Published in final edited form as:

Alcohol. 2020 November ; 88: 65–72. doi:10.1016/j.alcohol.2020.06.003.

Adolescent Alcohol Exposure Increases Orexin-A/Hypocretin-1 in the Anterior Hypothalamus

Leslie R. Amodéo^c, Wen Liu^b, Derek N. Wills^a, Ryan P. Vetreno^b, Fulton T. Crews^b, Cindy L. Ehlers^a

^aDepartment of Neuroscience, The Scripps Research Institute, La Jolla CA 92037

^bBowles Center for Alcohol Studies, School of Medicine, University of North Carolina, Chapel Hill, NC 27599

^cDepartment of Psychology, California State University San Bernardino, San Bernardino, CA 92407

Abstract

Adolescence is a time of marked changes in sleep, neuromaturation, and alcohol use. While there is substantial evidence that alcohol disrupts sleep and that disrupted sleep may play a role in the development of alcohol use disorders (AUD), there is very little known about the brain mechanisms underlying this phenomenon. The orexin (also known as hypocretin) system is fundamental for a number of homeostatic mechanisms including the initiation and maintenance of wakefulness that may be impacted by adolescent alcohol exposure. The current study investigated the impact of adolescent ethanol exposure on adult orexin-A/hypocretin-1 immunoreactive (orexin-A+IR) cells in hypothalamic nuclei in two models of adolescent intermittent ethanol (AIE) exposure. Both models assess adult hypothalamic orexin following either an AIE vapor exposure paradigm, or an AIE intragastric gavage paradigm during adolescence. Both AIE exposure models found that binge levels of ethanol intoxication during adolescence significantly increased adult orexin-A+IR expression in the anterior hypothalamic nucleus (AHN). Further, both AIE models found no change in orexin-A+IR in posterior hypothalamic area (PH), perifornical nucleus (PeF), dorsomedial hypothalamic nucleus, dorsal (DMD) or lateral hypothalamic area (LH). However, AIE vapor exposure reduced orexin-A+IR in the paraventricular nucleus (PVN), but not AIE gavage exposure. These findings suggest AHN orexinergic system is increased in adults following binge-like patterns of intoxication during adolescence. Altered adult AHN orexin could contribute to long-lasting changes in sleep.

Keywords

Adolescence; Hypocretin/Orexin; Hypothalamus

Corresponding author: Cindy L. Ehlers Ph.D. Department of Molecular and Cellular Neuroscience, The Scripps Research Institute, 10550 N Torrey Pines Road, La Jolla CA 92037, cindye@scripps.edu.

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Introduction

Adolescence is a time marked by physiological, behavioral, and neuro-maturational changes, which may contribute to a unique vulnerability for alcohol misuse (Barron et al., 2005; Crews, He, & Hodge, 2007). Consistent with these findings, early alcohol use can lead to a higher risk of alcohol-related problems in adulthood (Aiken et al., 2018; Patrick, Evans-Polce, & Terry-McElrath, 2019). Alcohol consumption during adolescence typically occurs in a “binge” pattern, characterized by high quantities of intermittent ethanol consumption over a short period. On average, adolescents consume less frequently, but in higher quantities per binge episode compared to adults (Masten, Faden, Zucker, & Spear, 2009). This behavior has been linked with several adverse consequences, including risky sexual behaviors, interpersonal violence, and higher rates of automobile accidents (Hingson & Zha, 2018). Out of the 1 in 5 youth (12 to 20 years) who reported alcohol consumption in the last month, 12% also reported binge drinking (SAMHSA, 2016). The relationship between adolescent maturation and alcohol use is often attributed to mediating social and environmental factors. However, changing biological homeostatic mechanisms could also contribute to the vulnerabilities in alcohol drinking during adolescence. For example, endogenous circadian rhythms naturally shift with puberty, causing a mismatch in wake-sleep schedules leading to reductions in sleep quantity and quality (Hagenauer, Perryman, Lee, & Carskadon, 2009). Additionally, there is a growing body of evidence to support a relationship between sleep disturbances and relapse to alcohol drinking (Brower & Perron, 2010; Clark et al., 1998, 1999; Drummond, Gillin, Smith, & DeModena, 1998; Foster & Peters, 1999; Gillin et al., 1994).

While there is substantial evidence that alcohol disrupts sleep and may play a pivotal role in the development of alcohol use disorder (Brower, 2015; Brower & Perron, 2010; Clark et al., 1998; Conroy et al., 2006; Stein & Friedmann, 2005), however, there is less known about the homeostatic mechanisms by which this occurs. The orexinergic system (also known as hypocretin) is fundamental for the initiation and maintenance of wakefulness, since depleting orexin neurons or knocking down orexin receptors results in the sleep-wake dysfunction (McCarley, 2007). Orexin-A/hypocretin-1 and orexin-B/hypocretin-2 containing cell bodies are predominantly found in the lateral (LH) and perifornical nuclei (PeF) of the hypothalamus with extensive projections throughout the central nervous system (Peyron et al., 1998). Some of the most dense projections from orexin-A neurons are to regions of the hypothalamus, brainstem, and spinal cord (Date et al., 1999; Elias et al., 1998; Peyron et al., 1998), whereas orexin-B fibers are sparsely distributed in the hypothalamus (Cutler et al., 1999). Under normal conditions, activity in orexin neurons is closely tied to arousal, occurring more frequently during wake or just prior to waking and less frequent or absent during sleep (Lee, Hassani, & Jones, 2005; Takahashi, Lin, & Sakai, 2008). Additionally, higher levels of orexin-A peptide in the hypothalamus occur during wakefulness compared to sleep (Kiyashchenko et al., 2002; Yoshida et al., 2001).

Apart from its role in the control of sleep and arousal, the orexin-A system has also been implicated in multiple factors including, but not limited to, energy homeostasis and food intake (Barson & Leibowitz, 2017), neuroendocrine regulation (Valassi, Scacchi, & Cavagnini, 2008), cardiovascular system control (Shahid, Rahman, & Pilowsky, 2011), and

the modulation of pain (Razavi & Hosseinzadeh, 2017). Not surprising, the positive relationship between orexin and palatable food is also evident with ethanol consumption (Barson & Leibowitz, 2017). Orexin has been found to promote ethanol intake, acting through various hypothalamic nuclei (Schneider, Rada, Darby, Leibowitz, & Hoebel, 2007). Exposure to ethanol during adulthood has been shown to alter hypothalamic orexin immunoreactivity and mRNA expression (Lawrence, Cowen, Yang, Chen, & Oldfield, 2006b; Morganstern et al., 2010; Olney, Navarro, & Thiele, 2015). The hypothalamic orexin-A cells have also been directly implicated in other drug seeking behaviors, evidenced by increased activation of the LH cells to cocaine and morphine associated stimuli (Harris, Wimmer, & Aston-Jones, 2005a). However, orexin cells in the PeF and dorsomedial (DM) hypothalamus regions seem to be more involved in stress and arousal states, demonstrating heterogeneity within the hypothalamic orexin system (Harris & Aston-Jones, 2006; Harris, Wimmer, & Aston-Jones, 2005b).

A growing body of literature demonstrates that ethanol produces age-related effects on behavioral and physiological responses that could influence and/or interact with mechanisms regulating sleep and arousal, amongst other homeostatic mechanisms. For instance, studies have shown that adolescent rats are less sensitive than adults to ethanol-induced motor incoordination and sedation ((Acevedo, Molina, Nizhnikov, Spear, & Pautassi, 2010; Little, Kuhn, Wilson, & Swartzwelder, 1996; Pian, Criado, & Ehlers, 2008). We have found that AIE in the rat vapor model can have lasting reductions in slow wave sleep (SWS) and are more susceptible to disruption of SWS during withdrawal (Criado, Wills, Walker, & Ehlers, 2008; Ehlers, Desikan, & Wills, 2013; Ehlers, Wills, & Gilder, 2018; Sanchez-Alavez, Wills, Amodeo, & Ehlers, 2018). The current study used established models of adolescent intermittent alcohol exposure (AIE) (an alcohol vapor inhalation and a gastric lavage model) to investigate their impact on orexin-A/hypocretin-1 immunoreactive (orexin-A+IR) cells in hypothalamic nuclei in adulthood in order to determine the long-term impact AIE may have on the adult orexinergic system.

Methods

Adolescent Intermittent Ethanol (AIE)

Vapor Exposure.—Male Wister rats arrived on postnatal day (PD) 21 (Charles River, USA) and were group-housed in standard polycarbonate cages at the Scripps Research Institute animal facilities. Vivarium was both temperature- (20°C) and humidity-controlled with a 12-hour light/dark cycle (lights on 08:00). Food and water were available *ad libitum*. Subjects were cared for in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

The ethanol vapor inhalation procedure and the chambers used in this study have been previously described (Ehlers, Liu, Wills, & Crews, 2013). Ethanol vapor chambers were calibrated to produce high to moderate blood ethanol concentrations between 175 and 225 mg/dL. Rats were randomly divided into adolescent intermittent exposure (AIE; n = 13) or control (CONT; n = 15) groups. The AIE rats were housed in sealed chambers, infused with vaporized 95% ethanol in a 14 hr-on/10 hr-off pattern (vapor beginning at 20:00). Rats were

exposed to this nightly vapor cycle for 5 weeks (PD 22–57). Every 3 to 4 days during this 5-week exposure period, blood samples (200 μ L) were collected from the tip of the tail to assess blood ethanol concentrations (BEC; mean = 214.5 ± 6.65 mg/dL). Blood samples were immediately centrifuged at 1500rpm for 15 minutes. Plasma was then extracted and stored at -80°C until further analysis. BECs were determined using the Analox microstatAM1 (Analox Instr. Ltd., Lunenburg, MA). Control animals were handled identically to ethanol rats, except for ethanol vapor exposure.

Intragastric Exposure.—Young time-mated pregnant female Wistar rats (embryonic day 17; Harlan Sprague-Dawley Inc., Indianapolis, IN) were acclimated to the animal facility prior to birthing at the University of North Carolina at Chapel Hill. On PD 21, pups were weaned and group-housed. Vivarium conditions were consistent with those in the vapor AIE study. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill, and conducted in accordance with the National Institutes of Health regulations for the care and use of animals in research.

Male Wistar rats were randomly assigned to either the AIE ($n = 8$) or water control (CONT; $n = 8$) group. To minimize the impact of litter, no more than one subject from a given litter was assigned to any experimental condition. From P25 to P55, AIE animals received a single daily intragastric (i.g.) gavage administration of ethanol (5.0 g/kg, 20% ethanol w/v) in the AM on a two-day on/two-day off schedule and CON subjects received comparable volumes of water. Tail blood was collected to assess blood ethanol concentrations (BECs) one hr after ethanol administration on P38 (mean = 195 ± 8 mg/dL) and P54 (mean = 215 ± 11 mg/dL), with BECs assessed using a GM7 Analyzer (Analox, London, UK). While there was an expected increase in body weight across adolescence, there was no observable effect of ethanol exposure on body weight ($p = 0.9$).

Immunoreaction orexin/hypocretin cells

On PD80, rats were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 4.0% paraformaldehyde in PBS. Brains were excised and post-fixed in 4.0% paraformaldehyde for 24 hr at 4°C followed by 4 days of fixation in 30% sucrose solution. Following tissue collection, orexin-A+IR for the vapor AIE samples were processed in-house at The Scripps Research Institute and intragastric gavage AIE samples were processed at NeuroScience Associates (Knoxville, TN).

For the vapor AIE samples, coronal sections were obtained at a thickness of 40 μ m in 1:12 series. Every 12th section was incubated in 0.6% hydrogen peroxide (H_2O_2) for 30 minutes to remove endogenous peroxidase activity, and blocked in 5% goat serum (0.2% Triton X-100, Sigma, Saint Louis, MO) for 1 hour at room temperature. Samples were then incubated overnight with Orexin A/Hypocretin-1 antibody (mouse monoclonal, 1:200, MAB763–500, R&D system, Minneapolis, MN) at 4°C . On the second day, sections were rinsed in PBS, and incubated with biotinylated secondary goat anti-mouse (1:200, Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. Subsequently, avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories) was added for 1 h at room

temperature. The positive expression was visualized using DAB (nickle-enhanced diaminobenzidine).

Intragastric AIE samples were treated overnight with 20% glycerol and 2.0% dimethylsulfoxide to prevent freezing artifacts, and embedded (4×6) in a gelatin matrix block (MultiBrain™). The block was rapidly frozen by immersion in isopentane chilled to -70°C with crushed dry ice and mounted to the freezing stage of an AO 860 sliding microtome. The MultiBrain block was sectioned into $40\text{ }\mu\text{m}$ coronal slices, bathed in Antigen Preserve Solution (50% PBS [pH 7.0], 50% ethylene glycol, 1.0% polyvinyl pyrrolidone). Free-floating MultiBrain™ sections were washed in Tris-buffered saline (TBS), incubated in H_2O_2 , and blocked in goat serum diluted in TBS (0.3% TritonX-100). Sections were incubated overnight at room temperature in the primary antibody Orexin A/Hypocretin-1 antibody (Calbiochem, catalog number PC 362) at 4°C . Sections were then washed with TBS, incubated in a goat anti-rabbit secondary antibody solution, and incubated in an avidin-biotin-HRP complex solution (Vectastain ABC Kit; Vector Laboratories). After washing with TBS, the sections were treated with diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 to visualize immunoreactivity.

Quantification

Following orexin-A+IR staining, all samples from both experiments were subsequently shipped to the University of North Carolina at Chapel Hill for immunohistological quantification. Bioquant Nova Advanced Image Analysis (R&M Biometric, Nashville, TN) was used for image capture and analysis (Crews et al., 2004). Images were captured by using an Olympus BX50 Microscope and Sony DXC-390 video camera linked to a computer. For orexin-A+IR analysis, pixel densities were measured for the outlined area expressed as pixels per square millimeter (pixels/mm²) with both sides of 3–5 section per animals, and the average value per mm² was used. The location brain regions of interest and abbreviations are shown below (Bregma): anterior hypothalamic nucleus (AHN), periventricular hypothalamic nucleus (Pe) and paraventricular hypothalamic nucleus (PVN) at -1.80 mm ; lateral hypothalamic area (LH), dorsomedial hypothalamic nucleus, dorsal part (DMD) and perifornical nucleus (PeF) at -2.80 mm ; and posterior hypothalamic area (PH) -4.18 mm (Paxinos & Watson, 1998).

Results

Hypothalamic orexin-A+IR was found in multiple nuclei. Figure 1 depicts those hypothalamic regions of interest at a depth of Bregma -1.8 mm , -2.8 mm , and -4.16 mm . The density of AHN hypothalamic orexin-A+IR ($\times 1000$, pixels/mm²) expression was analyzed in young adult rats after exposed to either vapor (Figure 2A, top) or intragastric gavage (Figure 2A, bottom) during adolescence. Adolescent ethanol vapor exposure significantly increased orexin-A+IR expression in the AHN at -1.8 mm ($\text{AIE} = 1.92 \pm 0.44$ pixels/mm²) compared to air-vapor controls [$\text{CONT} = 0.89 \pm 0.22$ pixels/mm²; $F(1,21)=4.997$, $P=0.036$]. These results were replicated using adolescent intragastric ethanol exposure, with a significant upturn in AHN orexin-A+IR expression ($\text{AIE} = 8.73 \pm 1.29$ pixels/mm²) compared to water-treated controls [$\text{CONT} = 5.45 \pm 0.78$ pixels/mm²; $F(1,14)$

=4.740, $P=0.047$]. Figure 2B includes representative examples of orexin-A+IR staining in AHN for AIE and CONT rats after vapor (top) or intragastric (bottom) exposure models.

Figure 3 depicts the orexin-A+IR density expressed in hypothalamic nuclei at a depth of -1.8 mm (Figure 3A), -2.8 mm (Figure 3B), and -4.16 mm (Figure 3C) for young adult rats after adolescent vapor (top) and intragastric gavage (bottom) exposure models. AIE vapor significantly decreased PVN orexin-A+IR at -1.8 mm ($AIE = 0.76 \pm 0.18$ pixels/mm²) compared to controls [$CONT = 1.42 \pm 0.20$ pixels/mm²; $F(1,21)=6.052$, $P=0.023$; Figure 3A, top]. However, these results were not replicated by the gavage model, demonstrating no difference between AIE exposed ($AIE = 22.21 \pm 3.50$ pixels/mm²) and controls ($CONT = 16.14 \pm 1.90$ pixels/mm²), (Figure 3A, bottom). There was no significant effect of AIE vapor on orexin-A+IR in the LH ($AIE = 11.09 \pm 2.21$ pixels/mm²; $CONT = 14.97 \pm 4.70$ pixels/mm²), DMD ($AIE = 6.51 \pm 1.59$ pixels/mm²; $CONT = 3.97 \pm 0.66$ pixels/mm²), or PeF ($AIE = 46.18 \pm 7.57$ pixels/mm²; $CONT = 42.55 \pm 8.35$ pixels/mm²) at a depth of -2.8 mm (Figure 3B, top). There were also no significant differences in the gavage model between AIE and CONT rats in orexin-A+IR expression at -2.8 mm in the LH ($AIE = 47.94 \pm 3.69$ pixels/mm²; $CONT = 36.98 \pm 4.03$ pixels/mm²), DMD ($AIE = 46.14 \pm 7.30$ pixels/mm²; $CONT = 29.40 \pm 4.71$ pixels/mm²), or PeF ($AIE = 149.08 \pm 24.78$ pixels/mm²; $CONT = 157.24 \pm 27.85$ pixels/mm²), (Figure 3B, bottom). Figure 3C depicts orexin-A+IR expression in the PH at Bregma 4.16 mm was no different for either the vapor (top; $AIE = 1.61 \pm 0.38$ pixels/mm²; $CONT = 1.10 \pm 0.41$ pixels/mm²) or gavage (bottom; $AIE = 12.80 \pm 5.32$ pixels/mm²; $CONT = 6.16 \pm 1.93$ pixels/mm²) model.

Discussion

Overall, these results demonstrate that, across exposure models, binge levels of ethanol intoxication during adolescence can significantly increase orexin-A+IR expression in the ANH. While adolescent ethanol vapor exposure reduced orexin-A+IR in the PVN, these results were not replicated using the gavage exposure method. Difference in orexin expression after adolescent vapor ethanol exposure compared to control were not observed in any of the other hypothalamic regions of interest, suggesting a distinct vulnerability of the AHN to binge-like patterns of intoxication during adolescence.

Previous research has demonstrated that exposure to ethanol produces alterations in the hypothalamic orexin system. The increases in orexin-A+IR in the AHN after two intermittent ethanol exposure paradigms is consistent with previous studies reporting increased hypothalamic orexin mRNA and/or orexin-A+IR expression after chronic ethanol consumption in alcohol-preferring iP rats (Lawrence, Cowen, Yang, Chen, & Oldfield, 2006a), intermittent ethanol consumption in Long-Evans rats (Barson, Ho, & Leibowitz, 2015), and acute ethanol gavage (Morganstern et al., 2010). Others have also found reductions in hypothalamic orexin mRNA expression after chronic ethanol consumption (Morganstern et al., 2010) and orexin IR following intermittent ethanol consumption in mice (Olney et al., 2015). Together, these findings demonstrate ethanol exposure can alter orexin levels and different methodological approaches, such as voluntary versus involuntary or the length of administration, may influence the nature of this effect. The current study was

conducted in animals pre-exposed to ethanol during adolescence and could indicate an age-related vulnerability of the orexin system to ethanol.

We found that ethanol reduced orexin IR in the PVN following intermittent ethanol vapor, however these results were not replicated by gavage administration. Orexin-A injected in the PVN has been shown to stimulate voluntary ethanol intake without significantly altering intake of food and water (Schneider et al., 2007). Conversely, orexin peptide expression in the PVN increased after both chronic and acute ethanol consumption (Chang et al., 2007; Leibowitz et al., 2003). While these results demonstrate a bidirectional relationship between orexin and ethanol exposure, not much is known about the impact ethanol exposure has on orexin neurons or fiber density in the PVN. What is known is that the PVN is densely innervated by orexin fibers (Cutler et al., 1999; Peyron et al., 1998; Plaza-Zabala, Flores, Maldonado, & Berrendero, 2012), which originate from hypothalamic neurons (Peyron et al., 1998). Orexin containing cells in the PVN seem to play a role in sympathetic regulation of cardiovascular function during arousal (Dergacheva, Yamanaka, Schwartz, Polotsky, & Mendelowitz, 2017). Interestingly, dysfunction of GABAergic pathway which originates from orexin neurons, may play a role in increased sympathetic activation of the heart and blood vessels which has been reported in individuals with cataplexic narcolepsy (Dergacheva et al., 2017; Grimaldi et al., 2012; van der Meijden et al., 2015). Further, sleep-related decreases in blood pressure were blunted in orexin-deficient mice compare to wild-type (Bastianini et al., 2011). The PVN has also been implicated in the release of stress hormones (Swanson, Sawchenko, Rivier, & Vale, 1983). Intracerebroventricular administration of orexin-A in rats resulted in Fos-like immunoreactive expression in almost all of the corticotropin-releasing factor (CRF) containing neurons in the PVN (Sakamoto, Yamada, & Ueta, 2004). Fos expression in orexin-A containing neurons has also been demonstrated after the application of two differing stressors (Sakamoto, Yamada, & Ueta, 2004). In sum, the differences in PVN orexin-IR activation could be attributed to methodological differences in ethanol administration, not AIE exposure itself. Further replication of our finding is necessary to better elucidate the impact ethanol may have on the orexin system in the PVN.

Our results indicated an increase in orexin-A+IR expression in the AHN after binge-levels of ethanol exposure during adolescence. This finding was subsequently replicated with gavage ethanol administration during adolescence suggesting a unique impact ethanol might be having on the anterior hypothalamus orexin system. The AHN/preoptic area (PO), two hypothalamic nuclei typically studied together, play a critical role in sleep-wake cycles (Hara & Sakurai, 2011). Orexin-A+IR fibers are moderately abundant in this area, with dense mRNA expression of both orexin-1 and orexin-2 receptors in the ANH/PO (Cutler et al., 1999; Marcus et al., 2001; Peyron et al., 1998). Activation of GABAergic neurons located in the PO send direct inhibitory projections, suppressing orexin arousal-regulatory systems and promote sleep (Saito et al., 2013; Saper, Cano, & Scammell, 2005; Szymusiak, Gvilia, & McGinty, 2007). Interestingly, increasing orexin-A peptide into the PO has been shown to increase wakefulness and suppressed both REM and slow-wave sleep (Mavanji et al., 2015; Methippara, Alam, Szymusiak, & McGinty, 2000). These data suggest that the AHN/PO may be an important anatomical site at which some agents may alter the sleep-wake cycle, but the mechanisms by which this occurs remain unclear. Previous studies have

shown that a wide range of sedative/hypnotic agents, including ethanol, induce sleep when microinjected acutely into the PO (Mendelson, 1996, 2001; Mendelson & Martin, 1992). We speculate that while ethanol may initially increase sleep through activation of GABAergic pathways to hypothalamic orexin nuclei, continual use may result in impairment in this pathway, leading to increase release of orexin-A peptide and reductions in slow-wave sleep (Criado et al., 2008).

However, there are multiple brain systems that are responsible for sleep homeostasis, that could potentially be impacted by ethanol. For instance, in adult rodents, acute binge alcohol has been shown to have sleep disrupting effects (Sharma, Sahota, & Thakkar, 2014), associated with reductions in the expression of equilibrative nucleoside transporter 1 (ENT1) (Sharma, Gonda, & Tarazi, 2018). Chronic ethanol administration has also been shown to lead to sleep disruption in adult rodents (Irwin, Miller, Christian Gillin, Demodena, & Ehlers, 2000; Mukherjee, Kazerooni, & Simasko, 2008; Mukherjee & Simasko, 2009; Sanchez-Alavez, Benedict, Wills, & Ehlers, 2019; Sanchez-Alavez, Wills, Amodeo, & Ehlers, 2018; Sharma, Engemann, Sahota, & Thakkar, 2010; Veatch, 2006), and it has been suggested that both cholinergic and adenosinergic mechanisms in basal forebrain may in part be responsible for alcohol-induced sleep disruption in adults (Ehlers, Criado, Wills, Liu, & Crews, 2011; Sharma, Sahota, & Thakkar, 2017). However, few studies have investigated the mechanisms underlying the effects of *adolescent* alcohol exposure on sleep.

Adolescence is frequently a time when the developing brain is first exposed to potentially neurotoxic levels of ethanol, and this early exposure has been demonstrated to enhance risk for the later development of dependence (Ehlers, Slutske, Gilder, Lau, & Wilhelmsen, 2006; Grant & Dawson, 1997; Johnston LD, O'Malley PM, Bachman JG, 2009). Recent findings have confirmed that adolescents and young adults with drug and alcohol problems also have sleep difficulties (Bartel, Gradisar, & Williamson, 2015; Cohen-Zion et al., 2009; Criado & Ehlers, 2010; Ehlers, Gilder, Criado, & Caetano, 2010; Ehlers, Wills, & Gilder, 2018; Hagenauer et al., 2009; Meltzer & Mindell, 2008; Mindell & Meltzer, 2008; Roberts, Roberts, & Chen, 2002). Current theory suggests that these sleep disturbances may further contribute to adolescent substance involvement (Hasler, Casement, Sitnick, Shaw, & Forbes, 2017; Hasler & Clark, 2013; Hasler, Franzen, et al., 2017; Hasler, Martin, Wood, Rosario, & Clark, 2014; Hasler, Soehner, & Clark, 2015, 2014; Logan et al., 2018). One short coming of the present study is the absence of female rats and the potential sex-dependent differences of AIE on orexin. We have previously shown that AIE does not impact adulthood expression of OxR1 and OxR2 mRNA in the frontal cortex, however baseline differences were higher for females compared to males (Amodeo et al., 2018). Thus, further research is needed to better elucidate the impact ethanol may have on adolescent brain development in general and specifically homeostatic mechanisms such as the orexin system. It is critical to the understanding of the mechanisms underlying alcohol-induced sleep pathology and the potential role of the orexingenic system as a therapeutic target.

Acknowledgments

This work was supported by National Institute of Health (NIH) grants, U01 AA019969; R01 AA006059 to Cindy L. Ehlers from the National Institute on Alcohol Abuse and Alcoholism (NIAAA). The authors thank Phil Lau for help in statistical analyses.

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Highlights

- Adolescent ethanol exposure increased orexin-A/hypocretin-1 immunoreactive (orexin-A+IR) cells in hypothalamic nuclei.
- Both intragastric and vapor models found that significantly increased expression in the anterior hypothalamic nucleus.
- Adolescent vapor exposure reduced orexin-A+IR in the paraventricular nucleus, but not adolescent gavage exposure.
- Altered adult AHN orexin could contribute to long-lasting changes in sleep seen following adolescent alcohol exposure.

Figure 1. Schematic drawings of the hypothalamic regions

Schematic drawings of the hypothalamic regions analyzed for orexin-A+IR expression in adolescent intermittent ethanol (AIE) and control (CONT) rats. The regions of interest included (A) anterior hypothalamic nucleus (AHN), periventricular hypothalamic nucleus (Pe) and paraventricular hypothalamic nucleus (PVN) at -1.80 mm; (B) lateral hypothalamic area (LH), dorsomedial hypothalamic nucleus, dorsal part (DMD) and perifornical nucleus (PeF) at -2.80 mm; and (C) posterior hypothalamic area (PH) at -4.18 mm. Figures have been adapted from the Paxino and Watson (1998) rat atlas.

Figure 2. Orexin-A+IR Expression in AHN after AIE Exposure

(A) The density of hypothalamic orexin-A+IR expression (x1000, pixels/mm²) was analyzed in young adult rats after intermittent ethanol vapor (top) or intragastric gavage (bottom) during adolescence. Orexin-A+IR in the AHN was significantly increased in AIE rats compared to controls (CONT) for both methods of exposure. (B) Example orexin-A+IR staining in AHN for AIE and CONT rats after vapor (top) or intragastric (bottom) exposure models. Scale bar = 50µm.

*p < 0.05 vs. controls.

Figure 3. Orexin-A in various hypothalamic nuclei after AIE Exposure

The density of hypothalamic orexin-A+IR expression (x1000, pixels/mm²) was analyzed after adolescent rats were exposed to either adolescent intermittent vapor (top) or gavage (bottom) models with ethanol (AIE) or non-ethanol controls (CONT). (A) Orexin-A+IR expression was analyzed in the Pe and PVN at Bregma -1.8 mm. AIE vapor exposure significantly decreased PVN orexin-A+IR when compared to air-matched controls, however this result was not replicated in the gavage model. No difference in orexin-A+IR were seen in the Pe of the hypothalamus for either model. (B) There was also no significant effect of AIE vapor or gavage model on orexin-A+IR density at Bregma -2.8 mm in the LH, DMD, or PeF of the hypothalamus. (C) Orexin-A+IR (pixel/mm²) in the PH at a depth of -4.16mm was no different for AIE compared to CONT for either vapor (top) or gavage (bottom) exposure model. *p < 0.05 vs. controls