

**EFFECTS OF SLEEP DEPRIVATION IN ADOLESCENT RATS ON ANXIETY-LIKE
BEHAVIOR AND ALCOHOL CONSUMPTION INTO ADULTHOOD**



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EFFECTS OF SLEEP DEPRIVATION IN ADOLESCENT RATS ON ANXIETY-LIKE
BEHAVIOR AND ALCOHOL CONSUMPTION INTO ADULTHOOD

A Senior Comprehensive Project

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Abstract

Alcohol use disorder (AUD) remains a significant global health concern, contributing to millions of preventable deaths annually. Despite extensive research on AUD, the interplay between sleep disturbances during critical developmental periods, such as adolescence, and subsequent alcohol consumption and anxiety-like behaviors in adulthood remains inadequately understood. Chronic sleep deprivation in adolescence is increasingly recognized as a risk factor for heightened susceptibility to substance use disorders, as this period is crucial for neurodevelopment, marked by significant changes in brain function related to decision-making, emotional regulation, and reward processing. Disruptions in sleep during adolescence can disturb neurotransmitter balance and neurocircuitry, leading to lasting alterations in behavior and mental health. Thus, the intricate relationship between chronic sleep deprivation, alcohol use, and subsequent anxiety-like behaviors is crucial to study given the enduring impact of early-life experiences on long-term health outcomes. The present study explores the potential link between chronic sleep deprivation during adolescence and subsequent alcohol consumption and anxiety-like behavior in female rats during adulthood. Adolescent female Sprague Dawley rats underwent sleep deprivation or served as controls, followed by anxiety assessment using the elevated plus maze (EPM). In adulthood, rats were provided access to alcohol, and anxiety-like behaviors were retested post-alcohol exposure. Sleep deprived rats showed heightened anxiety-like behaviors during adolescence but did not exhibit exacerbated anxiety in adulthood. However, they displayed increased alcohol intake and preference, indicating an impact on reward-seeking behaviors. These findings underscore adolescents' vulnerability to early-life stressors like sleep deprivation and highlight the importance of addressing sleep health and stress management to prevent the onset of substance use disorders later on in life.

Introduction

Alcohol use disorder (AUD) is one of the leading causes of preventable death worldwide, as it takes the lives of around 3 million people per year, making it an urgent public health issue and one of the most prevalent disorders globally. The disorder is characterized mainly by impaired control over the consumption of alcohol, as well as continued use despite negative consequences to one's health and the lives of others (Daviet et al. 2022). A multitude of physiological processes are disrupted or altered due to AUD, as the disorder is associated with functional declines in processes like memory and cognition and structural changes like the loss of brain volume in gray and white matter (Daviet et al. 2022). Alongside these well-known risks associated with AUD, there is a notable aspect of alcohol's effects that attracts individuals: its depressant and sleep-related properties (Roehrs & Roth, 2001). Many people use alcohol as a means of relaxation and stress relief, as it can induce feelings of calmness and reduce anxiety temporarily. Additionally, alcohol's sedative properties may help individuals cope with sleep disturbances, promoting drowsiness and facilitating sleep initiation (Roehrs & Roth, 2001). However, it's crucial to recognize that reliance on alcohol for these reinforcing effects can contribute to the development of dependency, tolerance, and exacerbation of AUD-related health issues over time. This risk is especially pronounced among vulnerable groups such as adolescents, given that adolescence is a critical period for the onset of substance use disorders (Logan et al. 2018). Thus, it is important to determine how sleep disturbances over the unique developmental period of adolescence can affect alcohol drinking and anxiety-like behaviors later in life.

Because of its central nervous system depressant properties, alcohol has been the subject of many studies that demonstrate its ability to induce sedative effects in individuals who

consume it, and 67% of people with sleep disorders report that using alcohol is effective in helping them sleep (Stein & Friedmann, 2006). Paradoxically, excessive alcohol consumption can actually lead to poorer sleep quality, disrupting essential sleep stages (Stein & Friedmann, 2006). Specifically, rapid eye movement sleep (REM) is suppressed in individuals who consume large amounts of alcohol, and the REM stage of sleep is particularly restorative, as it is important for one to wake up feeling refreshed and is vital for maintaining cognitive functions and memory processes (Shapiro & Flanigan, 1993). This paradox underscores the importance of addressing misconceptions about alcohol's sleep-inducing effects, especially among vulnerable populations like adolescents who may resort to alcohol as a solution for sleep disturbances.

Adolescents are especially susceptible to both sleep disturbances and alcohol use, with many underage drinkers citing that they consume alcohol to experience pleasure, relax, alleviate stress, and facilitate sleep (O'Malley et al. 1998). Recent research indicates that more adolescents are engaging in underage drinking, as evidenced by the fact that the average age of initial alcohol use was 14 in 2003 (National Institute on Alcohol Abuse and Alcoholism, 2006). Adolescent alcohol use can present adverse consequences to one's health, decision-making, and susceptibility to alcoholism later in life, making adolescents a particularly vulnerable population in alcohol research and underage drinking an overall public health crisis (National Institute on Alcohol Abuse and Alcoholism, 2006). Furthermore, studies revolving around sleep have also focused on adolescents, as they have become increasingly at risk for experiencing sleep deprivation because changes in the transition from childhood to adolescence can produce many adverse effects on their quality and quantity of sleep (Roberts et al., 2009). It appears evident that consuming alcohol and experiencing sleep deprivation during adolescence are closely linked, separately, to the development of anxiety disorders later in life (Jeanblanc, 2015). Therefore,

because issues like sleep disruptions and alcohol abuse can lead to enduring changes in adolescent brain structures and functions that can result in the development of anxiety disorders, it is vital to study how essential physiological processes like sleep at this critical period of neurodevelopment can potentially exacerbate one's propensity to drink more in the future.

Overview of Sleep Neurobiology

Sleep plays a fundamental role in maintaining human health, contributing significantly to cognitive function, immune resilience, and overall physiological well-being (Luyster et al. 2012). From brain function and cardiovascular health to metabolism, mood regulation, and disease resistance, sleep influences a wide array of physiological processes. Scientific evidence links insufficient or poor-quality sleep to heightened risks of various disorders, including hypertension, heart disease, diabetes, mood disorders, and obesity (Luyster et al. 2012). Understanding the intricacies of sleep has become a rapidly expanding field, shedding light on how it regulates our functioning and shapes our overall well-being.

Anatomical Regions Involved in Sleep-Wake States and Circadian Rhythm

Various anatomical regions play crucial roles in regulating sleep-wake states. The hypothalamus, situated deep in the brain, houses nerve cell clusters that control sleep and wakefulness, with the suprachiasmatic nucleus (SCN) managing circadian rhythms based on light input (Falup-Pecurariu et al. 2021). The SCN consists of a pair of compact nuclei located in the ventral and medial regions of the hypothalamus, positioned adjacent to the third ventricle and situated just above the optic chiasm. It receives a significant retinal input through the retinohypothalamic tract (RHT), and employs neuroendocrine and autonomic signals to align rhythms in various tissues, such as cardiovascular, hepatic, pancreatic, adipose, and

gastrointestinal tissues, with its own rhythm (Falup-Pecurariu et al. 2021; Scammell et al. 2017) (Figure 2).

Situated between the brain's hemispheres is the pineal gland, which receives signals from the SCN, leading to heightened production of melatonin derived from the essential amino acid tryptophan (Figure 2)—a hormone crucial for inducing sleep after sunset (Luyster et al. 2012). Both the SCN and pineal gland are responsible for helping to maintain the body's circadian rhythm, as they interact in a coordinated manner to regulate the internal clock of an organism. The circadian rhythm, controlled by clock genes like *BMAL1/BMAL2*, *CLOCK*, *CRY1/CRY2*, and *PER1/PER2/PER3*, orchestrates transcription and translation processes, impacting various signaling pathways within cells (Potter et al. 2016). Autonomously, circadian processes synchronize with the Earth's 24-hour rotation, adjusting sleep/wake behavior and physiological functions in response to environmental changes (Reddy et al. 2023). Melatonin release from the pineal gland is governed by the circadian rhythm, and it influences the SCN, creating a feedback loop within the body's timekeeping system (Scammell et al. 2017). Acting as the circadian pacemaker, the SCN receives inputs from the retinohypothalamic pineal pathway, adjusting melatonin secretion from the pineal gland based on light-dark cycles. As night approaches, the SCN activates the paraventricular nucleus (PVN), stimulating the sympathetic nervous system to induce sleepiness (Scammell et al. 2017). Conversely, during the day, cortisol secretion from the adrenal glands is higher and the inhibitory pathways involving GABA and sympathetic nerve activity suppress melatonin release (Reddy et al. 2023) (Figure 1).

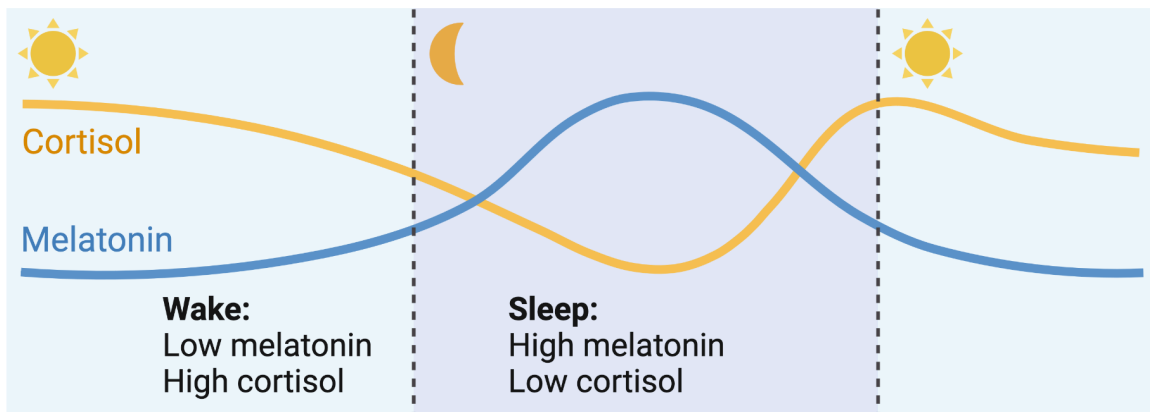


Fig. 1 The diurnal variation in cortisol and melatonin levels over a 24-hour period. The y-axis represents hormone concentration, while the x-axis represents the time of day. The yellow line depicts cortisol levels, showing a peak during the day and a gradual decline towards night, reflecting the typical pattern of higher cortisol levels during wakefulness and lower levels during sleep. The blue line represents melatonin levels, which exhibit an opposite pattern to cortisol. Adapted and made on Biorender.

The basal forebrain is near the front and bottom of the brain and also plays a role in promoting sleep and wakefulness, particularly through the release of the neurotransmitter gamma-aminobutyric acid (GABA) and the hormone somatostatin (Falup-Pecurariu et al. 2021). GABA is an inhibitory neurotransmitter that promotes sleep by reducing the activity of arousal centers in the hypothalamus and brainstem (Bishir et al. 2020); notably, the primary mechanism of action of alcohol is through increasing GABAergic tone (Banerjee, 2014). GABA helps quiet the brain's activity, preparing it for sleep onset and inhibiting any cortical activity. Somatostatin works to regulate sleep by inhibiting wake-active neurons, thus modulating the activity of other neurotransmitters and neuronal circuits involved in the sleep-wake cycle (Bishir et al. 2020) (Figure 2).

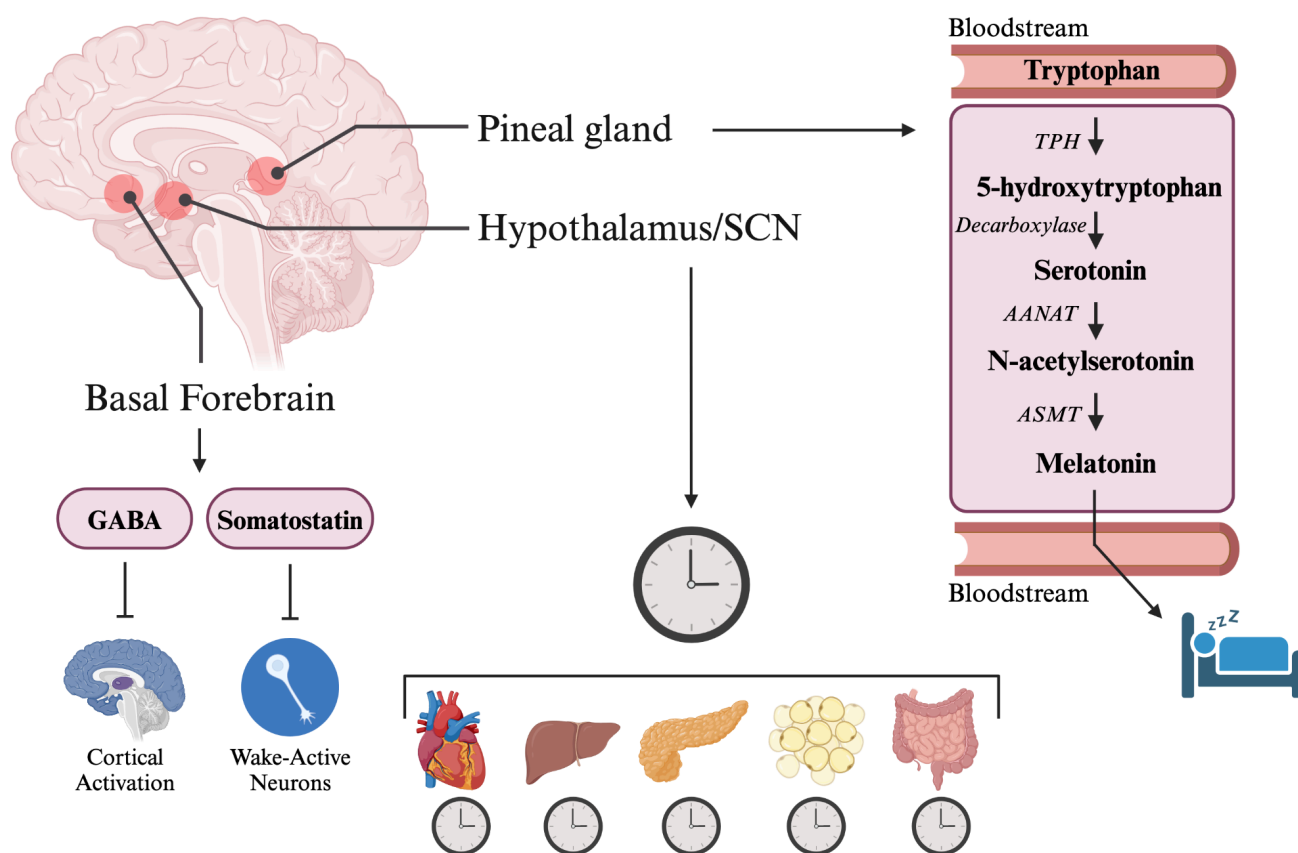


Fig. 2 Main regions of the brain involved in sleep-wake states. The pineal gland synthesizes melatonin from tryptophan, initially converting it to 5-hydroxytryptophan (5-HTP) via tryptophan hydroxylase (TPH). Subsequent enzymatic steps transform 5-HTP into serotonin, then N-acetylserotonin by arylalkylamine N-acetyltransferase (AANAT or ASMT), culminating in melatonin production released into the bloodstream and cerebrospinal fluid. The SCN in the hypothalamus coordinates autonomic and neuroendocrine signals, syncing peripheral clocks in various tissues in the heart, liver, pancreas, adipose, and gastrointestinal tract. The basal forebrain has GABAergic neurons that inhibit cortical activation, while somatostatin inhibits wake-active neurons in this region. Made on Biorender.

Stages of Sleep

Sleep comprises two main types: rapid eye movement (REM) sleep and non-REM (NREM) sleep, which includes three stages (N1, N2, and N3) marked by specific brain waves, neuronal activity, and physiological characteristics (Figure 3) (Luyster et al. 2012). Throughout a

typical night, one cycles through these stages multiple times, with REM sleep becoming more prolonged and intense towards the morning (Luyster et al. 2012).

Stage 1 of non-REM sleep, also known as N1, represents the transition from wakefulness to sleep (Patel et al. 2024). During this phase, which typically lasts 1 to 5 minutes and accounts for about 5% of total sleep time, theta waves with low voltage are recorded on EEG. In N1, there is a shift from alpha waves to low-amplitude mixed-frequency (LAMF) activity, accompanied by muscle tone in skeletal muscles, regular breathing, and a decrease in heart rate, breathing rate, and eye movements (Patel et al. 2024). This light sleep stage is characterized by the initial slowing down of brain waves from their wakeful patterns, leading to the relaxation of muscles with occasional twitches (Spielman et al. 2020).

During stage 2 or N2 sleep, which constitutes about 45% of total sleep time, the body transitions into a deeper state of relaxation. This stage is characterized by the presence of sleep spindles and K-complexes on EEG recordings (Patel et al. 2024). Sleep spindles are rapid bursts of neuronal firing in specific brain regions, believed to play a role in synaptic plasticity and memory consolidation (Fogel & Smith, 2010). K-complexes, on the other hand, are long delta waves associated with maintaining sleep and memory functions (Steriade & Amizica, 1998). As one progresses through stage 2 sleep, one's heart rate and body temperature decrease, muscles relax further, and eye movements cease. This stage is essential for memory consolidation, and its duration increases across sleep cycles, making up a significant portion of the sleep cycle (Spielman et al. 2020).

The last stage of NREM sleep is N3, also known as slow-wave sleep (SWS), which is crucial for feeling refreshed in the morning as it represents the deepest stage of sleep (Patel et al. 2024). During this stage, characterized by delta waves with low frequencies and high amplitudes,

one's heartbeat and breathing reach their lowest levels, making it challenging to be awoken.

Stage 3 sleep is essential for physical restoration, including tissue repair, bone and muscle growth, and immune system strengthening. However, it is also associated with phenomena like sleepwalking, night terrors, and bedwetting. Awakening from this stage may lead to transient mental foginess known as sleep inertia, impacting cognitive performance for a short period after waking up (Patel et al. 2024).

The final stage of the sleep cycle is REM sleep or rapid eye movement sleep, and it is a unique stage of the sleep cycle characterized by rapid movements of the eyes behind closed eyelids (Patel et al. 2024). This phase typically begins about 90 minutes after falling asleep and is associated with vivid dreaming. Interestingly, the brain activity during REM sleep resembles that of wakefulness, with mixed-frequency brain waves similar to beta waves. It is particularly associated with irregular breathing, increased heart rate and blood pressure, and temporary paralysis of the skeletal muscles, except for those involved in circulation and respiration (Patel et al. 2024). REM sleep also plays a critical role in various physiological functions, making it a crucial stage of the sleep cycle. It is associated with brain development through motor learning from muscle twitches, and memory consolidation, particularly in spatial and emotional memory (Peever & Fuller, 2017). It also facilitates synaptic regulation and maintenance, enhancing learning and memory processes (Peever & Fuller, 2017). Furthermore, alcohol's sedative effects have been studied to suppress REM sleep, as during the first half of the night, REM sleep is decreased, leading to a longer time to enter that stage of sleep (Colrain et al. 2014). Thus, understanding the importance of both SWS and REM sleep in the brain underscores the critical need to address how sleep disruptions may negatively impact one's physiological well-being,

including cognitive function, mental health, and susceptibility to issues such as affective disorders or substance abuse, emphasizing the comprehensive impact of sleep on overall health.

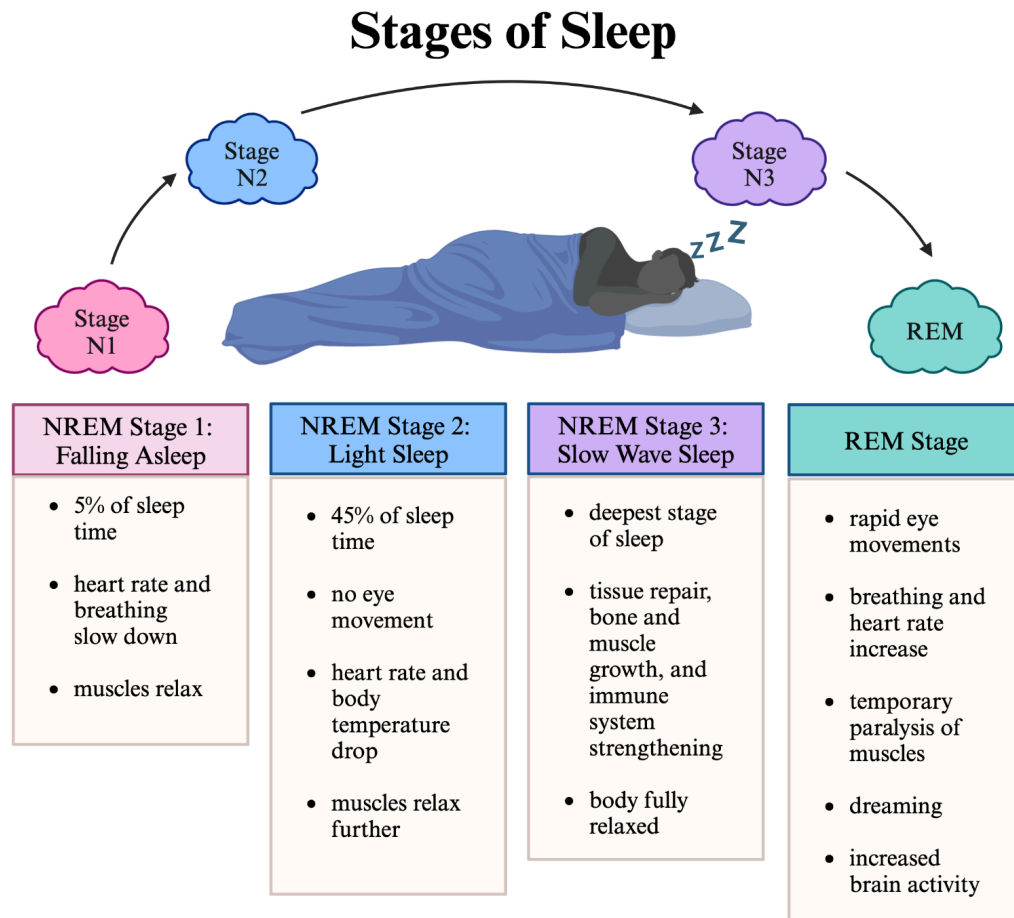


Fig. 3 Summary illustration depicting the stages of sleep and each of their physiological characteristics. Made with Biorender.

Sleep Deprivation and Adverse Consequences of Sleep Loss

Sleep deprivation is characterized by a state of insufficient quantity or quality of sleep, resulting in circadian rhythm sleep disorders, both voluntary and involuntary sleeplessness and negative effects on cognitive functions (Elmenhorst et al., 2009). It affects the brain through various complex mechanisms, influencing both structural and functional aspects of neural activity. Sleep deprivation can disrupt the distinct restorative functions of slow-wave sleep (SWS) and REM sleep, with SWS being essential for macromolecular synthesis and REM sleep

playing a role in clearing the byproducts of SWS to maintain synaptic connections (Shapiro & Flanigan, 1993). Furthermore, there is a suggestion that during REM sleep, neuronal connections within the catecholamine system, which regulates neurotransmitters like dopamine and norepinephrine, are formed, which is believed to be crucial for preserving cognitive function. Experiencing sleep deprivation can alter the balance of these neurotransmitters in the brain, therefore affecting communication between nerve cells (Shapiro & Flanigan, 1993). Another one of the critical neurotransmitters affected is adenosine, which normally promotes sleep and relaxation (Elmenhorst et al., 2009). As an individual experiences sleep deprivation and extended wakefulness over prolonged periods, adenosine levels show a notable increase. This continuous production and accumulation of adenosine within the brain contribute to an elevation in adenosine receptor density, reflecting the brain's response to the prolonged wakefulness, signaling a growing need for rest, and emphasizing the interplay between adenosine and its receptors in regulating the sleep-wake cycle (Elmenhorst et al., 2009). Additionally, adenosine's involvement in alcohol use disorders is evident, as acute ethanol exposure amplifies extracellular adenosine levels, contributing to intoxication, reward, and the sedative effects of alcohol (Nam et al. 2013).

Effects of Sleep Deprivation on Cognitive Function

The impaired cognitive functions caused by sleep deprivation are most likely due to its obstructive effects on synaptic homeostasis, where synaptic connections are strengthened or weakened based on their importance (Davey et al. 2008). Because sleep deprivation disrupts this process, declines in cognitive functions such as attention, decision-making, and problem-solving can occur (Davey et al. 2008). Furthermore, the prefrontal cortex, a region associated with executive functions, is particularly sensitive to sleep deprivation, leading to decreased

performance in tasks requiring higher-level cognitive processing (Tononi and Cirelli, 2005). This highlights the importance of adequate sleep for optimal cognitive functioning, especially during adolescence when brain development and synaptic pruning in the prefrontal cortex are crucial for cognitive abilities (Davey et al. 2008). Throughout adolescence, the prefrontal cortex undergoes significant development, particularly in synaptic pruning, myelination, and dopaminergic input, crucial for decision-making. This phase of brain maturation extends into the mid-twenties, with structural changes enhancing neural efficiency and the integration of salient connections, improving decision-making informed by affective information. Sleep deprivation during this critical period can disrupt these processes, leading to impaired cognitive functions in adolescence, especially in tasks requiring executive functions and decision-making skills (Davey et al. 2008). Furthermore, deficits in prefrontal cortex related cognitive functions significantly contribute to AUD, as the prefrontal cortex regulates goal-directed behavior and is vulnerable to damage from excessive alcohol exposure, impairing executive functions such as decision-making, memory formation, emotion regulation, and attention (Pahng et al. 2017). Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and heightened glucocorticoid signaling due to alcohol exposure can damage prefrontal cortex architecture and function, leading to impaired cognitive functions and negative affective states that promote the alcohol-dependent state (Pahng et al. 2017). The interactions between sleep deprivation, adolescent brain development, alcohol use, and the prefrontal cortex underscores the critical need for adequate sleep during this crucial developmental period, as disruptions can lead to impaired cognitive functions and contribute to alcohol use disorder by compromising executive functions and emotional regulation.

Sleep Deprivation's Impact on Anxiety and Stress Levels

Perhaps the most common consequence of sleep deprivation is increased anxiety levels, as it has the potential to impair our emotional well-being, reduce positive feelings, and increase the likelihood of experiencing symptoms of anxiety (Palmer et al. 2023). It is noteworthy that anxiety disorders frequently coincide with AUDs since alcohol is sometimes used to self-medicate or alleviate anxiety symptoms (Turner et al. 2018). Moreover, sleep deprivation can also disrupt the release of hormones that play a crucial role in regulating various physiological processes (Babson et al., 2010). Elevated cortisol levels, in particular, contribute to intensified emotional responses, rendering individuals more susceptible to negative stimuli and less adept at emotional regulation. This heightened emotional reactivity may contribute to mood disturbances, depression, and general distress (Babson et al., 2010). Furthermore, a comprehensive analysis by Palmer et al. in 2023 consisted of 154 studies involving 5,715 participants over five decades. These studies involved various methods of sleep disruption, including extended periods of wakefulness, shortened sleep durations, and intermittent awakenings during the night. The researchers measured emotion-related variables such as mood, response to emotional stimuli, and symptoms of depression and anxiety following sleep manipulations. Their findings revealed that all forms of sleep loss led to decreased positive emotions like joy and happiness, along with increased anxiety symptoms such as elevated heart rate and heightened worrying (Palmer et al. 2023). Thus, the interactions between disrupted sleep and its cascading effects on emotional well-being underscores the importance of prioritizing healthy sleep patterns for overall mental health.

Adolescents as a Vulnerable Population for Sleep Deprivation

Adolescence is a uniquely vulnerable phase marked by significant changes in reward circuitry, behavior, emotions, and sleep patterns, making it a prime time for initiating substance use (Logan et al. 2018). The co-occurrence of affective disorders and substance use disorders among adolescents ranges from 9.0% to 47.9%, with depression and anxiety often predating substance-related problems. Concurrently, adolescents undergo shifts in sleep timing, experiencing reduced sleep duration, increased daytime sleepiness, and more sleep disturbances. These changes, which involve a misalignment of the body's internal clock known as circadian misalignment, contribute to sleep disruption and overall sleep loss, all of which are linked to heightened substance use and its associated issues. Additionally, characteristics indicative of circadian misalignment in sleep and circadian rhythms are also associated with affective disorders and substance use disorders during adolescence (Logan et al. 2018).

The study by Palmer et al. (2023) described in the previous section found that up to 90 percent of teens don't get the recommended amount of sleep, so studies investigating sleep deprivation have specifically honed in on adolescents as a population vulnerable to its effects, as the changes during the transition from childhood to adolescence can disrupt or alter sleep patterns (Roberts et al. 2009). These changes include earlier start times for school, late-night activities or jobs, and pubertal phase delays, which all contribute to constricted hours of sleep available to adolescents (Roberts et al. 2009). In one study, adolescent mood and mood regulation were examined after chronic sleep restriction. It was found that shortened sleep duration (6.5 hours/night for five days) equivalent to that encountered by millions of adolescents on school nights resulted in worsened mood along with an inability to properly regulate negative emotions (Baum et al. 2013). Another study investigated the impact of sleep deprivation on

adolescents (10-13), mid-adolescents (13-16), and adults (30-60) to measure affect and stress levels (Talbot et al. 2010). Individuals in each stage of development who were sleep deprived had a less positive affect compared to those who were well-rested, reported higher anxiety levels during a catastrophizing task, and rated the likelihood of potential catastrophes as higher, indicating that the individuals who were sleep deprived were more stressed (Talbot et al. 2010). In particular, adolescents not only underwent the aforementioned impacts of sleep deprivation on affect and anxiety, but they also perceived their unique concerns as more threatening, suggesting that adolescents are prone to heightened negative emotions, such as stress, compared to other age groups. (Talbot et al. 2010). Adolescents are also already at a higher risk of developing anxiety disorders due to brain vulnerability, the unsynchronized development of anxiety-relevant brain functional systems, and the immaturity of neural networks underlying emotional regulation (Xie et al. 2021). The reward and stress response systems are hypersensitive during adolescence too, meaning adolescents can engage in risky behaviors like alcohol abuse to cope with stress or seek reward, potentially exacerbating their vulnerability to developing AUD in the future (Xie et al. 2021). Because sleep deprivation and increased susceptibility to anxiety highly impacts adolescents specifically, the future consequences of sleep deprivation should be considered to understand how these negative emotions may lead to problems later on in life, including affective and substance use disorders.

Alcohol and Sleep Quality

Mechanisms of Alcohol Use on Neurotransmitter Release

The effects of alcohol on the brain have significant consequences on an individual's physical and mental well-being, as AUD results in a multitude of emotional changes, stress, and disruptions to physiological processes. The brain malfunctions due to prolonged alcohol use,

affecting the sympathetic and parasympathetic components and the neurochemical system vital for emotional and stress functions (Griffith & La France, 2018). Alcohol also interferes with neurological processes, leading to impaired muscular coordination, peripheral neuropathy, impaired perception, learning challenges, memory loss, and personality changes (Griffith & La France, 2018). Simultaneously, alcohol's impact on neural communication is evident as interactions initially commence at the synaptic level, where neurotransmitters are released, either inhibitory or excitatory (Valenzuela, 1997). Short-term alcohol consumption disturbs this balance, depressing brain function and altering the equilibrium between inhibitory and excitatory neurotransmission. Acting as a depressant, alcohol influences the GABA system and inhibits excitatory neurotransmission through effects on glutamate, contributing to alcohol's depressant effects and ability to induce cognitive impairment (Valenzuela, 1997). By activating a specific receptor subtype known as GABA_A, GABA induces sedation and reduces anxiety levels in the central nucleus of the amygdala, which is an area involved in the regulation of emotional states (Banerjee, 2014). Additionally, glycine serves as the primary inhibitory neurotransmitter in the spinal cord and brain stem and can contribute to the further reduction of anxiety levels in conjunction with GABA (Valenzuela, 1997). Alcohol has also been studied to affect the same systems that regulate sleep, as alcohol enhances inhibitory neurotransmission by elevating the activity of the inhibitory neuromodulator adenosine, which induces sedation. Therefore, the rise in adenosine levels triggered by alcohol contributes to its sedative effects (Valenzuela, 1997). In terms of the rewarding and reinforcing effects of drinking, alcohol affects serotonin levels in the brain in various ways, with acute and short-term exposure altering synaptic functions and increasing serotonin release from the raphe nuclei (Lovinger, 1997). This increase in serotonin can lead to enhanced signal transmission at serotonergic synapses, influencing brain regions

involved in substance consumption and leading to feelings of happiness (Lovinger, 1997).

Finally, alcohol has been found to activate dopamine systems in important brain regions such as the limbic system, as alcohol seems to promote dopamine release by stimulating opioid release, disinhibiting dopaminergic neurons by blocking GABAergic neurotransmission through receptors in the ventral tegmental area and in the nucleus accumbens (Valenzuela, 1997; Cowen & Lawrence, 1999). This activation contributes to the short-term positive reinforcement of alcohol's effects by triggering feelings of pleasure and reward. Furthermore, dopamine systems seem to be suppressed following alcohol withdrawal, and this suppression can be alleviated by consuming more alcohol, leading to dependence (Valenzuela, 1997).

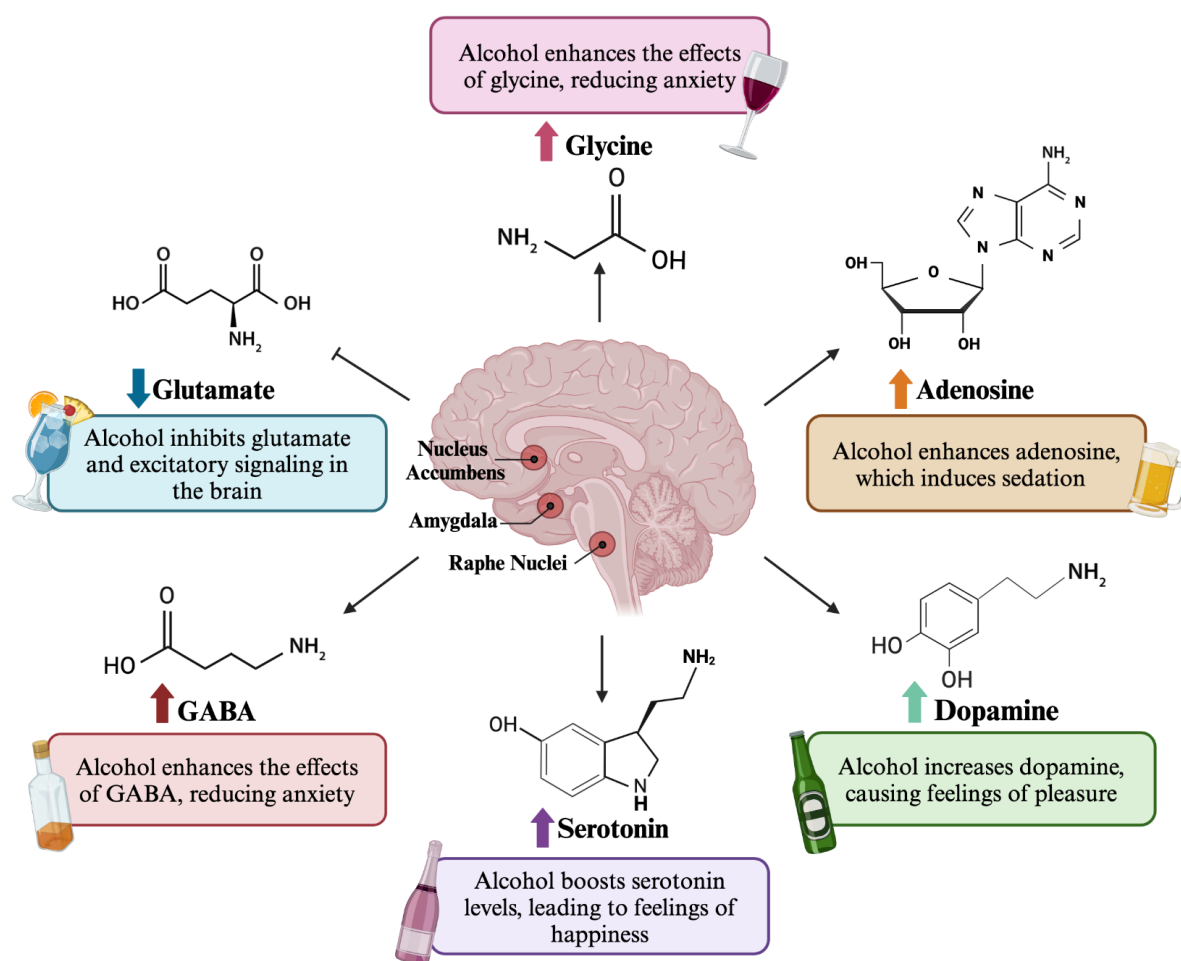


Fig. 5 Summary of the main neurotransmitters affected from short-term alcohol use with three of the main anatomical areas associated highlighted. Made with Biorender.

While the present study focuses more on short-term and acute alcohol exposure, long-term alcohol exposure can prompt compensatory changes, leading to decreased GABA_A receptor function and increased excitatory activity of glutamate receptors (Valenzuela, 1997). Tolerance develops, necessitating one to have to intake a higher volume of alcohol to produce the same effects. Sleep patterns, appetite, concentration, and memory are also all negatively impacted by alcohol use, contributing to an increased desire to drink (Valenzuela, 1997). In terms of sleep, many studies have specifically investigated the effects of alcohol use on the quality of sleep and the consequent results of alcohol use at each stage of the sleep cycle.

Stages of Alcohol Use Disorder and their Characteristics

In a review by Koob and Colrain (2020), the consequences of AUD on sleep were examined in three different stages characterized by the events and feelings that occur during AUD: (1) binge/intoxication, (2) withdrawal/negative affect, and (3) preoccupation/anticipation. The authors create a strong argument for the significance of the interactions between alcohol dependence and sleep, suggesting that the combinations of every stage of alcohol use result in addiction, and the effects on sleep are just some of the repercussions of addictive behaviors. Too, their framework explores the reciprocal relationship between sleep dysregulation and AUD, suggesting that AUD can cause sleep disturbances and vice versa, leading to a cycle of exacerbation (Figure 5). Overall, in each stage, the authors explain that alcohol use can have effects on the onset of sleep— inducing sleep quicker or making it more difficult to fall asleep—, wakefulness, sleep quality, and the time spent in slow-wave sleep (SWS) and REM sleep throughout the night. Their results ultimately suggest that disrupted sleep homeostasis may be a primary cause of sleep disturbances and lack of restorative sleep due to alcohol use, but the article does not address how poor sleep quality or sleep deprivation may have potential outcomes

on an individual's alcohol intake, especially during the binge/intoxication phase which is the phase in which this study focuses.

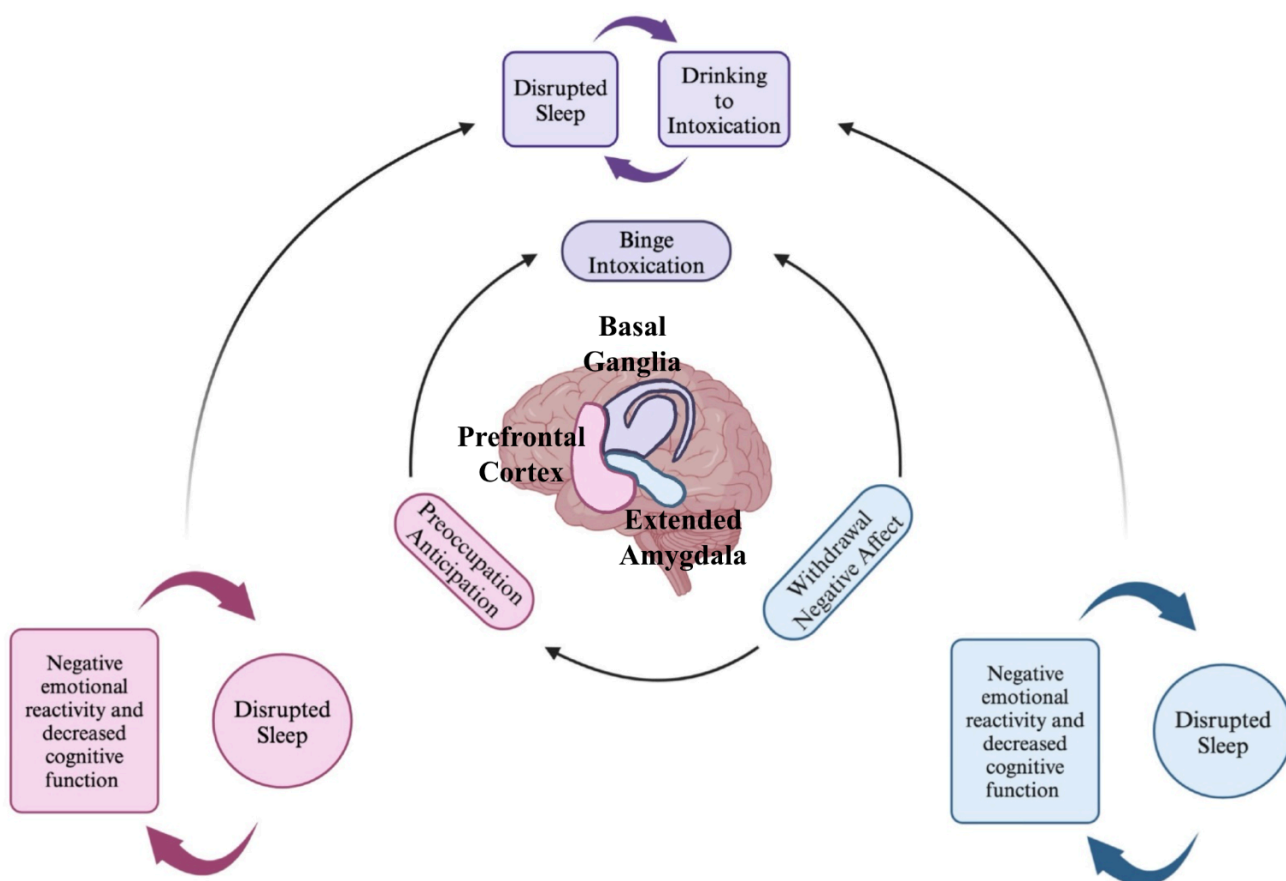


Fig. 5 Summary of each stage of AUD adapted from Koob and Colrain (2020). The framework delves into the bidirectional relationship between sleep dysregulation and AUD, proposing that AUD can induce sleep disruptions and conversely, sleep issues can contribute to AUD, creating a cycle of mutual aggravation. Made with Biorender.

The predominant focus of studies revolving around sleep and alcohol use have almost always centered on delineating the consequences of alcohol use at various stages of the sleep cycle, but even that area requires further exploration. As mentioned, many drugs of abuse, including alcohol, suppress REM sleep, but the significance of this suppression and its relationship to substance use outcomes are not fully understood (Roehrs & Roth, 2015). The relationship between sleep disturbance and all substance use disorders is an area of ongoing

research, with various factors contributing to the complexity of understanding their connection. While many studies have highlighted lack of sleep as a risk factor for substance abuse, the direct impact of sleep problems on the development of new cases of alcoholism or drug abuse is not fully elucidated. Overall, while there is evidence suggesting a link between sleep disturbances and increased vulnerability to substance abuse, more research is needed to determine the precise mechanisms and the effectiveness of targeted interventions in addressing sleep-related issues in individuals with substance use disorders (Roehrs & Roth, 2015). There has specifically been a relative lack of attention to investigating how disrupted sleep may influence drinking behaviors or contribute to the development of AUD later in life. This potential link creates a concerning cycle, as heightened alcohol use in adulthood has the potential to aggravate pre-existing sleep issues, furthering the likelihood of developing or worsening AUD for an individual. Exploring this aspect becomes crucial in understanding the interactions between adolescent sleep patterns, adult alcohol use, and the risk of AUD.

Risk Factors Involved in the Interactions Between Sleep Deprivation and Alcohol Use

In studies of alcohol abuse, stress has been seen to increase anxiety, resulting in increased consumption of alcohol to try and reduce or “treat” those negative emotions. The self-medication hypothesis (SMH) posits that individuals may use substances such as alcohol or drugs as coping mechanisms to alleviate symptoms associated with mood and anxiety disorders (Turner et al. 2018). This hypothesis suggests that as substance use becomes a more frequent coping strategy, it can lead to the development of substance use disorders independent of the original psychiatric condition (Turner et al. 2018). Thus, because sleep deprivation studies have found that anxiety is induced in individuals who are sleep deprived, it is vital to consider sleep deprivation as a potential factor that could cause an increase in alcohol intake. The effects of chronic alcohol

exposure on sleep have been examined in animals, finding that adult rats who were exposed to alcohol during adolescence had reduced measures of SWS with variations in peak frequencies that correlate with cognitive performance, attentional demands, and arousal (Criado et al. 2008). A study addressing how the introduction of sleep deprivation in adolescent rats would affect alcohol drinking found that sleep deprived adolescent rats exhibited more anxiety and depression-like behaviors, as well as consumed more alcohol overall when compared to control rats (Atrooz et al. 2022). However, the rats were only tested for their anxiety-like behaviors after the period of sleep deprivation in adolescence, but the researchers did not consider how these effects may have been endured or compounded after the period of alcohol consumption into adulthood. The authors also highlight that their study represents a pioneering effort in the preclinical domain, being the first to document the impact of sleep deprivation during early-life on alcohol consumption in adolescent rats. This novel exploration underscores the importance of investigating this understudied phenomenon to deepen the understanding of the dynamics between sleep patterns, developmental phases, and substance use tendencies among adolescents.

Limitations to Existing Studies

The study by Atrooz et al. (2022) only utilized male rats, meaning the results cannot be generalizable to all individuals, as sex was not considered a biological variable in the experiments. In neuroscience studies, growing evidence has disproven the assumed variability of female hormonal fluctuations from the estrous cycle that causes many researchers to exclude female subjects in their studies (Becker et al. 2016). Men and women experience disorders differently, specifically concerning alcohol use, as men are more likely to develop alcohol dependence, while women can develop a dependence on the drug of abuse in a shorter period of time (Albrechet-Souza et al. 2020). Too, recent research into gender disparities in alcohol use

disorders indicates that women experience intoxication with just half the amount of alcohol compared to men, and metabolize alcohol in a distinct manner, leading to a faster development of liver cirrhosis and an increased risk of fatalities due to alcohol-related accidents (Greenfield, 2002). Numerous studies have also indicated that women are more likely than men to experience comorbid anxiety disorders and AUDs, as women may use alcohol as a means to self-medicate for mood problems, particularly anxiety (Smith & Randall, 2012; Brady & Randall, 1999). Empirical evidence suggests that women report higher stress levels and a stronger connection between stress and alcohol consumption, highlighting their tendency to rely on alcohol to manage anxiety (Rice & Van Arsdale, 2010; Timko et al. 2005). A study by Verlinden et al. (2022) also found that among heavy drinkers experiencing insomnia, the correlation between poor sleep and alcohol-related issues is more pronounced in women compared to men. However, further studies are necessary to explore the possible mechanisms that underlie this association. Thus, while many studies have investigated how alcohol can affect sleep deprivation or the compounded effects of both sleep deprivation and alcohol in mainly males, much remains to be determined about how sleep deprivation may also inversely have consequences on alcohol consumption in females and how the two together may also induce long-lasting anxiety-like behaviors from adolescence into adulthood.

Present Study

The present study aimed to explore how chronic sleep deprivation at an early age may have an effect on alcohol consumption and anxiety-like behavior in female rats later on into adulthood. Adolescent female Sprague Dawley rats (PND37-PND40 depending on date of birth) either served as controls or were sleep deprived, and their anxiety-like behaviors were assessed directly after the sleep deprivation period utilizing the elevated plus maze (EPM). Then, going

into adulthood, some of the rats were given access to alcohol to see if early exposure to sleep deprivation produced any effects on consumption, as well as being re-tested for their anxiety-like behaviors to measure the effects of alcohol on anxiety-like behavior and to see if there were any residual effects of the sleep deprivation. Because of the confounding problem with repeating the EPM due to “one-trial tolerance” where the rats will spend less time in the open arms if introduced to the apparatus a second time, it is suggested to have a 28-day inter-trial interval as well as change the lighting and room the EPM is in to reliably retest animals for anxiety-like behaviors (Schneider et al., 2011). These suggested measures were implemented during the re-testing of anxiety-like behaviors using the EPM once the rats were through with their period of alcohol access in adulthood. It was predicted that adolescent exposure to sleep deprivation would increase anxiety-like behaviors in the female rats, as evidenced in the initial EPM test. Further, it was predicted that adolescent sleep deprivation would increase alcohol consumption, and would persist in increasing anxiety-like behavior in adulthood, given that females primarily are more susceptible to affective dysfunction and display an increased sensitivity to stress (Klinger et al., 2019). Finally, an interaction between sleep deprivation and alcohol drinking was predicted in the EPM, such that sleep-restricted rats would show enhanced alcohol drinking and “normalized” anxiety-like behavior in line with the self-medication hypothesis while the sleep deprived rats who were not given access to alcohol would exemplify heightened anxiety-like behaviors. Overall, the goal of this study was to determine whether or not the residual effects of sleep deprivation– like anxiety – can influence the consumption of alcohol in female Sprague Dawley rats over a long period of time and perhaps further magnify anxiety-like behaviors, providing more insight into the sex differences associated with sleep and alcohol research, as well as how age can determine how alcohol-motivated behaviors develop.

Method

Subjects

A total of 32 adolescent (PND37-PND40 depending on date of birth) female Sprague Dawley rats were used in this experiment. The rats were housed in pairs for the sleep deprivation period and remained pair-housed later on for measures of alcohol consumption with alcohol-drinking rats being in the same cage with dividers. The rats were given an acclimation period of around 7 days with a 15:9 hr light/dark cycle before the beginning of the experiment with *ad libitum* food and water. The Allegheny College Animal Research Committee approved all procedures.

Materials

In this experiment, two vibrating mats were used beneath the rat cages, both of which were on a timer. Additionally, a timed white noise device was employed to deprive the rodents of sleep at an early age. A standard elevated plus maze (EPM) was used to assess anxiety-like behaviors, which is a plus sign-shaped maze made out of wood elevated 84 cm above the floor. The EPM contains two arms (51.5 cm x 11 cm) which are enclosed with walls that are 42 cm high and two opposing arms (50.5 cm x 11 cm) that are open. To measure alcohol consumption in adulthood, 200 proof ethanol was dissolved in tap water to make a 20% V/V solution. Custom plexiglass dividers tailored to fit each cage were installed, featuring numerous approximately ½ inch holes. These dividers facilitated physical interaction, such as nose-to-nose and paw contact, while ensuring precise measurement of water and alcohol consumption and reducing the confound of social isolation .

Design

This study was conducted as a 2x2 between-subjects design. The independent variables included whether the rats were sleep deprived during adolescence and whether they had intermittent access to alcohol during adulthood. Eight of the rats were sleep restricted beginning at PND37-40 (mid-adolescence) and given access to alcohol in adulthood, 8 of the rats were sleep restricted and not given access to alcohol, 8 of the rats were not sleep restricted and were given access to alcohol, and 8 rats were not sleep restricted and were not given access to alcohol in adulthood. The dependent variables measured in the EPM were the time the rats spent in the open and closed arms of the apparatus and the number of entries in both the closed and open arms. Percent open and closed arm entries were also calculated to account for any differences in general locomotor activity (total arm entries). For alcohol consumption, alcohol and water-containing bottles were weighed before and after each drinking session to calculate both alcohol preference ($\text{mL ethanol} / \text{mL ethanol} + \text{mL water}$) and alcohol intake in grams per kilogram of the rats' body weight.

Procedure

Experimental Timeline

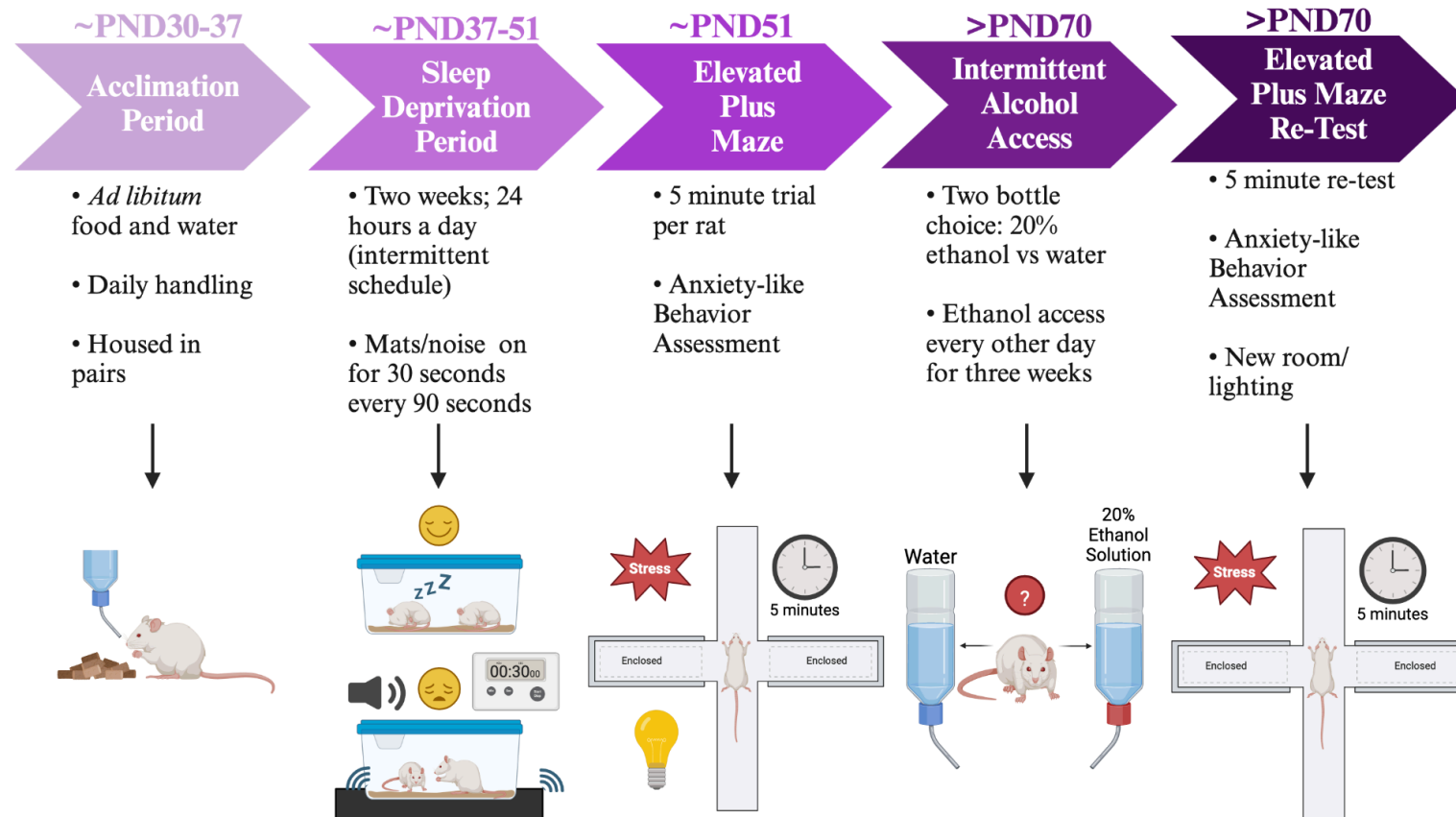


Fig. 6 Experimental procedure timeline. Rats were acclimated from postnatal days 30-33 for a week, followed by sleep deprivation or undisturbed conditions for two weeks beginning at PND37-40. Anxiety-like behaviors were assessed after sleep deprivation (PND51-54), followed by alcohol consumption measurement using an intermittent access paradigm from adulthood (>P70). Following the drinking period, all rats underwent a single EPM trial to reassess anxiety-like behaviors. Made with Biorender.

Beginning on PND30-33, the rats were given a 7-day period of acclimation where they were able to adjust to their environment and handled daily. Following acclimation beginning on PND37-40, 16 rats served as controls and were left undisturbed (other than routine animal care), while the rest were sleep deprived for two weeks (n=16). Directly after the sleep deprivation period at PND51-54, anxiety-like behaviors were assessed in the EPM for each of the rats to determine whether sleep deprivation increased anxiety-like behavior. In adulthood (aged >P70), 8 rats that were sleep deprived and 8 rats that were left undisturbed were pair-housed using dividers to measure alcohol consumption using the intermittent access paradigm with the two-bottle choice of either the 20% ethanol solution or water for three weeks, every other day. Rats in the water drinking control group received similar manipulations but were regularly pair housed and their bottles contained water. Following the three week drinking period, all of the rats underwent a single EPM trial to assess once more for anxiety-like behaviors (Figure 6).

Sleep Deprivation

The experimental (n= 16 female) rats were sleep deprived for two weeks 24 hours a day on an intermittent schedule with inter-stimulus-intervals by using 67 x 22 inch vibrating mats under their cages and a noise device to be activated on a timer for 30 seconds every 90 seconds, beginning at PND37-40. Rats were housed in a small room within the animal facility and maintained on a 12:12 light/dark cycle, consistent with other sleep studies. The sleep deprivation period was constant throughout the day from the beginning of the light cycle when the rats would typically be inactive throughout the night when they would typically be asleep for a cumulative total of 6 hours of sleep deprivation per day.

Anxiety Assessment

To assess anxiety-like behavior, the EPM was used, and each rat was given the opportunity to explore the maze for one session after the sleep deprivation period in adolescence and following the period of alcohol access in adulthood. At PND51-PND54, the rats were each individually transferred to a dimly lit room and placed in the intersection area of the EPM facing an open arm. The behavior of the animal was observed both live and on recorded footage for 5 minutes to determine the time spent in both the open and closed arms of the apparatus ($\text{time spent in open arms} / (\text{time spent in open} + \text{time spent in closed}) \times 100$), as well as the number of entries in both the closed and opened arms. All four paws had to be present in an arm of the EPM to be considered an entry. One session in the EPM was repeated to measure the same behaviors for each rodent after the three weeks of intermittent alcohol access in a different room with even dimmer and weaker lighting and different spatial cues to account for the one-trial tolerance of the EPM (Schneider et al., 2011).

Intermittent Alcohol Access

Once rats reached adulthood ($>P70$), half of the sleep deprived, and half of the non-sleep deprived rats ($n=16$ total) were given intermittent access to a 20% ethanol solution along with a water bottle to measure alcohol consumption and preference. The animals drinking alcohol were pair housed during this period using plastic dividers to ensure water and alcohol measurements were accurate, but to reduce the confound of social isolation. The ethanol solution was given to the rats every other day for 24 hours on and 24 hours off for three consecutive weeks (Carnicella et al. 2014). On days when alcohol was not given to the female rats, the dividers were removed to allow for social interaction. Assessed in g/kg, the amount of alcohol consumed was

determined for each rat by measuring the amount each rat drank at the end of each day relative to the concentration of alcohol and the rat's body weight. To determine if the rats had a preference for alcohol, the volume of alcohol consumed was divided by the total fluid intake of both the alcohol and water for each rat, multiplied by 100 to get a percentage.

Statistical Analysis

The statistical program JASP was used to analyze the results of this study. A 2x2x6 mixed factorial ANOVA with sleep deprivation condition (deprived vs. non-deprived) and drinking condition (alcohol drinking vs. water drinking) as between-subjects factors, and day of 2-bottle choice exposure (10 days total, though water was only measured in the control group for 6 days) was used to analyze water intake in all animals on days where alcohol access was not provided. A similar 2x10 ANOVA and a 2x9 ANOVA were used to measure alcohol consumption (g/kg) and preference in the alcohol-exposed group (α set at .05), respectively. To assess the manifestation of anxiety-like behaviors in each rat, four independent sample t-tests were performed following the initial EPM test, contrasting the responses of sleep-deprived rats against those of non-sleep deprived rats. Additionally, four 2x2 ANOVAs were conducted following the second EPM in adulthood to examine the effects of sleep deprivation versus normal sleep patterns and alcohol intake versus water consumption (α set at .05). Post-hoc tests were conducted where appropriate.

Results

Effects of sleep deprivation on anxiety-like behavior in adolescent rats

To assess anxiety-like behaviors following the two week sleep deprivation period, four independent sample t-tests were conducted to compare the open arm time, closed arm time, percent open arm entries, and percent closed arm entries between the experimental group (n=16) and non-sleep restricted group (n=16). The results revealed significant differences in anxiety-like behaviors between the two groups. Specifically, the sleep deprived rats spent significantly less time in the open arms of the EPM ($M= 60.19$, $SD= 30.98$) compared to the non-sleep-deprived rats ($M= 87.04$, $SD= 33.85$), $t(30)= 2.34$, $p= 0.026$, CI [0.098, 1.55], Cohen's $d= 0.83$ (Figure 7A). The sleep deprived rats spent significantly more time in the closed arms of the EPM ($M= 175.06$, $SD= 36.25$) as well compared to the non-sleep-deprived rats ($M= 126.15$, $SD= 25.60$), $t(30)= -4.41$, $p < .001$, CI [-2.35, -0.75], Cohen's $d= -1.56$ (Figure 7B). Additionally, the sleep deprived rats exhibited a significantly lower percentage of open arm entries ($M= 30.55$, $SD= 8.01$) compared to control rats ($M= 40.70$, $SD= 9.81$), $t(30)= 3.21$, $p= 0.003$, CI [0.38, 1.88], Cohen's $d= 1.13$ (Figure 7C). The sleep deprived rats also had a significantly higher percentage of closed arm entries ($M= 69.45$, $SD= 8.01$) compared to the non-sleep deprived rats ($M= 59.30$, $SD= 9.81$), $t(30)= -3.21$, $p= 0.003$, CI [-1.88, -0.38], Cohen's $d= -1.13$ (Figure 7D). Overall, the rats that experienced sleep deprivation exhibited increased entries into and spent more time in the closed arms of the maze, while the non-sleep restricted group showed the opposite trend, favoring the open arms.

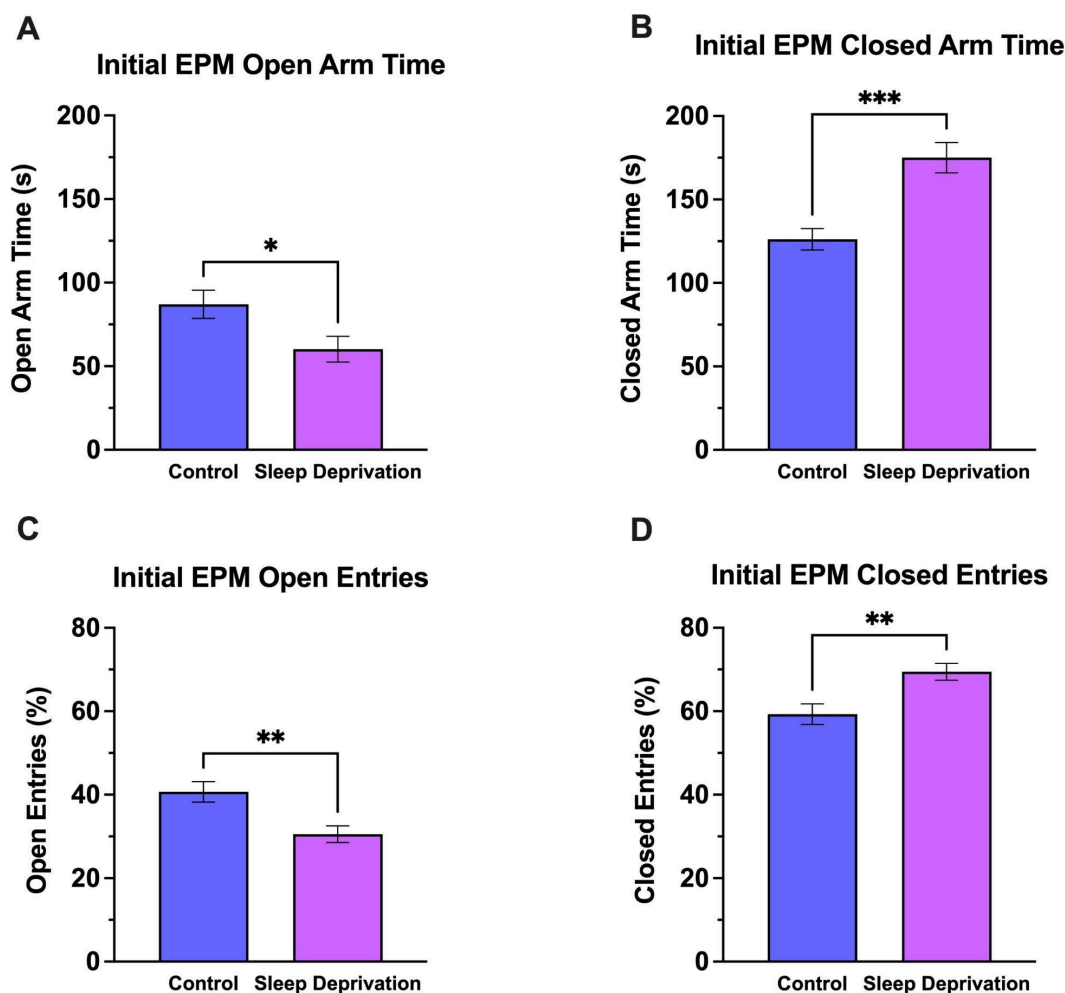


Fig. 7 The bar graphs illustrate the duration of time spent in seconds in the open arms (A) and closed arms (B), as well as the percentage of time spent in the open arms (C) and closed arms (D) in the Elevated Plus Maze (EPM) for the control rats (blue) and sleep-deprived rats (magenta). Error bars, constructed using one standard error from the mean, are included to indicate the variability and precision of the data within each group. Indicated above the bar graphs (*) are significant results with a $p < 0.05$.

Effects of adolescent sleep deprivation on alcohol drinking in adult rats

Ethanol Intake (g/kg)

Once all rats had reached adulthood (PND>70), half of the sleep deprived rats (n=8) and half of the non-sleep restricted rats (n=8) were given access to a 20% ethanol solution along with their water bottles every other day for three weeks. A 2x10 ANOVA was conducted to determine

the effects of adolescent sleep deprivation on ethanol intake based on each rats' weight and the amount of alcohol they drank per day (g/kg). The Greenhouse-Geisser test was employed to correct and adjust for the violation of the assumption of sphericity for ethanol intake (g/kg), as indicated by Mauchly's test of sphericity. There was a main effect of day found for alcohol intake (g/kg), $F(3.60, 50.43) = 3.10, p = 0.027, \eta^2 = 0.118$, with a large effect size. Bonferonni post-hoc tests indicated that the rats drank significantly more on day 1 of ethanol access (which was highly variable) than they did on day 5, $p = 0.002$, and the rats drank significantly less on day 5 than they did on day 8, $p = 0.041$. There was also a main effect of sleep deprivation between subjects, $F(1, 14) = 5.53, p = 0.034, \eta^2 = 0.061$ with a medium effect size, as rats that were sleep deprived drank more alcohol (g/kg) compared to those that were not sleep restricted in adolescence. An interaction between day of ethanol access and sleep deprivation was also present with a large effect size, though post-hoc tests did not reveal any systematic patterns or effects to explain why sleep deprivation would cause rats to drink more or less on certain days, $F(3.60, 50.43) = 3.44, p = 0.018, \eta^2 = 0.131$ (Figure 8).

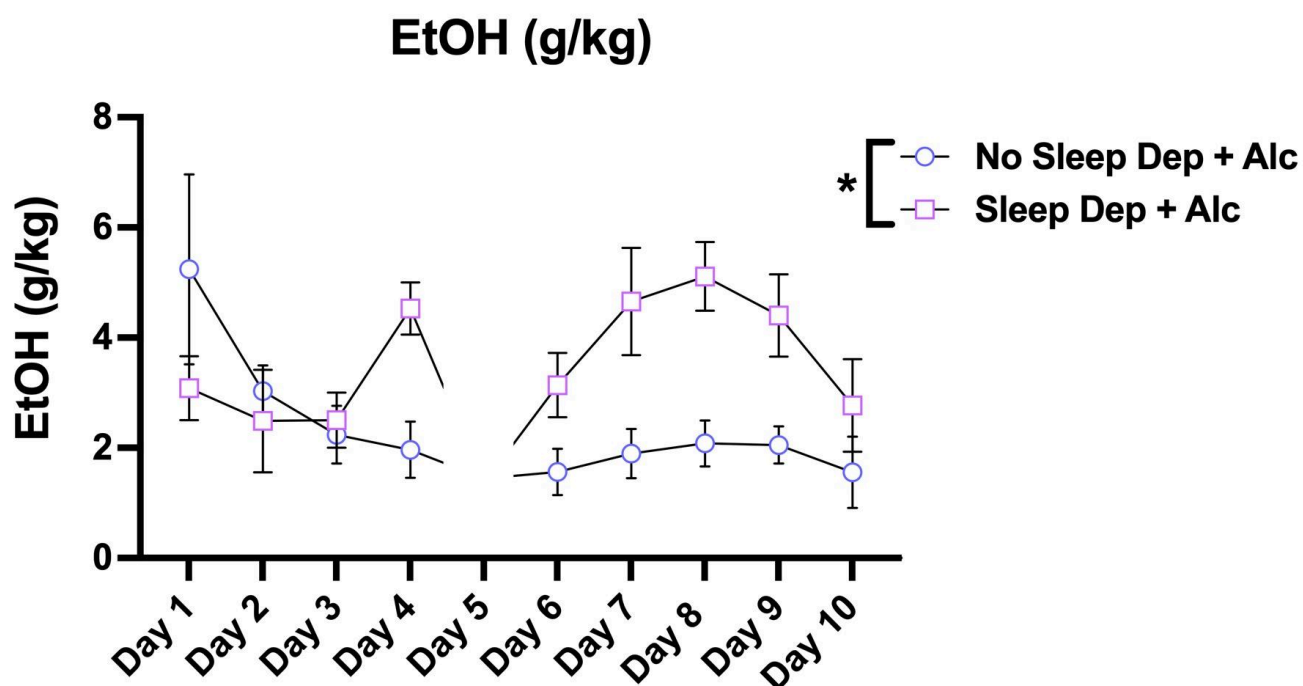


Fig. 8 Alcohol intake in adulthood (PND>70) for rats sleep deprived in adolescence (white boxes with magenta borders) and rats non-sleep restricted (white circle with blue borders). The break in the plot indicates a day where lab assistants may have incorrectly measured bottle weights. Indicated in the legend (*) is the main effect of sleep deprivation with a $p < 0.05$.

Ethanol Preference

Along with alcohol intake (g/kg), ethanol preference was analyzed using a 2x9 ANOVA to examine potential differences and interactions between sleep deprivation and access to ethanol in determining rats' preference for ethanol over water. The Greenhouse-Geisser test was utilized to rectify and account for the deviation from the assumption of sphericity observed in Mauchly's test concerning ethanol preference. There was no main effect of day of ethanol access $F(3.28, 45.91) = 1.81, p = 0.154, \eta^2 = 0.114$, but there was a main effect of sleep deprivation between subjects with a medium effect size, indicating that the rats sleep deprived in adolescents had a higher preference for alcohol compared to the rats that were non-sleep restricted, $F(1, 14) = 5.70, p = 0.032, \eta^2 = 0.289$ (Figure 9). There was not an interaction between day of EtOH access and sleep deprivation, $F(3.28, 45.91) = 1.46, p = 0.235, \eta^2 = 0.095$.

EtOH Preference

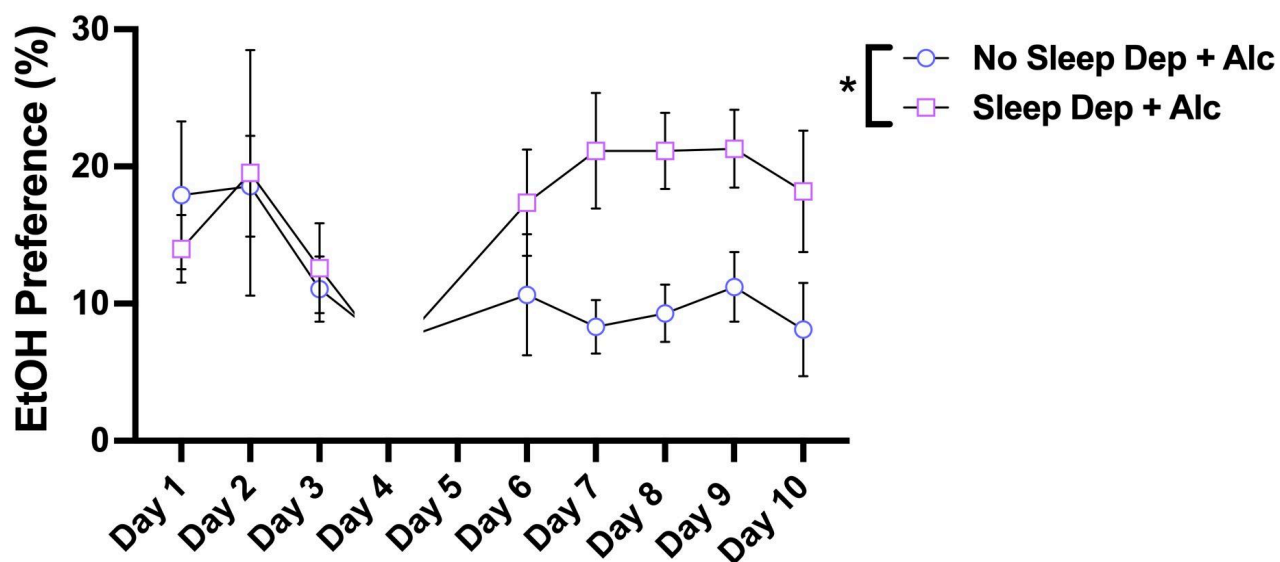


Fig. 9 Alcohol preference in adulthood (PND>70) for rats sleep deprived in adolescence (white boxes with magenta borders) and rats non-sleep restricted (white circles with blue borders). The break in the plot indicates a day where lab assistants may have incorrectly measured bottle weights. Indicated in the legend (*) is the main effect of sleep deprivation with a $p < 0.05$.

Water intake for all rats during alcohol-free days for rats with EtOH access

A 2x2x6 ANOVA was conducted to assess water intake for all rats during the days when rats given a two-bottle choice with ethanol did not have access to alcohol, allowing for the examination of interactions between sleep deprivation, ethanol access, and alcohol-free periods. The Greenhouse-Geisser test was employed to correct and adjust for the violation of the assumption of sphericity for ethanol intake (g/kg), as indicated by Mauchly's test of sphericity. Within subjects, there was a main effect of day of water with a large effect size, $F(3.55, 99.26) = 10.06, p < .001, \eta^2 = 0.125$. Post-hoc comparisons using Bonferroni corrections revealed significant differences in water intake among the rats across multiple days. Specifically, all rats consumed more water on day 1 compared to day 6, $p < .001$, and exhibited higher water intake on day 4 than on day 2, $p = 0.002$. Additionally, there was a significant increase in water consumption on day 4 compared to day 3, $p = 0.022$, whereas water intake on day 3 was higher than on day 6, $p = 0.009$. Furthermore, all rats showed increased water intake on day 4 compared to days 5, $p < .001$, and 6, $p < .001$. Within subjects, there were no significant interactions between day of water access only and sleep deprivation, $F(3.55, 99.26) = 1.18, p = 0.325, \eta^2 = 0.015$, day of water access only and alcohol, $F(3.55, 99.26) = 1.39, p = 0.247, \eta^2 = 0.017$, and day of water access only and sleep deprivation and alcohol, $F(3.55, 99.26) = 0.341, p = 0.828, \eta^2 = 0.004$. Between subjects, there was no main effect observed for sleep deprivation, $F(1, 28) = 2.64, p = 0.116, \eta^2 = 0.023$. However, rats with access to alcohol every other day exhibited reduced water intake on days without alcohol access, indicating a significant main effect of

alcohol consumption with a large effect size, $F(1, 28) = 21.47, p < .001, \eta^2 = 0.187$. There was also an interaction observed between subjects regarding sleep deprivation and alcohol consumption, with rats that experienced sleep deprivation during adolescence and had access to alcohol drinking significantly less water on off days compared to rats that were not sleep restricted and had access to alcohol, $F(1, 28) = 4.36, p = 0.046, \eta^2 = 0.038$ (Figure 10).

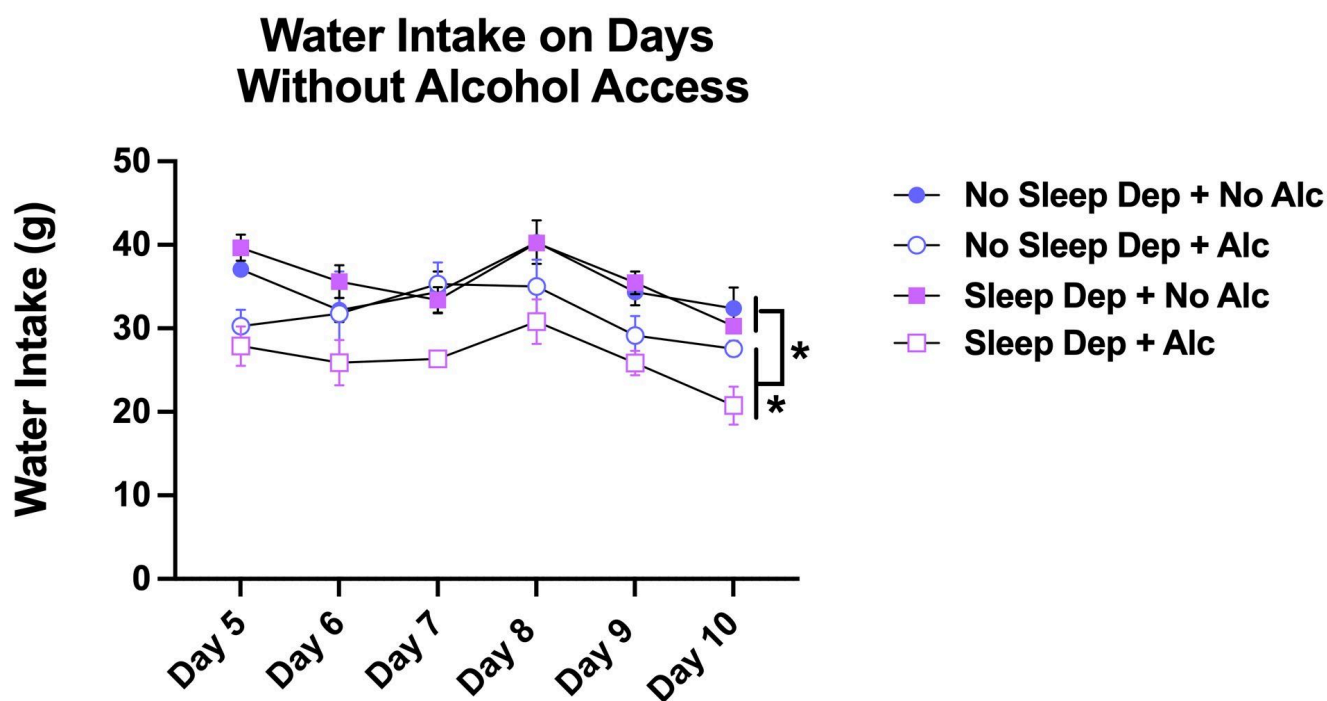


Fig. 10 Water intake on alcohol-free days for rats categorized into four groups: sleep-deprived with alcohol access (white boxes with magenta borders), sleep-deprived without alcohol access (solid magenta boxes), non-sleep-deprived with alcohol access (white circles with blue borders), and non-sleep-deprived without alcohol access (solid blue circles). Indicated on the graph (*) are the main effect of alcohol and the interaction of sleep deprivation and alcohol with $p < 0.05$.

Effects of adolescent sleep deprivation and alcohol access on anxiety-like behavior in adult rats

Separate 2x2 ANOVAs were conducted with sleep deprivation (sleep deprived vs. non-sleep deprived) and alcohol access (alcohol vs. no alcohol) as between-subjects factors,

analyzing open arm time, closed arm time, percent open entries, and percent closed entries in the second EPM following the alcohol drinking period during adulthood. There was a significant main effect of alcohol observed for open arm time with a large effect size, $F(1, 28) = 4.74, p = 0.038, \eta^2 = 0.145$, with all rats that had access to alcohol every other day spending more time in the open arms of the EPM compared to the rats who were not given access to alcohol (Figure 11A). No main effect of sleep deprivation, $F(1, 28) = 0.53, p = 0.474, \eta^2 = 0.015$, or interaction between sleep deprivation and alcohol access, $F(1, 28) = 0.71, p = 0.406, \eta^2 = 0.025$, were observed for open arm time. For closed arm time, there were no significant main effects of sleep deprivation, $F(1, 28) = 2.71, p = 0.111, \eta^2 = 0.088$, or alcohol access, $F(1, 28) = 0.12, p = 0.732, \eta^2 = 0.004$, nor an interaction between the two, $F(1, 28) = 5.81 \times 10^{-4}, p = 0.981, \eta^2 = 1.885 \times 10^{-5}$ (Figure 11B). A main effect of alcohol access was evident in the percentage of open arm entries with a large effect size, $F(1, 28) = 5.66, p = 0.024, \eta^2 = 0.168$, signifying that rats with access to alcohol every other day made more entries into the open arms compared to those without alcohol access (Figure 11C). However, no main effect of alcohol access, $F(1, 28) = 0.017, p = 0.897, \eta^2 = 5.089 \times 10^{-4}$, or interaction between sleep deprivation and alcohol access was observed, $F(1, 28) = 0.022, p = .884, \eta^2 = 6.433 \times 10^{-4}$. A main effect of alcohol access was also evident in the percentage of closed arm entries with a large effect size, $F(1, 28) = 5.66, p = 0.024, \eta^2 = 0.168$, signifying that rats with access to alcohol every other day made less entries into the closed arms compared to those without alcohol access (Figure 11D). No main effect of alcohol access, $F(1, 28) = 0.017, p = 0.897, \eta^2 = 5.089 \times 10^{-4}$, or interaction between sleep deprivation and alcohol access was observed, $F(1, 28) = 0.022, p = 0.884, \eta^2 = 6.433 \times 10^{-4}$, for percent closed arm entries.

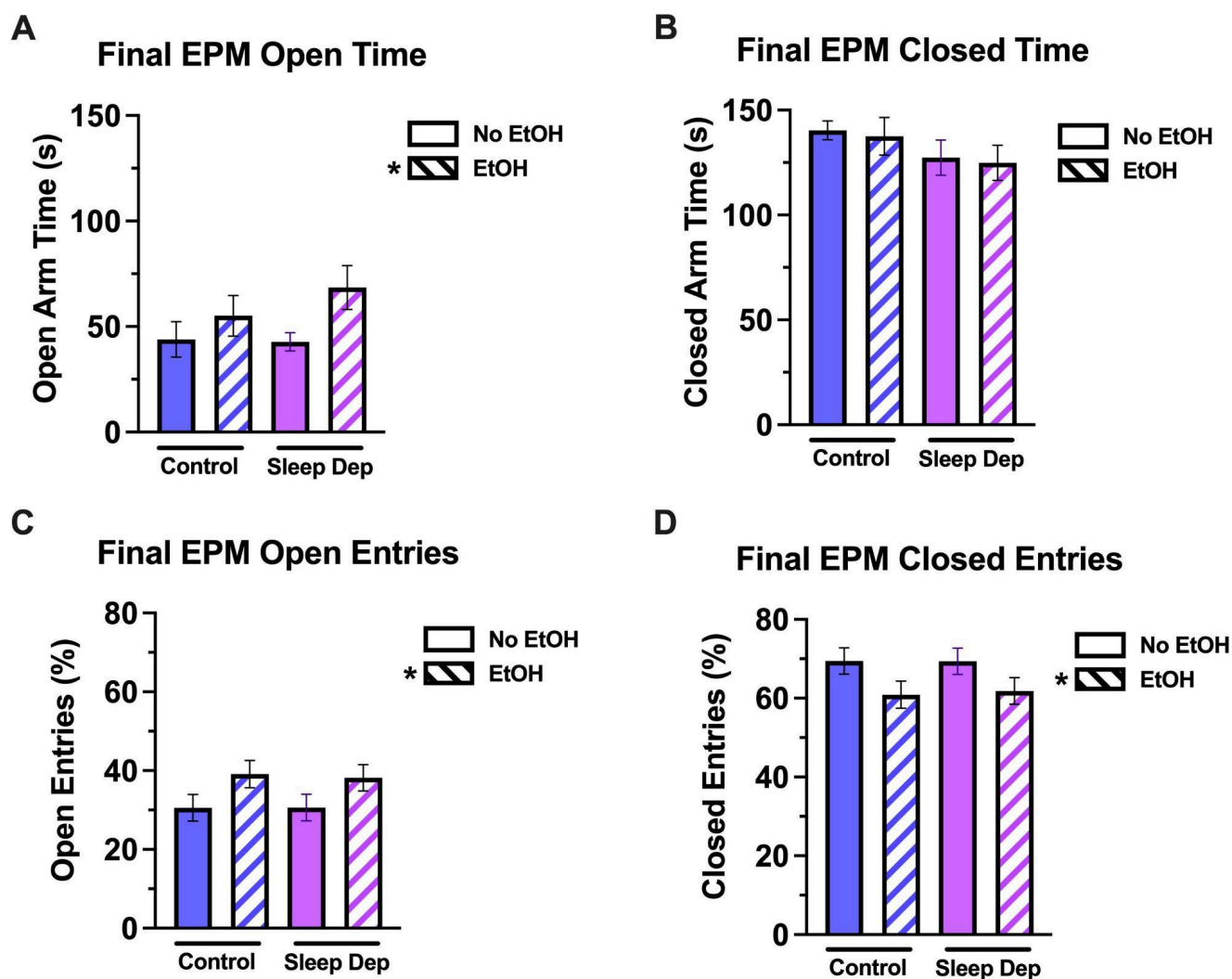


Fig. 11 The bar graphs illustrate the duration of time spent in seconds in the open arms (A) and closed arms (B), as well as the percentage of time spent in the open arms (C) and closed arms (D) in the Elevated Plus Maze (EPM) for the four groups: sleep-deprived with alcohol access (magenta with stripes), sleep-deprived without alcohol access (magenta), non-sleep-deprived with alcohol access (blue with stripes), and non-sleep-deprived without alcohol access (blue). Error bars, constructed using one standard error from the mean, are included to indicate the variability and precision of the data within each group. Indicated in the legends (*) are the main effects of alcohol with a $p < 0.05$.

Discussion

The primary objective of this study was to examine the enduring effects of sleep deprivation during adolescence on subsequent alcohol consumption in female Sprague Dawley rats during adulthood, aiming to contribute to the understanding of the interactions among sleep deprivation, anxiety, and alcohol consumption in female rats, and providing insights into developmental influences on alcohol-motivated behaviors. It was hypothesized that sleep deprived adolescent female rats would exhibit heightened anxiety-like behaviors, through the initial EPM assessment. Into adulthood, it was predicted that adolescent sleep deprivation would lead to increased alcohol intake and sustain anxiety-related behaviors into adulthood, particularly given the known susceptibility of female rats to affective disorders and stress-induced responses (Klinger et al., 2019). Additionally, it was anticipated that there would be an interactive effect between sleep deprivation and alcohol consumption, wherein sleep restricted rats would demonstrate escalated alcohol intake alongside potentially normalized anxiety-like behaviors, aligning with the self-medication hypothesis. However, the findings of this study did not entirely align with the hypotheses proposed. The female rats that experienced sleep deprivation during adolescence did display increased anxiety-like behaviors compared to the control rats during the initial EPM assessment, indicating that the sleep deprivation elicited an anxiogenic response in the rats. Furthermore, sleep deprivation appeared to influence the rats' ethanol intake (g/kg) and ethanol preference, with sleep deprived rats in adolescence exhibiting increased alcohol intake and a greater preference for alcohol compared to their non-sleep restricted counterparts. The rats that experienced sleep deprivation during adolescence and had access to alcohol every other day also exhibited a significant decrease in water consumption on days when alcohol was not available compared to the other groups. However, despite the anticipated self-medication effect,

there was no significant interaction observed between alcohol access and sleep deprivation during the second EPM assessment in adulthood. This result did not align with the hypothesis that rats that experienced sleep deprivation would exhibit reduced anxiety-like behaviors if they had access to alcohol, and sleep deprived rats without alcohol access would continue to display anxiety due to the compounded effects and stress of the adolescent sleep deprivation. However, alcohol consumption had an overall anxiolytic effect, evidenced by increased open arm time and percent open arm entries in alcohol drinking rats compared to non-alcohol exposed controls.

Adolescent Sleep Deprivation Increased Anxiety-Like Behaviors

During the initial EPM assessment, female rats subjected to sleep deprivation during adolescence exhibited increased anxiety-like behaviors in contrast to the control group, suggesting that the sleep deprivation had a stressful effect. The rats exposed to sleep deprivation using the novel method of vibrating mats and noise device spent significantly less time and made fewer entries into the open arms of the EPM. Conversely, they spent more time and had more entries into the closed arms of the EPM compared to the control group rats. These results are consistent with the general research that demonstrates sleep deprivation has been associated with negative mood states, including anxiety (Oginska & Pokorski, 2006; Sagaspe et al., 2006). The study by Atrooz et al. (2022) that investigated the effects of adolescent sleep deprivation on anxiety-like behaviors and alcohol consumption found that their sleep deprived rats also spent significantly less time in the open arms and had less entries in the open arm of the EPM. Although their study utilized male rats instead of females, both their study and this study found that anxiety-like behaviors were evident post adolescent sleep deprivation. Two studies also tested the effects of sleep deprivation on rat performance and anxiety-like behaviors in the EPM.

A study by Suchecki et al. (2002) aimed to reveal distinct alterations in behavioral responses in the elevated plus maze induced by paradoxical or REM sleep deprivation. Although it's uncertain whether the sleep deprivation induced in this study was paradoxical, as EEG data was not collected to assess if there was a disproportionate impact on REM sleep, the sleep deprived rats exhibited a notable increase in time spent in the closed segments of the EPM. In contrast, the control rats, showing less anxiety-like behavior, explored more of the open segments. Similarly, a study by Silva et al. (2004) sleep deprived rodents acutely for 72 hours and found a higher degree of anxiety-like behavior in the rodents that were sleep deprived, as evidenced by decreased time spent in the open arms of the EPM compared to control rodents. While both studies utilized different methods of inducing sleep deprivation in their rodents, the results from their experiments align with this study's, as the sleep deprived rats' performance in the EPM suggested that the rats who experienced sleep deprivation were more likely to exhibit anxiety-like behaviors.

No Exacerbation of Anxiety-like Behaviors in Adulthood Despite Adolescent Stress

In the second EPM in adulthood, there was no interaction between adolescent sleep deprivation and alcohol present in the female rats' behaviors as hypothesized. It was anticipated that adolescent rats subjected to sleep deprivation but not provided access to alcohol in adulthood would still display anxiety-like behaviors, while the sleep deprived rats given access to alcohol during adolescence would exhibit reduced anxiety-like behaviors, aligning with the self-medication hypothesis (Turner et al. 2018). It is possible that the stressful repercussions of sleep deprivation observed during adolescence did not persist into adulthood or perhaps were not sufficiently stressful to escalate into adulthood for the rats that were not given access to alcohol.

It is also important to consider that the rats were only sleep deprived in mid-late adolescence (~PND37-51), so the results may also reflect that a more intense sleep deprivation protocol or sleep deprivation that spanned a longer period of adolescence could reveal better results in the EPM. A study by Bertholomey et al. (2016) showed that chronic corticosterone exposure from PND30-50 increased alcohol self-administration in females compared to males, highlighting a notable sex-specific response to stress-induced alcohol-seeking behavior during adolescence. Therefore, prolonging the duration of stress exposure beyond two weeks, as explored in the study by Bertholomey et al. (2016) on chronic corticosterone exposure, might have heightened stress levels and potentially led to more pronounced effects in the EPM. Additionally, compared to the initial test during adolescence, there were notable changes in behavior patterns. Control rats spent slightly more time in the closed arms, while sleep deprived rats spent slightly less time in the closed arms. Furthermore, all groups showed a decrease in time spent in the open arms, which could be attributed to either repeat-testing effects or aging-related factors (Schneider et al. 2011). Despite these variations, the entries into different arms remained relatively consistent across the groups.

There was a main effect of alcohol in open arm time, open entries, and closed entries, as rats that were given access to alcohol spent more time and had more entries into the open arms of the apparatus, as opposed to the controls that spent less time in the open arms and had significantly more closed entries. The EPM takes advantage of rodents' natural inclination towards dark, enclosed areas and their innate fear of heights and open spaces, so it is possible that these results could be associated with the fact that acute alcohol exposure can modulate amygdala activity and reduce fear responses, contributing to its anxiolytic effects (Gorka et al. 2013; Walf & Frye, 2007). In a study by Gilman et al. (2008), the impact of alcohol on how the

brain responds to fearful and neutral facial expressions was conducted. Their studies revealed that administering alcohol intravenously led to reduced activity in the amygdala when participants viewed fearful faces. Surprisingly, the authors observed that alcohol increased amygdala activity when participants viewed neutral faces, suggesting a potential mechanism for alcohol's anxiety-reducing effects by altering how the brain processes threatening information or by dampening the amygdala's responsiveness to threats (Gilman et al., 2008). A study by Sripada et al. (2011) also found that alcohol reduced amygdala reactivity to faces conveying threat but did not affect responses to happy faces. Their findings suggest that alcohol's anxiety-reducing effects might be linked to its ability to alter the brain's response to threatening cues. These findings suggest that alcohol's anxiety-reducing effects may be tied to its ability to modify the brain's reaction to threatening stimuli, potentially explaining the unexpected behaviors observed in rats with alcohol access contradicting their typical fear responses in the EPM. However, it's important to note that this study did not measure amygdala activity, so this hypothesis cannot be confirmed definitively. In a study that investigated the role of delta opioid receptors (DORs) in the ventral tegmental area and their modulation of GABAergic signaling in the context of alcohol consumption, anxiety-related behaviors, and relapse prevention, it was suggested that alterations in DOR function due to chronic alcohol exposure may impact anxiety levels (Mitchell et al. 2012). Thus, higher levels of alcohol consumption as seen in this study could potentially lead to changes in DOR-mediated modulation of anxiety-related pathways, which might influence how animals perceive and respond to risky situations, such as navigating the EPM. Another study by Boerngen-Lacerda and Souza-Formigoni (2000) found that low doses of ethanol induces an anxiolytic effect, leading to increased open arm time in both acute and chronic administrations in the EPM. While their study utilized ethanol injections given at doses higher than most rodents

will self-administer, their results suggest that ethanol administration reduces fear and increases exploratory behavior, particularly in anxiety-provoking situations. Despite the use of ethanol injections instead of the self-administration protocol used in this study, their results align with the findings of the present study, as the rats that drank more alcohol spent more time in the open arms of the EPM.

Adolescent Sleep Deprivation Led to Higher Alcohol Intake and Preference in Rats

In adulthood, the female rats who were sleep deprived in adolescence exhibited a higher alcohol intake (g/kg) and preference in comparison to rats who were not sleep restricted. In terms of intake, a main effect of day of alcohol access and an interaction between sleep deprivation and day of alcohol access were observed, although there was a considerable level of variability and no consistent pattern contributing to these effects. For ethanol preference, there was not a main effect of day or an interaction between sleep deprivation and day of alcohol access. However, there was a significant main effect of sleep deprivation observed for both alcohol intake and alcohol preference. Rats that experienced adolescent sleep deprivation consistently consumed higher amounts of alcohol and showed a stronger preference for alcohol compared to the control rats in adulthood. This effect was noticeable from day 3 for alcohol intake and day 2 for alcohol preference onwards. These results align with other studies that investigated the effects of sleep deprivation on alcohol consumption. For example, a study by Cowan (2017) sleep restricted rats using forced exercise wheels for both 6 hours and 18 hours and found that sleep deprivation did enhance voluntary alcohol consumption in adult rats for both conditions. Similarly, research utilizing the flowerpot technique with modifications such as a cuff pedestal and electrified grid floor instead of water to induce REM-sleep deprivation also found an increase in alcohol intake and consumption (Aalto & Kiianmaa, 1984). Another investigation employing the gentle

handling technique for sleep deprivation revealed that adult rats subjected to sleep restriction consistently exhibited higher alcohol consumption than control rats over a period of seven days (García-García et al. 2021). Although the study by Atrooz et al. (2022) focused on male rats and provided alcohol during adolescence, it also reported an increase in alcohol intake following early-life sleep deprivation. These studies, excluding the one by Atrooz et al. (2022), primarily centered on examining the relationship between sleep deprivation and subsequent alcohol consumption in adult rats, coinciding with the phase when the rats in our study were provided access to alcohol. However, none of these studies used the developmental perspective utilized in this research, which explored how early-life sleep deprivation might contribute to increased alcohol consumption in adulthood.

The results of this study suggest that female rats subjected to sleep deprivation in adolescents are more likely to drink alcohol in adulthood compared to rats that are not sleep restricted. As evidenced by the results of the initial EPM test, the rats in this study seemed to experience stress from the sleep deprivation, which led to them exhibiting more anxiety-like behaviors. While it was initially hypothesized that the rats who were sleep deprived in adolescence would drink more as a form of self-medication to alleviate potential exacerbated stress in adulthood, the second EPM in adulthood did not support this hypothesis or potential reasoning. However, one potential explanation for rats that were sleep deprived in adolescence drinking more alcohol in adulthood without exhibiting more anxiety-like behaviors could be related to neuroadaptation and coping mechanisms. Because of several factors aligning during adolescence, the developing brain exhibits heightened sensitivity to stress-induced neurobehavioral issues, which can significantly impact an individual's present and future health and wellness (Eiland & Romeo, 2013). Specifically, adolescents are at a particularly vulnerable

period in their brain development, as their stress and reward systems are hypersensitive, meaning that they are more prone to exaggerated responses to stressors and rewarding stimuli, driven by neurobiological mechanisms such as heightened activity in the mesocorticolimbic dopamine pathway and increased synaptic plasticity in key brain regions like the prefrontal cortex and nucleus accumbens (Xie et al. 2021; Walker et al. 2017). The medial prefrontal cortex is a key component of the mesocorticolimbic pathway and plays a crucial role in regulating reward-directed behaviors and cognitive development during adolescence. It receives dopaminergic inputs from the ventral tegmental area and sends glutamatergic projections to the nucleus accumbens, an essential part of the brain for processing rewards. Disruptions in this circuitry, such as those induced by stress during adolescence, can lead to long-term changes in reward processing and increased susceptibility to addictive behaviors in adulthood (Walker et al. 2017). Thus, the fact that the adolescent sleep deprived rats drank more alcohol could be related to the fact that the sleep deprivation induced anxiety, leading to this neuroadaptation. It is possible that the sleep deprivation during adolescence led to alterations in the brain chemistry and function of the female rats, specifically involving changes in their reward pathways and stress response systems. These alterations could have led to a heightened sensitivity to the rewarding effects of alcohol while not necessarily increasing anxiety-related behaviors, although this explanation cannot be confirmed due to this study's inability to investigate these pathways and structures in the brain post-adolescent sleep deprivation. Too, the sleep deprived rats that did not receive alcohol exhibited EPM behaviors similar to those of the non-sleep restricted rats without access to alcohol. This lack of a persistent effect of sleep deprivation into adulthood would have made it challenging to detect any interaction between sleep deprivation and alcohol consumption.

The observed results regarding water intake during alcohol-free days reveal intriguing insights into interactions between sleep deprivation, alcohol, and water consumption behaviors in rats. There was a main effect of day, but there were no systematic patterns apparent that could explain that effect. There was also a main effect of alcohol, as rats who were given access to alcohol had reduced water intake on off days. This result does not align with the idea that alcohol is a diuretic, meaning that consumption of alcohol can lead to a negative water balance or loss of body water, causing dehydration (Stookey, 1999). This outcome could, however, potentially be caused by the rats developing a preference for alcohol, particularly evident in the sleep deprived rats provided access to alcohol, as they exhibited a greater preference for alcohol compared to the controls and consistently consumed less water on non-alcohol days.

The most notable result for water intake was the interaction between sleep deprivation and alcohol access on water intake during off days. Rats that experienced sleep deprivation during adolescence and had access to alcohol displayed significantly reduced water intake on days without alcohol access compared to all other groups. Once again, these results could reflect the physiological functions disrupted by sleep deprivation, such as hormone regulation and metabolic processes (Medic et al. 2017). Circadian rhythms are responsible for regulating metabolic activity, including food intake, body temperature, and heart rate, so it is possible that the sleep deprivation had an effect on the rats' metabolic function, causing them to display altered patterns of water intake, leading to a decreased sensation of thirst or altered water balance regulation (Medic et al. 2017). Hormone regulation is another physiological function affected by sleep deprivation, particularly satiety and stress hormones (Potter et al. 2016). Sleep disturbances have been shown to reduce levels of the hormone leptin, which normally suppresses appetite, and increase levels of ghrelin, a hormone that stimulates appetite (Potter et al. 2016). Thus, the sleep

deprived and alcohol drinking rats could have had decreased water intake due to other changes in hormone levels, such as vasopressin, which plays a crucial role in regulating water retention and urine concentration (Rosinger et al. 2019). Although the direct impact of sleep deprivation and alcohol consumption on vasopressin is not well-studied, it has been shown that the hormone is influenced by both hydration needs and circadian rhythms. During the late sleep period, vasopressin levels typically increase to prevent dehydration. However, if this sleep period is missed due to short sleep duration, it can affect vasopressin release, potentially leading to disruptions in the body's water balance, homeostasis, and thirst perception (Rosinger et al. 2019). Additionally, alcohol's diuretic effects also affect vasopressin release, potentially inhibiting it and leading to increased urine output. This combination of disrupted vasopressin levels due to sleep deprivation and alcohol's effects on vasopressin may contribute to the observed decreased water intake in sleep deprived and alcohol-drinking rats, as their physiological mechanisms for water retention and regulation could have been compromised. While the specific mechanisms behind these interactions remain unclear and were not investigated in this study, it is plausible that disrupted vasopressin levels due to sleep deprivation and alcohol consumption could have interacted, leading to reduced water intake by the rats on days without alcohol access.

Limitations

The novel method of sleep deprivation used in the present study influenced the behavior of adolescent rats, appearing to induce anxiety-like behaviors. However, it's challenging to definitively ascertain whether these behaviors stemmed directly from sleep deprivation or were primarily a result of heightened stress levels. Utilizing vibrating massage mats and intermittent noise devices on a timer represented a novel and cost-effective approach to induce sleep deprivation in the rats. Too, it has been shown that the most extensive criticism of usual methods

of sleep deprivation is that they can further compound the stress experienced by the experimental animals (Khan et al., 2021). Nonetheless, without confirming the sleep deprivation through electrical activity of electroencephalogram (EEG) and electromyogram (EMG) or measuring corticosterone levels and using neuroimaging techniques to study brain activity and changes in neural circuits associated with anxiety-related behaviors, it remains uncertain whether these behaviors directly resulted from the sleep deprivation or were primarily attributed to stress levels. Too, this study was unable to confirm the extent to which REM sleep was affected due to lack of EEG recordings, which is particularly important to consider in studies of substance abuse, as alcohol tends to suppress REM sleep. (Roehrs & Roth, 2015)

There were also some confounding variables present in regards to the alcohol-drinking period of the experiment that may have caused some variability in the results. The bottles provided to the rats during days of alcohol access varied in size, which prevented them from being switched every other day to prevent the rats from developing a preference for one side, as switching the bottles regularly would have facilitated unbiased drinking behavior. Furthermore, only the alcohol-drinking rats were separated throughout the experiment using dividers on drinking days, but the non-drinking rats remained entirely pair housed for the duration of the experiment. While the dividers were removed on off days in hopes of preventing the confound of social isolation, the potential influence of social dynamics and stress associated with the separation during drinking days on the behavior of the alcohol-drinking rats cannot be entirely ruled out. Unfortunately, the absence of dividers for the non-drinking rats daily and the alcohol-drinking rats on off days posed challenges in accurately measuring water intake, as their paired housing arrangement prevented individual intake measurement for each rat. As a result, water intake on off days was divided by two in this experiment, assuming equivalent intake by

both rats. However, this assumption cannot be confirmed, introducing a potential confound into the study.

There were also some potential miscalculations by lab assistants in the data (day 5 of ethanol intake and day 4 of ethanol preference) that could have resulted in skewed results. These days were taken out of the graphs in the results section but were still included in the statistical analyses. While the data on both of these days were outliers and did not follow the trend of the rest of the data, it is impossible to know the extent to which these calculations could have been wrong or have influenced the overall findings.

Conclusion and Future Directions

This study aimed to investigate the enduring effects of sleep deprivation during adolescence on subsequent alcohol consumption in female Sprague Dawley rats during adulthood, with a focus on understanding the interplay among sleep deprivation, anxiety, and alcohol consumption in female rats. The findings reveal that sleep deprived rats during adolescence displayed heightened anxiety-like behaviors, indicating a stress response to sleep disruption. These anxiety-like behaviors were not exacerbated into adulthood as predicted, but the sleep deprived rats still exhibited a higher alcohol intake and preference compared to the control rats, suggesting that sleep deprivation did have an effect on reward-seeking behaviors. These results underscore the significance of early-life sleep disturbances in shaping adult behaviors, particularly in the context of alcohol consumption and substance use disorders. Further exploration of the neurobiological mechanisms underlying these results is needed to fully understand the complexities of sleep deprivation, stress responses, and alcohol-motivated behaviors across different developmental stages.

Future directions for this study could include conducting a study with both sexes to be able to better delineate any sex-specific interactions or behaviors in terms of adolescent sleep deprivation and adult substance use. Too, utilizing advanced neuroimaging techniques such as functional magnetic resonance imaging (fMRI) or positron emission tomography (PET) would provide insights into the neural circuits and brain regions involved in stress, reward pathways, and alcohol consumption following sleep deprivation. It would be particularly interesting to conduct in vivo imaging or post-mortem tissue analyses of the brain regions mentioned in the discussion that could have led to the results of this study, such as the amygdala, the mesocorticolimbic dopamine pathway, and synaptic plasticity in the prefrontal cortex and nucleus accumbens. Incorporating measures of neurotransmitter levels, receptor densities, and gene expression related to stress and reward systems could also offer a more comprehensive understanding of the molecular basis of these behaviors. In terms of sleep deprivation, future studies could utilize EEG and EMG monitoring throughout the study duration to enhance the accuracy of sleep manipulation and provide a clearer link between sleep disruption and subsequent behavioral changes. Further investigation is also needed to understand the impact of sleep deprivation on vasopressin regulation and its potential effects on alcohol interactions, necessitating a deeper examination of vasopressin's role, function, and altered release mechanisms.

Ultimately, this study sought to underscore the vulnerability of adolescents during their developmental phase of neurodevelopment, behavior, and emotional regulation, particularly regarding the impact of early-life stressors like sleep deprivation on long-term health outcomes and substance use. The results of this study demonstrate that early exposure to sleep deprivation during adolescence can potentially lead to increased susceptibility to alcohol use in adulthood.

The study's focus on how sleep deprivation during this critical developmental period can contribute to long-lasting consequences, such as increased alcohol consumption in adulthood, reveals the importance of addressing sleep health and stress management strategies early on. These findings have significant implications for approaches aimed at preventing substance use disorders and promoting healthy sleep habits among adolescents, emphasizing the need for targeted interventions and further research that addresses the intersection of sleep, stress, and substance use during this vulnerable developmental stage.

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