrupt the mechanisms that underlie homeostatic regulation of sleep, but rather alcohol is likely to alter the mechanisms that control the timing and/or continuity of sleep. On the other hand, acute exposure to alcohol did alter the daily distribution of sleep states, with the effects of dark onset administration being different.
than light onset administration. This suggests that acutely alcohol does alter the manner in which
the homeostatic signals are interpreted, and that the specific effects of acute alcohol in this regard
are dependent upon the specific nature of the signal at particular times of the day, but that in the
chronic exposure paradigm there is adaptation to bring total sleep back into balance.

Effects of alcohol on the distribution between LDW and VC and behavior within VC. We
have previously [33] presented arguments that sleep-wake transitions within VC are likely the
domain of the sleep-wake flip-flop switch [31], whereas control of LDW episodes is likely to be
the domain of the hypothalamic integrator that balances circadian rhythms with sensory,
cognitive, and emotional cues [32]. Thus our findings that the main effect of chronic alcohol
was to alter the distribution of LDW and VC suggest that the main effect of chronic alcohol is to
alter the function of the hypothalamic integrator and not the behavior (and by extension, the
neurocircuitry) of state transitions driven by the sleep-wake flip-flop switch. Although the
character of VC in the dark period did change (appears to be light period-like), this change is
likely to be mediated by the balance between homeostatic drive for sleep with circadian (and
possibly other) inputs onto the VLPO. For example, the loss of VC in the light period increases
the homeostatic drive for sleep and thus dark period adaptations need to occur to keep total daily
sleep constant. On the other hand, the acute effect of alcohol given at dark onset does appear to
alter the behavior within the flip-flop switch to favor SWS over BW, but the animal seems to
adapt to these acute changes.

Another remarkable observation is the relative resistance of the BW and SWS behavior
within VC in the light period to alcohol effects. This behavior was not altered by either chronic
alcohol or acute light onset administration of alcohol. This suggests that the circuits that underlie
the distribution of BW and SWS, which we speculate is the flip-flop switch, is not inherently
sensitive to the acute presence of alcohol, at least not in the light period. However, dark onset administration of alcohol did cause decreases in duration of both BW and SWS in the following light period, an effect that occurs after alcohol has mostly cleared from the system. Again, this argues that within the switch (for example, the balance of GABAergic transmission between neuronal components) is relatively resistant to alcohol effects, but that this balance is upset by inputs onto the switch, which may be different at different times of day.

**REMS suppression and rebound.** Regardless of whether acute alcohol was given at light or dark onset, we observed an early REMS suppression followed by a profound REMS rebound. Interestingly, acute alcohol had no effect on REMS episode duration after either light or dark onset administration. Thus the acute presence of alcohol must increase the probability of entering into a REMS episode rather than alter the mechanisms that control REMS episode duration. With dark onset administration the REMS suppression and rebound balanced so there was no net change in total REMS, however with light onset administration, the REMS rebound was in excess of the suppression so there was a net increase in total REMS. These acute effects are in contrast to the effects observed after chronic alcohol treatment. After chronic alcohol the total amount of daily REMS remained normal, suggesting the homeostatic regulation of REMS was intact, but the distribution of REMS shifted from predominately in the light period to being about equally balanced between light and dark periods.

The chronic alcohol-induced alteration of REMS could be an action mediated within the REMS flip-flop switch in the brainstem or an action on the hypothalamic inputs onto the REMS flip-flop switch [20]. An argument in favor of the hypothalamic site of action is that durations of REMS episodes increased in the dark period but slightly decreased in the light period. If alcohol targeted mechanisms within the REMS flip-flop switch (e.g., enhanced suppression of REMS-on
by REMS-off mechanisms) one would expect a similar change (suppression) in both light and dark periods. This suggests that after chronic alcohol the two components of the brainstem REMS flip-flop switch are functioning normally, but that the inputs onto the switch from the hypothalamus which control the circadian differences in REMS episode duration and number, have changed. Another important conclusion from these observations is further support that the effects of chronic alcohol require adaptation and is not mimicked by the acute effects of alcohol on REMS control mechanisms.

**Circadian effects of alcohol.** One clear effect of chronic alcohol on sleep is that the normal circadian pattern of sleep is disrupted. Both the normal distribution between VC and LDW was altered as was the behavior of VC in the dark period, which became more light-period like. The durations of BW episodes were not altered by alcohol treatment, suggesting alcohol has no effect on the factors that regulate this parameter. While most sleep models proposed to date do a good job explaining circuits that control wakefulness, SWS, and REMS, they have yet to address the mechanisms of circadian influences that adjust the distribution between LDW and VC, as well as the specific patterning of vigilance states within VC. Thus possible mechanisms that might underlie the effects of alcohol on these patterns remain obscure. However, it is well documented that prolonged alcohol treatments can alter the circadian pattern of a variety of hormonal and behavioral rhythms [7, 15, 21, 28, 29, 36]. Also mutations in the clock Per2 gene have been shown to modulate alcohol consumption in both mice and humans [35]. How alcohol consumption alters circadian patterns and how clock genes influence alcohol consumption remain unknown.

**Negative affect produced by chronic alcohol.** In addition to circadian disruptions it is also possible that other actions of chronic alcohol exposure could impinge on sleep. In the
hypothalamic integrator model of Saper et al. [32], the hypothalamus not only integrates circadian influences on sleep, but is also the site where other environmental cues, including emotional inputs from limbic structures, influence sleep. In the allostatic state model of drug addiction put forth by Koob and Le Moal [16], compulsive drug seeking behavior is driven by both drug induced activation of brain reward systems and also negative affect due to drug induced upregulation of antireward systems. In the alcohol addicted state Heilig and Koob [14] have put forth that this antireward system originates by excess actions of corticotropin-releasing factor (CRF) caused by CRF1 receptor upregulation in the amygdala. CRF is known to disrupt sleep [10, 38] and CRF hyperactivity has been put forth as mediating the hyperarousal associated with primary insomnia [30]. These findings suggest that the negative affective state produced by chronic alcohol, perhaps mediated by CRF acting in the amygdala, may be a type of emotional input into the hypothalamic regulator that produces fragmentation of VC. Future studies will be needed to test this hypothesis.

**Conclusion.** Our analysis of sleep in rats that utilized the concepts of LDW and VC has enabled us to identify an extended sleep period in these polyphasic sleeping animals. Using this analysis we show that chronic alcohol treatment in rats also causes a disruption in the ability to maintain long sleep episodes (i.e., extended periods of VC in the light) similar to the insomniac effects observed in human alcoholics and recovered alcoholics [1, 5, 8, 12, 13, 34, 39]. In addition, we found that the normal circadian pattern of sleep was altered by alcohol. Another important aspect from this study is alcohol can have different actions on the distribution of LDW and VC versus the behavior of SWS and BW within VC. For example, in the light period VC is fragmented, but the behavior within VC is normal. This provides further support for our previous conclusion that LDW and BW are differently regulated [33] and demonstrates the
usefulness of the analysis of sleep patterns used in this study. Additional studies will be needed to fully explore the nature of alcohol induced alterations in sleep, but our findings begin to define the nature of these alterations, and with an increase understanding of the neuronal mechanisms that underlie the different components of sleep, will point to mechanisms that account for the actions of alcohol on sleep.
Fig. 1
Figure 1. Vigilance state diagram for alcohol treated (open symbols and bars; n = 8) and pair-fed (filled symbols and bars; n = 6) rats after 6 weeks of alcohol treatment. In this and all subsequent graphs shaded portion of graphs indicates dark period and unshaded area light period. Panels A, C, and E indicate percent of time spent in indicated vigilance state in two-hour time blocks. Panels B, D, and F summarize the distribution of vigilance states across dark and light periods. Panels G, H, and I illustrate the total amount of each vigilance state across the entire 22 recording period. Asterisks indicate that the alcohol treated value is statistically different from pair-fed value at the same time (*p<0.05, **p<0.005). Pound signs indicate the light period value is significantly different from the corresponding dark period value within the treatment group (#p<0.05, ##p<0.005).
Representative hypnograms of pairfed control (top two panels) and alcohol treated rats (bottom two panels) after 6 weeks of treatment. Panel A and B are hypnograms from two pair-fed control rats, PF1 and PF2. Panel C and D are hypnograms from two alcohol treated rats, ET8 and ET9. R, S, and W on the vertical axis indicates REMS, SWS, and wake, respectively. The lines above the hypnogram (labeled VC on the vertical axis) indicate periods of vigilance cycling.
Slow-wave amplitude (SWA) after 6 weeks of treatment for pair-fed (filled symbols, n = 6) and alcohol treated (open symbols, n = 8) animals are illustrated in panel A. Asterisks indicate data points from pair-fed animals that are significantly different from the peak value (data point with extra circle; one-way ANOVA; p<0.05). In the lower panels are illustrated the averaged power spectra for the 1st 2 hours in the dark period (panel B) and 1st 2 hours of the light period (panel C). Above the data points are lines that indicate the δ and θ frequency ranges.
**Figure 4.** Vigilance state diagram for alcohol treated (open symbols and bars; n = 6) and pair-fed (filled symbols and bars; n = 6) rats 16 days after complete cessation of alcohol exposure. Panels **A, C,** and **E** indicate percent of time spent in indicated vigilance state in two-hour time blocks. Panels **B, D,** and **F** summarize the distribution of vigilance states across dark and light periods. Panels **G, H,** and **I** illustrate the total amount of each vigilance state across the entire 22 recording period. Pound signs indicate the light period value is significantly different from the corresponding dark period value within the treatment group (###p<0.005).
Slow-wave amplitude (SWA) on day 16 after cessation of alcohol treatment for pair-fed (filled symbols, n = 6) and alcohol treated (open symbols, n = 6) animals are illustrated in panel A. Asterisks indicate data points from pair-fed animals that are significantly different from the peak value (data point with extra circle; one-way ANOVA; p<0.05). Pound signs indicate data points from alcohol treated animals that are significantly different from the peak value (data point with extra circle; one-way ANOVA; p<0.05). In the lower panels are illustrated the averaged power spectra for the 1st 2 hours in the dark period (panel B) and 1st 2 hours of the light period (panel C). Above the data points are lines that indicate the δ and θ frequency ranges.
Fig. 6

(A) % wake over time with error bars showing significant differences.
(B) Wake min/2h for different treatments.
(C) % SWS over time with error bars showing significant differences.
(D) SWS min/2h for different treatments.
(E) % REMS over time with error bars showing significant differences.
(F) REMS min/2h for different treatments.

** Data points significant at p < 0.05
## Data points significant at p < 0.01
**Figure 6.** Vigilance state diagram for alcohol treated (open symbols and bars; n = 8) and pair-fed (filled symbols and bars; n = 6) rats on day 2 after complete cessation of alcohol exposure. Panels **A, C,** and **E** indicate percent of time spent in indicated vigilance state in two-hour time blocks. Panels **B, D,** and **F** summarize the distribution of vigilance states across dark and light periods. Panels **G, H,** and **I** illustrate the total amount of each vigilance state across the entire 22 recording period. Asterisks indicate that the alcohol treated value is statistically different from pair-fed value at the same time (*p<0.05, **p<0.005). Pound signs indicate the light period value is significantly different from the corresponding dark period value within the treatment group (##p<0.005).
Vigilance state diagram for dark onset administration of alcohol (n = 20, left two sets of panels) and light onset administration of alcohol (n = 19, right two sets of panels) after water gavage control (filled symbols and bars) and alcohol gavage (open symbols and bars). The shaded portion of graphs indicates dark period and unshaded portion of the graph indicates light period. Graphs A, C, and E (dark onset alcohol administration) and graphs G, I, and K (light onset alcohol administration) indicate percent of time spent in indicated vigilance state in two-hour time blocks. Panels B, D, and F (dark onset alcohol administration) and H, I, and J (light onset alcohol administration) summarize the distribution of vigilance states across dark and light period. Asterisks indicate that the alcohol treated value is statistically different from pair-fed value at the same time (*p<0.05, **p<0.005). Pound signs indicate the light period value is significantly different from the corresponding dark period value within the treatment group (#p<0.05, ##p<0.005).
Representative hypnograms from two rats from dark onset (top two hypnograms) and light onset (bottom two hypnograms) recordings. Control (water gavage) hypnograms are E6 co and C4 co and alcohol gavages are E6 et and C4 et. R, S, and W on the vertical axis indicates REMS, SWS, and wake, respectively. The lines above the hypnogram (labeled VC on the vertical axis) indicate periods of vigilance cycling.
Slow-wave amplitude (SWA) after water gavage (filled circle) and alcohol gavage (open circle) after dark onset administration of alcohol (Panel A, n = 20) and after light onset administration of alcohol (Panel B, n = 19). Results were analyzed by two way ANOVA (treatment x time). After alcohol treatment the SWA increased in the first two hour in the dark period and decreased in the first two hours in the light period (** p< 0.005).
Table 1. Summary of the effect of 6 week treatment with alcohol on sleep parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pair-fed</th>
<th>Alcohol-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dark period</td>
<td>Light period</td>
</tr>
<tr>
<td>Long duration wake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of LDW episodes</td>
<td>17.0 ± 1.0</td>
<td>5.3 ± 0.8</td>
</tr>
<tr>
<td>Duration of LDW</td>
<td>1430 ± 150</td>
<td>1020 ± 160</td>
</tr>
<tr>
<td>% time in LDW</td>
<td>55.3 ± 4.2</td>
<td>14.9 ± 2.5 **</td>
</tr>
<tr>
<td>Vigilance cycling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of VC episodes</td>
<td>17.2 ± 0.9</td>
<td>5.2 ± 0.7</td>
</tr>
<tr>
<td>Duration of VC</td>
<td>1130 ± 100</td>
<td>6860 ± 1090 #</td>
</tr>
<tr>
<td>% time in VC</td>
<td>44.3 ± 4.3</td>
<td>85.1 ± 2.5 **</td>
</tr>
<tr>
<td>Brief wake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW episode duration</td>
<td>35.3 ± 2.3</td>
<td>33.6 ± 0.9</td>
</tr>
<tr>
<td>BW (% of VC)</td>
<td>35.2 ± 3.6</td>
<td>22.9 ± 1.7 **</td>
</tr>
<tr>
<td>Slow-wave sleep</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS episode duration</td>
<td>55.7 ± 9.4</td>
<td>89.3 ± 11.8 #</td>
</tr>
<tr>
<td>SWS (% of VC)</td>
<td>55.2 ± 2.7</td>
<td>58.7 ± 2.5</td>
</tr>
<tr>
<td>REMS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>REMS episode duration</td>
<td>48.0 ± 6.6</td>
<td>76.8 ± 5.9 **</td>
</tr>
<tr>
<td>REMS (% of VC)</td>
<td>9.6 ± 1.3</td>
<td>18.4 ± 1.1 **</td>
</tr>
</tbody>
</table>

Durations are reported in sec. No. of LDW episodes is the number of episodes assigned to LDW within the light or dark period. Duration of LDW is the average duration of LDW episodes within the period. Percent time in LDW and VC are relative to entire dark or light period. BW, SWS, and REMS (% of VC) are the percent of time within VC that is occupied by the respective state. BW, SWS, and REMS episode durations are the average duration of episodes that occurred within VC. Values are averages ± SE; N = 8 for alcoholic animals and N = 6 for pair-fed animals. Pound sign: significantly different from dark period for same treatment (#p<0.05, ##p<0.005). Asterisk: significantly different from pair-fed in same period (* p<0.05, ** p<0.005).
Table 2. Summary of sleep parameters on day 16 following after cessation of alcohol.

<table>
<thead>
<tr>
<th>parameter</th>
<th>pair-fed dark period</th>
<th>light period</th>
<th>alcohol-treated dark period</th>
<th>light period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long duration Wake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of LDW episodes:</td>
<td>18.7 ± 1.8</td>
<td>5.5 ± 0.6</td>
<td>14.7 ± 1.7</td>
<td>6.0 ± 0.9</td>
</tr>
<tr>
<td>Duration of LDW:</td>
<td>1310 ± 100</td>
<td>1100 ± 60 #</td>
<td>1730 ± 190</td>
<td>1050 ± 60 #</td>
</tr>
<tr>
<td>% time in LDW:</td>
<td>54.9 ± 2.6</td>
<td>17.1 ± 2.6 #</td>
<td>55.4 ± 3.4</td>
<td>17.8 ± 3.5 #</td>
</tr>
<tr>
<td>Vigilance Cycling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of VC episodes:</td>
<td>19.2 ± 1.8</td>
<td>5.3 ± 0.6</td>
<td>15.0 ± 1.8</td>
<td>6.5 ± 1.0</td>
</tr>
<tr>
<td>Duration of VC:</td>
<td>1090 ± 150</td>
<td>6940 ± 1720 #</td>
<td>1470 ± 330</td>
<td>5580 ± 890 #</td>
</tr>
<tr>
<td>% time in VC:</td>
<td>44.8 ± 2.7</td>
<td>82.8 ± 2.6 #</td>
<td>44.4 ± 3.5</td>
<td>82.2 ± 3.5 #</td>
</tr>
<tr>
<td>Brief wake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW episode duration:</td>
<td>33.5 ± 2.2</td>
<td>30.6 ± 1.4</td>
<td>32.6 ± 1.3</td>
<td>31.2 ± 1.5</td>
</tr>
<tr>
<td>BW (% of VC):</td>
<td>36.7 ± 2.0</td>
<td>24.2 ± 1.6 #</td>
<td>36.4 ± 1.2</td>
<td>27.5 ± 2.2 #</td>
</tr>
<tr>
<td>Slow-Wave Sleep</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS episode duration:</td>
<td>48.4 ± 5.1</td>
<td>79.2 ± 10.7 #</td>
<td>46.0 ± 2.5</td>
<td>68.1 ± 7.0 #</td>
</tr>
<tr>
<td>SWS (% of VC):</td>
<td>55.6 ± 1.9</td>
<td>60.1 ± 2.5</td>
<td>53.9 ± 1.4</td>
<td>57.9 ± 1.6</td>
</tr>
<tr>
<td>REMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REMS episode duration:</td>
<td>50.4 ± 4.9</td>
<td>82.1 ± 6.1 #</td>
<td>53.1 ± 4.7</td>
<td>72.4 ± 4.2 #</td>
</tr>
<tr>
<td>REMS (% of VC):</td>
<td>7.8 ± 0.9</td>
<td>15.7 ± 1.2 #</td>
<td>9.7 ± 1.5</td>
<td>14.6 ± 1.5 #</td>
</tr>
</tbody>
</table>

Durations are reported in sec. No. of LDW episodes is the number of episodes assigned to LDW within the light or dark period. Duration of LDW is the average duration of LDW episodes within the period. Percent time in LDW and VC are relative to entire dark or light period. BW, SWS, and REMS (% of VC) are the percent of time within VC that is occupied by the respective state. BW, SWS, and REMS episode durations are the average duration of episodes that occurred within VC. Values are averages ± SE; N = 8 for alcoholic animals and N = 6 for pair-fed animals. Pound sign: significantly different from dark period for same treatment (#p<0.05, ##p<0.005). Asterisk: significantly different from pair-fed in same period (* p<0.05, ** p<0.005).
Table 3. Summary of sleep parameters day 2 after cessation of alcohol treatment.

<table>
<thead>
<tr>
<th>parameter</th>
<th>pair-fed dark period</th>
<th>light period</th>
<th>alcohol-treated dark period</th>
<th>light period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long duration Wake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of LDW episodes:</td>
<td>20.5 ± 2.1</td>
<td>4.7 ± 0.7</td>
<td>13.9 ± 2.1 *</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td>Duration of LDW:</td>
<td>1280 ± 100</td>
<td>920 ± 60 #</td>
<td>1890 ± 300 *</td>
<td>1240 ± 130 *#</td>
</tr>
<tr>
<td>% time in LDW:</td>
<td>58.6 ± 2.4</td>
<td>12.3 ± 2.3 ##</td>
<td>49.7 ± 2.7 *</td>
<td>23.2 ± 3.2 *##</td>
</tr>
<tr>
<td>Vigilance Cycling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of VC episodes:</td>
<td>21.0 ± 2.1</td>
<td>5.0 ± 0.8</td>
<td>14.4 ± 2.3</td>
<td>7.3 ± 1.0</td>
</tr>
<tr>
<td>Duration of VC:</td>
<td>930 ± 140</td>
<td>8680 ± 1790 ##</td>
<td>1760 ± 260</td>
<td>5210 ± 1490 ##</td>
</tr>
<tr>
<td>% time in VC:</td>
<td>41.3 ± 2.5</td>
<td>87.7 ± 2.3 ##</td>
<td>48.3 ± 1.7</td>
<td>76.8 ± 3.2 ##</td>
</tr>
<tr>
<td>Brief wake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW episode duration:</td>
<td>30.2 ± 2.2</td>
<td>30.1 ± 1.8</td>
<td>28.5 ± 1.3</td>
<td>28.1 ± 0.9</td>
</tr>
<tr>
<td>BW (% of VC):</td>
<td>41.0 ± 2.8</td>
<td>24.7 ± 1.7 ##</td>
<td>26.7 ± 2.1 **</td>
<td>25.6 ± 1.1</td>
</tr>
<tr>
<td>Slow-Wave Sleep</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS episode duration:</td>
<td>38.2 ± 6.8</td>
<td>77.3 ± 8.2 ##</td>
<td>63.5 ± 6.5 *</td>
<td>64.7 ± 4.7</td>
</tr>
<tr>
<td>SWS (% of VC):</td>
<td>51.9 ± 2.4</td>
<td>59.0 ± 2.4 #</td>
<td>60.4 ± 2.1 *</td>
<td>58.2 ± 1.3</td>
</tr>
<tr>
<td>REMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REMS episode duration:</td>
<td>45.1 ± 3.8</td>
<td>71.4 ± 5.1 ##</td>
<td>61.4 ± 3.5</td>
<td>70.5 ± 4.5 ##</td>
</tr>
<tr>
<td>REMS (% of VC):</td>
<td>7.1 ± 1.6</td>
<td>16.2 ± 0.9 ##</td>
<td>12.9 ± 0.7 **</td>
<td>16.2 ± 0.6 #</td>
</tr>
</tbody>
</table>

Durations are reported in sec. No. of LDW episodes is the number of episodes assigned to LDW within the light or dark period. Duration of LDW is the average duration of LDW episodes within the period. Percent time in LDW and VC are relative to entire dark or light period. BW, SWS, and REMS (% of VC) are the percent of time within VC that is occupied by the respective state. BW, SWS, and REMS episode durations are the average duration of episodes that occurred within VC. Values are averages ± SE; N = 8 for alcoholic animals and N = 6 for pair-fed animals. Pound sign: significantly different from dark period for same treatment (#p<0.05, ##p<0.005). Asterisk: significantly different from pair-fed in same period (* p<0.05, ** p<0.005).
Table 4. Summary of sleep parameters acute dark onset administration of alcohol.

<table>
<thead>
<tr>
<th>parameter</th>
<th>control dark period</th>
<th>light period</th>
<th>acute alcohol dark period</th>
<th>light period</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Long duration wake</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of LDW episodes:</td>
<td>18.5 ± 0.9</td>
<td>5.0 ± 0.4</td>
<td><strong>17.3 ± 1.0</strong></td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>Duration of LDW:</td>
<td>1360 ± 70</td>
<td>1460 ± 110</td>
<td><strong>1100 ± 80</strong></td>
<td>1220 ± 110</td>
</tr>
<tr>
<td>% time in LDW:</td>
<td>56.0 ± 2.0</td>
<td>15.4 ± 0.8</td>
<td><strong>41.6 ± 2.3</strong></td>
<td>15.0 ± 0.7</td>
</tr>
<tr>
<td><strong>Vigilance cycling</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of VC episodes:</td>
<td>18.2 ± 0.9</td>
<td>5.1 ± 0.3</td>
<td><strong>17.4 ± 0.9</strong></td>
<td>6.4 ± 0.3</td>
</tr>
<tr>
<td>Duration of VC:</td>
<td>1110 ± 100</td>
<td>8100 ± 600</td>
<td><strong>1560 ± 100</strong></td>
<td>6430 ± 310</td>
</tr>
<tr>
<td>% time in VC:</td>
<td>43.8 ± 2.0</td>
<td>84.5 ± 0.8</td>
<td><strong>58.3 ± 2.3</strong></td>
<td>84.9 ± 0.7</td>
</tr>
<tr>
<td><strong>BW</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW episode duration:</td>
<td>35.9 ± 1.5</td>
<td>37.7 ± 1.5</td>
<td>31.8 ± 1.4</td>
<td>34.2 ± 1.3</td>
</tr>
<tr>
<td>BW (% of VC):</td>
<td>37.1 ± 1.6</td>
<td>23.7 ± 0.7</td>
<td><strong>30.2 ± 1.7</strong></td>
<td>27.7 ± 1.3</td>
</tr>
<tr>
<td><strong>Slow-Wave Sleep</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS episode duration:</td>
<td>53.7 ± 4.0</td>
<td>96.0 ± 4.5</td>
<td><strong>64.2 ± 3.9</strong></td>
<td>73.2 ± 4.5</td>
</tr>
<tr>
<td>SWS (% of VC):</td>
<td>56.8 ± 1.4</td>
<td>59.4 ± 0.7</td>
<td><strong>62.1 ± 1.5</strong></td>
<td>56.9 ± 0.9</td>
</tr>
<tr>
<td><strong>REMS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REMS episode duration:</td>
<td>53.3 ± 3.7</td>
<td>88.6 ± 3.6</td>
<td><strong>53.2 ± 3.3</strong></td>
<td>77.7 ± 3.2</td>
</tr>
<tr>
<td>REMS (% of VC):</td>
<td>6.2 ± 0.7</td>
<td>17.0 ± 0.6</td>
<td><strong>7.8 ± 0.7</strong></td>
<td>15.4 ± 0.9</td>
</tr>
</tbody>
</table>

Durations are reported in sec. # of LDW episodes is the number of episodes assigned to LDW within the light or dark period. Duration of LDW is the average duration of LDW episodes within the period. Percent time in LDW and VC are relative to entire dark or light period. Percent AR, SWS, and REMS within VC are the percent of time within VC that is occupied by the respective state. Duration of BW, SWS, and REMS episodes within VC are the average duration of episodes that occurred within VC. Values are averages ± SE; N = 20 animals. Asterisks: significantly different from control for a particular circadian period. Pound symbol: significantly different from dark for a particular treatment (*p<0.05, **p<0.005, 2 way ANOVA, time x treatment).
Table 5. Summary of sleep parameters after acute light onset administration of alcohol.

<table>
<thead>
<tr>
<th>parameter</th>
<th>control Light period</th>
<th>dark period</th>
<th>acute alcohol light period</th>
<th>dark period</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of LDW episodes:</td>
<td>5.8 ± 0.4</td>
<td>15.9 ± 1.1 *</td>
<td>9.2 ± 0.5 **</td>
<td>14.1 ± 0.9 **</td>
</tr>
<tr>
<td>Duration of LDW:</td>
<td>1180 ± 80</td>
<td>1660 ± 110 *</td>
<td>990 ± 60</td>
<td>1590 ± 130</td>
</tr>
<tr>
<td>% time in LDW:</td>
<td>15.8 ± 1.3</td>
<td>57.0 ± 1.9</td>
<td>20.4 ± 1.1</td>
<td>48.0 ± 2.5 **</td>
</tr>
<tr>
<td>Vigilance cycling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of VC episodes:</td>
<td>5.9 ± 0.4</td>
<td>16.1 ± 1.1 *</td>
<td>9.3 ± 0.5 **</td>
<td>14.7 ± 1.0 **</td>
</tr>
<tr>
<td>Duration of VC:</td>
<td>6840 ± 430</td>
<td>1310 ± 140 *</td>
<td>4160 ± 390 **</td>
<td>1650 ± 130 *</td>
</tr>
<tr>
<td>% time in VC:</td>
<td>84.1 ± 1.3</td>
<td>42.9 ± 1.9 *</td>
<td>79.5 ± 1.1</td>
<td>51.9 ± 2.5 **</td>
</tr>
<tr>
<td>BW</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW episode duration:</td>
<td>30.3 ± 1.1</td>
<td>33.2 ± 0.9</td>
<td>32.4 ± 2.6</td>
<td>31.2 ± 1.1</td>
</tr>
<tr>
<td>BW (% of VC):</td>
<td>29.2 ± 1.0</td>
<td>40.8 ± 1.1 **</td>
<td>29.2 ± 1.3</td>
<td>37.3 ± 1.4 **</td>
</tr>
<tr>
<td>Slow-Wave Sleep</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWS episode duration:</td>
<td>63.9 ± 3.7</td>
<td>39.0 ± 1.7 *</td>
<td>58.1 ± 2.7</td>
<td>39.4 ± 1.7 **</td>
</tr>
<tr>
<td>SWS (% of VC):</td>
<td>56.7 ± 1.0</td>
<td>48.9 ± 1.0 *</td>
<td>54.3 ± 1.4</td>
<td>47.6 ± 1.5 **</td>
</tr>
<tr>
<td>REMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REMS episode duration:</td>
<td>68.6 ± 2.9</td>
<td>56.7 ± 5.2 *</td>
<td>73.4 ± 2.0</td>
<td>57.2 ± 2.2 **</td>
</tr>
<tr>
<td>REMS (% of VC):</td>
<td>14.1 ± 1.0</td>
<td>10.3 ± 0.9 *</td>
<td>16.5 ± 0.7 **</td>
<td>15.1 ± 0.9 **</td>
</tr>
</tbody>
</table>

Durations are reported in sec. # of LDW episodes is the number of episodes assigned to LDW within the light or dark period. Duration of LDW is the average duration of LDW episodes within the period. Percent time in LDW and VC are relative to entire dark or light period. Percent AR, SWS, and REMS within VC are the percent of time within VC that is occupied by the respective state. Duration of BW, SWS, and REMS episodes within VC are the average duration of episodes that occurred within VC. Values are averages ± SE; N = 19 animals. Asterisks: significantly different from control for a particular circadian period. Pound symbol: significantly different from dark for a particular treatment (*p<0.05, **p<0.005, 2 way ANOVA, time x treatment).
ACKNOWLEDGEMENTS

This work was supported by grant no. AA13248 from the NIAAA awarded to S.M.S.
REFERENCES


169


Chapter 5 General discussion and conclusion

Six percent alcohol in liquid diet for 6 weeks is the minimum protocol required to see robust sleep alterations in the rats. This includes decrement in the circadian variation in the distribution of wake, SWS, and REMS across light and dark period. Six weeks of alcohol treatment causes fragmentation of VC and decreases REMS in the light period (sleep period) similar to the findings in human literature [Brower et al., 2001, Mello et al., 1970]. This alteration in the sleep patterns was due to the adaptation to chronic alcohol treatment and not due to the acute presence of alcohol. This is supported by the observation that the sleep pattern was still altered 2 days after stopping the alcohol treatment. Furthermore, acute alcohol induced alteration of sleep was different from disturbed sleep after chronic alcohol treatment. However, the altered sleep patterns in these animals was not long-lasting as the sleep patterns returned to control level around 16 days after cessation of alcohol treatment.

A more accurate description of sleep in rats can be made by the inclusion of the concepts of LDW versus BW and periods of VC. The duration of LDW does not change between light and dark period, although the characteristic of VC alters across day and night. The sleep pressure within VC wanes as VC episode progresses, which suggests that VC is highly regulated. Although rats are thought of as episodic sleepers, prior to our study a formal definition of when these sleep episodes begin and end has not been put forth. One of the outcomes of this analysis is defining a sleep unit in rats, the VC. Like rats, human also shows brief wakes [Lo et al, 2004]. However, unlike the repeated LDW episodes observed in rats throughout the day, humans show only one LDW episode of ~16 h duration. One suggestion is that a single VC is similar to an 8 h sleep episode in human. An alternative view would be that a VC is equivalent to a sleep
cycle (REMS-REMS cycle) in humans. In the later case the difference between sleep in rats and humans would be that in humans the cycles are not interrupted by LDW episodes, as is observed in the rat.

It is reasonable to conclude that because episode stability is different between LDW and BW, they are likely to have some difference in the brain structures that support this stability. Support for this conclusion comes from the observations of Blanco-Centurion et al. (2007) and Mochizuki et al. (2004) in which different lesions caused selective disruption of what we would label BW episodes and LDW episodes. Using these studies as guides we speculate that the relatively rapid transitions between SWS and BW, and between SWS and REMS, within a VC would be the domain of sleep-wake and REMS flip-flop switches [Saper et al., 2002; Lu et al., 2006]. However, the regulation between VC and LDW is likely to take place at a different level, perhaps within the DMH, a nuclei that has been proposed to be the integrator of circadian, emotional, visceral, and sensory information [Saper et al., 2005]. On top of the DMH integrator would be sleep homeostatic drive, possibly mediated by the accumulation of adenosine in the forebrain [Porkka-Heiskanen et al., 1997] or cytokines [Krueger et al., 1999]. Both homeostatic control and hypothalamic integrator impinge on the VLPO, which in turn, appears to be an important switch in the control of the sleep-wake flip-flop switch [Pace-Schott and Hobson, 2002]. Futher investigation is required to explore these hypotheses and understand the mechanistic basis of the timing of VC and LDW and the timing between SWS, BW, and REMS.

An important reason for understanding these mechanisms is that in addition to REMS suppression in the early light period, the major effect of chronic alcohol was to disrupt the normally long periods of VC that occur in the light period with LDW episodes. We speculate that the increased sleep in the dark period was in part due to the need for the animals to make up
for the loss of sleep in the light period because of these disruptions. Thus it would also appear that the homeostatic drive for sleep is intact after the alcohol treatment. Once a mechanistic basis for the distribution of LDW and VC is obtained, it might lead to plausible mechanisms that underlie the alcohol-induced disturbed sleep patterns. A possible speculation is that this is due to increases in CRF action in the amygdala. For example, alcohol treatment in rats increases CRF 1 receptor expression in the amygdala, which has been suggested to have a role in mediating the negative affect of alcohol addiction [Heleig and Koob, 2007]. In support of this others have shown that CRF can disturb sleep [Ehler et al., 1997; Uchida et al., 2007; Roth et al., 2007]. Our hypothesis would be that CRF dysregulation in the amygdala impinges on the DMH (a nucleus thought to integrate the effects of emotional and affective states on the sleep regulatory system), to cause the disturbed sleep in the light period. Perhaps with increasing periods of time without alcohol, the negative affective state produced by alcohol withdrawal eventually impinges on the LDW-VC regulator to intrude on VC episodes. The rat then wakes in response to this dysphoria and proceeds to drink to relieve the distress, and in so doing generates a LDW episode. Because the amygdala is also an important mediator of stress on behavior, this linkage through the amygdala can account for multiple effects of alcohol withdrawal and the compulsion to continue drinking. Therapies specifically directed toward repairing this circuit might be able to relieve the dysphoria of alcohol withdrawal, and in so doing, also resolve some of the sleep issues caused by alcoholism. These speculations are testable in future experiments.

There are several additional areas that it would be useful to fully develop a rat model for the effects of chronic alcohol on sleep. One of the shortcomings of the model used in this study is that the motivation for alcohol consumption is mixed with the motivation for seeking food or water. Thus future investigations should be designed to separate the motivation for seeking food
and water from the motivation for seeking alcohol. This could be achieved by getting rats to spontaneously drink alcohol via a period of force drinking followed by offering alcohol in a two-bottle free-choice paradigm.

A second shortcoming was that after chronic alcohol treatment altered sleep parameters returned to control level by 16 days after the cessation of alcohol treatment. This is in contrast to protracted sleep impairment observed in human alcoholics after complete abstinence from drinking. Additional studies to explore the possibility of inducing more enduring changes in the sleep regulatory circuitry in the brain should be explored. These might be accomplished by exposing rats to even longer periods of alcohol treatment (e.g. 12 weeks or 6 months), by increasing the dose of alcohol, or by inducing recurrent withdrawal from alcohol treatment, a manipulation thought to produce more profound physiological alterations.

Future studies addressing the regulation of VC and LDW could be designed in several ways. For example, lesioning various arousal centers of the brains, such as chateholaminergic neurons of LC, histaminergic neurons of TMN and cholinergic neurons in the basal forebrain, either alone or in combinations, could provide as insight as how these activation centers of the brain modulate VC and LDW. We predict that these will alter brief wakes because we speculate that the BW and SWS within VC are generated by mutual inhibitory interaction between the sleep promoting neurons in the vLPO and wake promoting histaminergic neurons in the TMN and other arousal systems in the brain (flip-flop switch). It would be interesting to see how these lesions will alter SWS and REMS within vigilance cycling. Again it would be exciting to see the effects on VC and LDW after lesioning DMH. We predict that it will decrease the duration of LDW since we theorize that the input from this hypothalamic integrator locks the vigilance state into long duration wake. To determine the effects of circadian input to the characters of VC and
LDW, we could lesion SCN. We predict that the light and dark period variation in the characters of VC will be lost after this lesion. We could also determine the character of VC and LDW after keeping rats at constant darkness to determine the effect of light and dark on VC and LDW characters.

Another interesting study could be determining the effects of sleep restrictions on the behavior of VC and LDW. We could impose a period of sleep restriction (e.g. six hours) at the beginning of the dark period or light period and determine the effects on VC and LDW. We predict that after acute sleep restriction VC will be longer in duration. But it will be interesting to know how the SWS and REMS will alter within VC. Thus we might have insight how altering homeostatic sleep pressure alters VC and its characters. We could also impose a chronic sleep restriction with a definite sleep patterns, for example by allowing rats to go periodically to VC ~43% of time in the light period like they have in dark period and see how it alters the characters of their VC in the dark period. We predict that rats will have light period like VC character in the dark period.

This novel analysis shows a more complete description of rat’s sleep pattern. However, we need to use this analysis beyond rat to determine the sleep patterns of other species with polyphasic sleeping patterns such as mouse. We could then determine how the character of long duration wake and vigilance cycling vary across species with polyphasic sleeping habit.
REFERENCES


