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Sex Differences in Photic Entrainment and Sensitivity to Ethanol-Induced Chronodisruption in Adult Mice after Adolescent Intermittent Ethanol Exposure

DR. Christina L. Ruby^{1,*}, Gerneleh Paye¹, Jason L. Fabi¹, Jiawen Zhang¹, Megan O. Risinger¹, Kaitlyn N. Palmer¹, Natalie M. Verbanes¹, Ariana D'Angelo¹, Tia M. Watts¹, Lauren Mabe¹, and DR. H. Scott Swartzwelder²

¹Department of Biology, Indiana University of Pennsylvania, Indiana, PA 15705

²Department of Psychiatry and Behavioral Sciences, Duke University, Durham, NC 27708

Abstract

Background: Evidence supports a role for the circadian system in alcohol use disorders, but the impact of adolescent alcohol exposure on circadian timing later in life is unknown. Acute ethanol attenuates circadian photic phase-resetting in adult, but not adolescent, rodents. However, nearly all studies have focused on males and it is unknown whether this adolescent-typical insensitivity to ethanol persists into adulthood after adolescent drinking.

Methods: Circadian activity was monitored in C57BL6/J mice receiving adolescent intermittent ethanol (AIE) exposure (15% ethanol and water every other day throughout adolescence) or water alone followed by 24 days wherein ethanol was not available (washout). Mice then received a challenge dose of ethanol (1.5 g/kg, i.p.) or saline 15 min prior to a 30-min phase-delaying light pulse, then were released into constant darkness (DD). To control for possible phase-shifting by ethanol challenge alone, a separate group of mice underwent AIE exposure (or water-only) and washout, then received an ethanol or saline injection, but did not receive a light pulse prior to DD.

Results: Striking sex differences in nearly all measures of circadian photic entrainment were observed during adolescence but AIE effects were subtle and few. Only ethanol-naïve adult male mice showed attenuated photic phase-shifts with ethanol challenge, while all other groups showed normal phase-resetting responses to light. AIE-exposed females showed a persistent delay in activity offset.

Conclusions: Adult male AIE-exposed mice retained adolescent-like insensitivity to ethanol-induced suppression of photic phase-resetting, suggesting AIE-induced 'lock-in' of an adolescent behavioral phenotype. Adult AIE-exposed females showed delayed initiation of the rest phase. Our results also indicate that intermittent ethanol drinking has subtle effects on circadian activity in mice during adolescence that differ from previously reported effects on adult males. The observed sex differences in circadian activity, ethanol consumption and preference, and responses to ethanol challenge merit future mechanistic study.

*Corresponding Author: Christina L. Ruby, Ph.D., Department of Biology, Indiana University of Pennsylvania, 114 Weyandt Hall, 975 Oakland Avenue, Indiana, PA 15705, Tel: (724)357-2352, Fax: (724)357-5524, cruby@iup.edu.

Keywords

alcohol; circadian; adolescent; photic phase-resetting

INTRODUCTION

Alcohol (ethanol) has long been known to disrupt circadian behavioral, physiological, and gene expression rhythms, and a large body of research supports a central role for circadian timing in the pathophysiology of alcohol use disorders (Rosenwasser, 2010). In mammals, circadian rhythms are regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus, a clock whose timing is regulated by oscillatory gene expression and synchronized to the environment by photic (light), and nonphotic (behavioral) stimuli (Pickard, 1982, Webb et al., 2014). These stimuli elicit either advances or delays of the circadian rhythm based upon the time of day at which they are presented. Ethanol disrupts circadian timing by inhibiting both photic and nonphotic phase-resetting in several adult rodent species (Brager et al., 2010, Brager et al., 2011, Ruby et al., 2009a, Ruby et al., 2009b, Seggio et al., 2009). However, we recently reported that adolescent male mice are insensitive to the attenuating effect of ethanol on photic phase-resetting (Ruby et al., 2017), adding to the growing body of evidence that age of exposure determines in large part the effects and addictive potential of ethanol.

In humans, circadian activity and sleep patterns change throughout life and are generally delayed during adolescence (Hagenauer et al., 2009). The basis for this delayed chronotype is unknown, though it has been speculated that adolescents may exhibit enhanced sensitivity to phase-delays and/or reduced sensitivity to phase-advances compared to adults (Hagenauer et al., 2009). “Eveningness” (peak alertness occurring in the evening rather than the morning hours) is a very strong predictor of alcohol and drug use (Kanerva et al., 2012, Kervran et al., 2015, Lemoine et al., 2013, Robinson et al., 2013, Tavernier and Willoughby, 2014, Taylor et al., 2011, Urban et al., 2011, Watson et al., 2013, Whittier et al., 2014), particularly within adolescent and college-age populations (Tavernier and Willoughby, 2014, Taylor et al., 2011). Moreover, behavioral traits commonly associated with addiction and typified by adolescents, such as reward dependence, impulsivity, and novelty-seeking, are also linked with eveningness (Hsu et al., 2012). Thus, a better understanding of the relationship between ethanol and circadian phase regulation during adolescence may lead to new insights into addictive liability.

Animal models have proven key to understanding age-related differences in drinking and ethanol sensitivity as well as the long-term impact of adolescent alcohol exposure later in life. Adolescent rodents drink more alcohol than adults, which is thought to be related to their greater sensitivity to many reinforcing properties of acute ethanol (e.g. anxiolysis, social facilitation, locomotor stimulation), as well as memory impairments, and reduced sensitivity to many of its undesirable effects (e.g. sedation, conditioned taste aversion, alcohol deprivation effect (Garcia et al., 2001, Hefner and Holmes, 2007, Holstein et al., 2011, Lacaille et al., 2015, Linsenhardt et al., 2009, Little et al., 1996, Lopez et al., 2003, Markwiese et al., 1998, Melendez, 2011, Melon and Boehm, 2011, Moore et al., 2010,

Moore et al., 2013, Quoilin and Boehm, 2016, Spanos et al., 2012). Interestingly, some adolescent-typical alcohol sensitivities persist into adulthood after adolescent intermittent ethanol (AIE) exposure even long after the ethanol exposure has ceased (see Spear and Swartzwelder, 2014 for review).

Studies of alcohol's effects on circadian rhythms during development are hampered by the lack of research on developmental changes in rodents' circadian rhythmicity, despite the fact that circadian activity measures translate easily between rodents and humans. We have recently begun to address this gap in the literature, reporting age-related changes in photic entrainment and larger phase-delays of circadian activity in adolescent mice compared to adults (Ruby et al., 2017), which is consistent with previous reports (Weinert et al., 1994, Weinert and Kompauerova, 1998) and supports one of the proposed mechanisms underlying eveningness in human adolescents (Hagenauer et al., 2009). We also observed insensitivity to the inhibitory effect of acute, moderate-dose ethanol on photic phase-resetting among adolescent mice, suggesting this as a permissive factor for adolescent drinking (Ruby et al., 2017). The present study aimed to characterize the effects of adolescent intermittent ethanol (AIE) exposure on circadian activity in mice both during adolescence and later, in adulthood. We also compared male and female mice, as the majority of circadian rhythm studies have used males only. Specifically, we hypothesized that mice of both sexes would be relatively resistant to chronodisruption during AIE and that they would retain insensitivity to acute ethanol-induced attenuation of photic phase-resetting into adulthood after AIE exposure.

MATERIALS AND METHODS

Animals

Male and female C57BL6/J mice were bred in-house and weaned at P21. Weanlings were transferred to the testing room, where they were held in group-housed cages for the first 4 days to minimize stress. After the 4-day acclimation period, mice were individually caged and placed under infrared sensors to monitor their circadian activity for the remainder of the 70-day experimental procedure (Figure 1A). Three cohorts of approximately 24 mice each were used in serial experiments for a total of 32 females and 36 males ($n \approx 12$ per sex in each cohort). All mice were tested in the same testing room under the same environmental conditions (except the experimental variables, described in the following sections). For the first 55 days, the testing room was maintained on a 12-hour light, 12-hour dark (LD) photocycle, with lights-on at 1:00 AM, and lights-off at 1:00 PM. This photocycle allowed the phase-resetting experiments, which take place during the dark phase, to occur at a convenient time for the investigators. LD entrainment measures were taken from the 24-day period during adolescent intermittent ethanol (AIE) exposure (or water-only handling control; see below). Following the phase-resetting experiments on day 56, the testing room was then maintained in constant darkness (DD) for an additional 14 days to assess phase-resetting responses (described below). The entire experimental procedure and ages of mice during each segment are illustrated in Figure 1A. Figure 1B shows the phase-resetting experiment design relative to the photocycles and which segments of the procedure are represented by the data in subsequent Figures. Food and water were available ad libitum

throughout the experimental procedure. Animal care/handling and experimental procedures were approved by the Indiana University of Pennsylvania IACUC, consistent with NIH guidelines.

Adolescent Intermittent Ethanol (AIE) Exposure

Approximately half of the mice ($n \approx 6$ per sex) in each cohort were given access to 15% ethanol (v/v) in two bottle choice with water every other day during the periadolescent period (Melendez, 2011) for a 24-day period (days 8-31; Figure 1A). The other half of the mice ($n \approx 6$ per sex) in each cohort were maintained on water. All mice and bottles were weighed daily during this time to monitor ethanol intake in the AIE group, and in water drinkers, to control for the daily handling experienced by the AIE group. To avoid the possibility that this daily handling would act as a nonphotic Zeitgeber, we did it at ZT13 under dim red light, which does not elicit phase-resetting in mice. Bottle positions were switched at each exposure to control for possible side-preferences. Ethanol consumption (g/kg/day) and preference (% ethanol solution/total fluid consumption) were averaged over the 12 exposures.

LD Entrainment

General circadian locomotor activity rhythms for each individually-housed mouse were monitored using infrared motion detectors interfaced with a computerized data acquisition system (Clocklab; Coulbourn Instruments, Whitehall, PA). All data was analyzed using the ClockLab circadian toolbox (Actimetrics, Wilmette, IL) for MATLAB (Mathworks, Natick, MA). The following entrainment measures were taken for all mice ($n = 32$ females, $n = 36$ males) during the 24-day period of AIE (or control) exposure and during the 24-day washout period. Circadian period (τ) was determined using ClockLab's chi-squared periodogram analysis. The peak magnitude (amplitude) of the periodogram was used to estimate the robustness of the rhythm. Activity onset was defined as the first 10 min activity that 1) exceeded 10% maximum daily rate, 2) was preceded by 4 h inactivity, and 3) was followed by 30 min sustained activity. Onsets were then calculated relative Zeitgeber Time 12 (ZT12), the beginning of the dark phase (active phase for nocturnal rodents). Activity offset was defined as the final 10 min that was preceded by 60 min sustained activity and followed by 4 h inactivity and was calculated in relation to ZT0, the time of lights-on (inactive/rest phase for nocturnal rodents). Alpha (the duration of the active phase) was calculated as the period between activity onset and activity offset (h).

Photic Phase-Resetting

Two of the 3 cohorts of mice were used to determine whether there were lasting changes in the effect of ethanol on photic phase-delays. After AIE (or control) exposure, mice underwent a 24-day Washout period during which they had only water available (or continued to have only water available; days 32-55). The final ~14 days of this period were used to determine baseline activity onsets by which phase-shifts are calculated (described below). On day 56 (mice age = P76, age of adult mice in (Ruby et al., 2017), mice were divided into treatment groups and received a challenge injection of ethanol (1.5 g/kg, i.p. $n \approx 6$ per sex X exposure group) or of saline ($n \approx 6$ per sex X exposure group) 15 min preceding a 30-min phase-delaying light pulse (25 lux) at ZT14.5 (2.5 hours into the dark phase).

Injections were undertaken in the dark using dim red light. Immediately after the light pulse, mice were released into DD for ~14 days to assess phase shifting using an Aschoff Type II procedure (Aschoff, 1965). Phase-shifts were calculated as the difference between the projected times of activity onset (defined as above) on the day after stimulation as determined by 1) back extrapolation of the least-squares line through activity onsets on days 2-10 after treatment and 2) extrapolation of the least-squares line calculated from activity onset data collected during the 10 days before treatment.

Phase-Resetting Control

The 3rd cohort of mice was used to rule out the possibility that the ethanol challenge injection had a phase-resetting action on its own at ZT14.5. Once mice showed stable entrainment to the LD photocycle for at least 14 days (as described above), they were divided into treatment groups. On the day of the experiment, mice received an i.p. injection of either ethanol (1.5 g/kg, n = 3 per sex X exposure group) or saline (n = 3 per sex X exposure group) 15 min preceding ZT14.5, but no light pulse was given. Injections were undertaken in the dark using dim red light. Following the injection, mice remained in DD for ~14 days to assess phase shifts (as described above).

Statistical Analysis

LD entrainment measures were analyzed using two-way ANOVA with factors of sex (male vs. female) and exposure (AIE vs. control) to compare possible differences in circadian activity during adolescence (AIE) and during adulthood (washout) arising from AIE exposure and/or sex. Pairwise comparisons between adolescence and adulthood were not made due to the differences in handling between these two periods of the experiment (daily handling during AIE vs. no handling during washout). Within the AIE group, some entrainment measures were analyzed using an additional two-way RM ANOVA with factors of sex (male vs. female) and ethanol availability (on ethanol vs. off ethanol). Phase-resetting data were analyzed within each sex using a two-way ANOVA with factors of exposure (AIE vs. control) and injection (ethanol challenge vs. saline). A separate two-way ANOVA with factors of exposure (AIE vs. control) and injection (ethanol challenge vs. saline) was performed for the phase-resetting control experiment because this experiment addressed a separate question, as posed above (e.g. the presence or absence of a light pulse was not an experimental condition *per se*). ANOVA were followed by Tukey's multiple comparisons post-hoc tests where interactions between variables were found. Results were considered significant when $p < 0.05$.

RESULTS

Female Adolescent Mice Consume More Ethanol Than Males.

Female adolescent mice (n = 18) consumed more ethanol than their male counterparts (n = 19) over the 12 sessions of ethanol availability ($t = 2.249$, $p = 0.0309$), averaging 21.13 ± 1.14 g/kg/day and 17.16 ± 1.34 g/kg/day, respectively (Figure 2A). Preference for the ethanol solution was comparable between males and females ($t = 1.311$, $p = 0.1983$), averaging 59.29 ± 4.31 % for males and 66.87 ± 3.82 % for females (Figure 2B).

Pronounced Sex Differences in Photocycle Entrainment Measures in Adolescent Mice.

Female adolescent mice generally showed more robust circadian activity than males during the 24 day exposure period regardless of whether they received ethanol. On average, females had a higher amplitude rhythm in LD than males, with no differences attributed to AIE exposure and no sex X exposure interaction (Figure 3A). Females also showed a longer active-phase duration (alpha) compared to males, with no influence of AIE exposure and no interaction between the two variables (Figure 3B). Activity onsets (calculated relative to the time of lights-out, Zeitgeber Time 12, ZT12) were earlier (less negative values) in females compared to males with no influence of AIE exposure and no interaction between the two variables (note that negative values indicate that the activity occurred after, as opposed to before lights-out; Figure 3C). There was a trend for later offsets (calculated relative to ZT0, lights-on) among female adolescent mice compared to males, with no exposure effect or sex X exposure interaction (Figure 3D).

In addition to the above measures, we also compared nightly (active phase) and daily (rest phase) activity between AIE-exposed mice and controls. We found that adolescent female mice had fewer nightly activity bouts compared to adolescent males, with no effects of AIE exposure and no sex X exposure interaction (Figure 3E). Female mice were active for more time at night and showed greater levels of nightly activity, with no AIE effects or interactions in either measure (Figure 3F-G). Taken together, these results show that adolescent female mice showed more consolidated and robust activity at night compared to adolescent males.

Daytime activity was also greater in adolescent females compared to their male counterparts. Daytime activity bouts were more numerous in females than in males, with no AIE-related effect or interaction (Figure 3H). The time spent active during the day was also greater in females, with no effect of AIE or interaction between the two variables (Figure 3I). Activity intensity during the daytime was also higher in female mice compared to males with no main effect of AIE, but a trend for a sex X exposure interaction (Figure 3J). Taken together, these data show that adolescent females are more active during the rest phase compared to adolescent males. Means and statistical results for all circadian entrainment measures taken during adolescence are summarized in Table 1.

Ethanol Has Subtle Effects on Circadian Entrainment in Adolescent Mice.

The above analyses of circadian entrainment were between AIE-exposed mice and water-drinking controls and encompassed the entire 24-day exposure period. Because ethanol was available every other day, we also compared circadian activity patterns within AIE-exposed mice during the 12 sessions when ethanol was available (on EtOH) versus the 12 sessions when it was not (off EtOH). These analyses revealed subtle but intriguing differences in circadian activity due to ethanol consumption. Alpha (the length of the active phase) was longer on average when ethanol was available compared to when it was not. A trend for sex differences in this measure remained, and though there was no interaction between variables (Figure 4A), pairwise analysis showed alpha changed within subjects ($t = 5.810$, $p < 0.0001$). Analysis of activity onsets revealed an interaction between variables that was driven by earlier onsets in females during ethanol availability, whereas no change was seen in

males. A trend was observed for a main effect of sex (Figure 4B). Activity offsets were similarly delayed in both sexes during ethanol availability ($t = 5.366$, $p < 0.0001$ within subjects), with no interactions (Figure 4C).

Adolescent female mice had fewer nightly activity bouts compared to adolescent males, and there was an interaction between sex and ethanol availability. Post-hoc testing revealed that female mice had more nightly bouts (about 1 more on average) on EtOH compared to off EtOH (Figure 4D). There were main effects of sex and ethanol availability on time spent active at night, with an interaction between variables and a within-subject difference ($t = 8.997$, $p < 0.0001$). Post-hoc testing showed that both males and females were less active at nighttime when ethanol was available versus when it was not (Figure 4E). Main effects of sex and ethanol availability were seen in nightly activity intensity, as was a within-subject difference ($t = 5.355$, $p < 0.0001$) indicating that mice were less active when ethanol was available than when it was not and that females were more active than males (Figure 4F). These results show a subtle but significant decrease in nighttime activity during ethanol availability in both adolescent female and male mice.

Daytime activity was higher in adolescent females compared to their male counterparts and was not effected by ethanol availability. Daytime activity bouts were more numerous in females versus males, with no effect of ethanol availability or interaction between variables (Figure 4G). The time spent active during the day was also greater in females regardless of ethanol availability, with no interaction between the two variables (Figure 4H). Activity intensity during the day was also higher in female mice compared to males with no main effect of ethanol availability or interaction between variables (Figure 4I). These data show that rest phase activity was unaffected by ethanol availability in adolescent mice. Means and statistical results for all On vs. Off measures are summarized in Table 2.

Sex Differences in Photocycle Entrainment Remained in Adult Mice, but Activity Offset Was Delayed in Females with Prior AIE Exposure.

Adult female mice continued to be more active than adult males during the 24 day washout period, with a higher amplitude rhythm in LD than males, with no differences attributed to AIE exposure and no sex X exposure interaction (Figure 5A). Females also showed a longer active-phase duration (alpha) compared to males, with no influence of AIE exposure. There was an interaction between the two variables, but post-hoc testing did not show significant differences due to AIE in either sex (Figure 5B). Activity were earlier in females compared to males with no influence of AIE exposure and no interaction between the two variables (Figure 5C). Activity offsets were later in female mice compared to males, and there was a sex X exposure interaction. Post-hoc testing revealed that adult female mice with prior AIE exposure had delayed activity offsets relative to control females, with no such difference in males (Figure 5D).

Nightly (active phase) and daily (rest phase) activity between adult mice after AIE or control exposure showed that female mice had fewer nightly activity bouts compared to males, with no effects of AIE exposure and no sex X exposure interaction (Figure 5E). Female mice were active for more time at night and showed greater levels of nightly activity, with no AIE effects or interactions in either measure (Figure 5F-G). Taken together, these results show

that adult female mice continue to show more consolidated and robust activity at night compared to adult males.

Daytime activity was also greater in adolescent females compared to their male counterparts. Daytime activity bouts were more numerous in females versus males, with no AIE-related effect or interaction (Figure 5H). The time spent active during the day was also greater in females, with no effect of AIE or interaction between the two variables (Figure 5I). Activity intensity during the daytime continued to be higher in adult female mice compared to males with no main effect of AIE or sex X exposure interaction (Figure 5J). These data show that adult females remain more active during the rest phase compared to adult males. Means and statistical results for all entrainment measures during adulthood are summarized in Table 3.

Insensitivity to Ethanol-Induced Attenuation of Photic Phase-Resetting in Adult Females and Adult Males Previously Exposed to AIE.

Because we had previously found that adolescent male mice were insensitive to the inhibitory effect of acute ethanol on photic phase-resetting compared to adult males, we hypothesized that this insensitivity would persist in adults with prior AIE exposure. Indeed, among adult males, we found main effects of ethanol challenge and exposure and an interaction between the two variables on photic phase-shifts, with only ethanol-naïve (control) males that received the ethanol challenge injection as adults showing attenuated photic phase-delays, while adult males with prior AIE exposure showed phase-resetting responses after ethanol challenge that were consistent with the responses of the saline-treated groups. Males previously exposed to AIE were significantly less sensitive to the effects of ethanol challenge, consistent with the previously reported insensitivity during adolescence (Figure 6A; note that negative values indicate a phase-delay, whereas positive values would denote a phase-advance of the rhythm). Representative actograms of the entire experimental procedure from 2 males in each group are shown in Figure 7. Interestingly, among adult females, we found no main effects and no interaction between variables, indicating that females were unaffected by this moderate challenge dose of ethanol regardless of whether or not they had prior AIE exposure (Figure 6B). Representative actograms of the entire experimental procedure from 2 females in each group are shown in Figure 8. Finally, ethanol challenge did not induce phase-shifts in the absence of a light pulse in adult mice (Figure 6C).

DISCUSSION

The present results confirm the hypothesis that prior exposure to adolescent intermittent ethanol (AIE) leads to persistent insensitivity to the inhibitory effect of acute, moderate-dose ethanol on photic phase-resetting of circadian activity rhythms in adult male mice. In contrast, adult females were insensitive to acute ethanol-induced inhibition of photic phase-resetting whether they consumed ethanol during adolescence or not, but showed persistent delays in activity offset (e.g. beginning of rest phase) with prior AIE exposure. Additionally, our study reveals a significant influence of sex on several aspects of photocycle entrainment of circadian activity in mice during adolescence and early adulthood. We confirm and extend previous reports showing higher activity levels and more robust rhythmicity in young female

mice compared to males. We also show, for the first time, subtle and reversible changes in circadian activity during AIE that appear different from the better-studied effects of chronic ethanol on adult male rodents. The similarities and differences between male and female mice in our study provide important clues for future behavioral and mechanistic investigations and further support the necessity of including females in alcohol and chronobiology research.

Sex differences in various alcohol-related endpoints have been increasingly reported in adolescent rodents, yet relatively few circadian studies to date have examined female adolescent animals. In general, our female mice were more active than our males, indicated by their higher amplitude activity, longer active-phase duration (alpha), and increased nightly and daily activity levels (time spent active and counts of activity). These relative differences were evident in adolescence (Fig. 3) and remained during young adulthood (Fig. 5). Stowie and Glass (2015) also noted longer alpha and greater bout duration and intensity in adolescent and young adult female C57BL6J mice compared to age-matched males (Stowie and Glass, 2015). Similarly, Royston et al. (2016) observed more wheel-running activity in adult female, compared to adult male mice of the same strain, a difference which was enhanced by aromatase deletion and dampened by estradiol (Royston et al., 2016). Longer activity duration has also been observed during DD in young adult (2-3 month old) female *Per2^{Luc}* mice on a C57BL6J background (Kuljis et al., 2013). These authors also correlated duration of activity with chromosomal sex (XX) independent of gonadal phenotype whereas gonadal hormones influence activity levels in gonadal males independent of chromosomal sex (Kuljis et al., 2013). We were also able to confirm findings relating to earlier onsets in female adolescents versus males (Stowie and Glass, 2015). However, these authors showed similar phase-angles of entrainment in males and females, which occurred prior to ZT12, whereas our mice became active after lights-off. It is noteworthy that advanced onsets in female mice echo what is seen in humans, as women of child-bearing age showed advanced core body temperature rhythm compared to men (Boivin et al., 2016). Subjective alertness in women is also advanced relative to men, differences which disappear at menopause (Bailey and Silver, 2014, Boivin et al., 2016). Melatonin rhythms are advanced in women in several studies (Bailey and Silver, 2014), but not when controlling for menstrual phase and hormonal contraceptive use (Boivin et al., 2016). We did not measure sleep *per se*, but we and others (Stowie and Glass, 2015) have observed later offsets of activity and greater rest-phase activity in young females compared to males. Similar sex differences in habitual bedtimes and nighttime awakenings were recently shown among a large sample (n = 800) of Mexican American and American Indian youth (Ehlers et al., 2018).

Interestingly, we did observe a subtle impact of ethanol drinking during adolescence on murine circadian entrainment which only became evident when comparing days when ethanol was available to when it was not within the treatment group. For some measures, the direction of change was the same in males and females, while for others, a change was only evident in females. Of note, these changes bear potentially important similarities and differences with prior studies primarily conducted in adult males. The active phase was longer in our adolescent mice during ethanol availability, which was apparently driven by a delay in activity offset, as onsets were not markedly changed (although advanced onsets in

females reached significance). This contrasts with the reduction in alpha that has been reported for adult male C57BL6J mice during chronic ethanol drinking (Brager et al., 2010), although we did see a decrease in nocturnal activity in male and female mice when drinking ethanol, consistent with what is seen in adult male mice and hamsters (Brager et al., 2010, Ruby et al., 2009a). Advanced onsets in females on days with ethanol may reflect ethanol-induced shortening of circadian period (Seggio et al., 2009) and/or slightly shorter sleep duration. Because ethanol availability began for mice an hour after the dark phase began (to avoid experimenter entry into the room affecting onset), onsets occurred prior to ethanol availability during “On” days, and during the last hour of ethanol availability during “Off” days. However, the onset difference in females, though significant, was less than one minute and we saw no impact of ethanol availability on rest-phase activity in adolescent mice, contrasting with increased daytime activity reported in adult male rodents (a possible proxy for fragmented sleep; (Brager et al., 2010, Ruby et al., 2009a).

These observations do not preclude an effect of ethanol availability (or deprivation) on sleep, as Ehlers et al. (2013) have noted high susceptibility to SWS disruption during hangover from acute ethanol in adolescent male rats (Ehlers et al., 2013). It is interesting that we observed even subtle changes in circadian behavior, given that ethanol was administered to mice every other day in free-choice with water, as others have shown only forced daily ethanol affects rhythmicity in adult male C57BL6J mice (Brager et al., 2010, Seggio et al., 2009). Thus, it will be important to repeat this experiment in adult mice for comparison. It will also be important to establish whether or not this paradigm affects sleep, as studies using different ethanol administration paradigms report variable results, suggesting they may differentially affect circadian versus homeostatic sleep. For example, in many rodent species, oral ethanol shortens free-running circadian period (Rosenwasser et al., 2014, Seggio et al., 2009, Seggio et al., 2007) and disrupts circadian activity levels (Brager et al., 2010, Ruby et al., 2009a), while chronic intermittent ethanol (CIE) via vapor chamber does not (Logan et al., 2010). Ethanol vapor alone or mixed vapor-drinking designs markedly affect homeostatic SWS sleep but not always REM (Ehlers and Slawecki, 2000, Huitron-Resendiz et al., 2018, Veatch, 2006), while ethanol liquid diets (Kubota et al., 2002, Mukherjee et al., 2008, Mukherjee and Simasko, 2009) disrupt REM sleep in the same manner as alcohol drinking does in humans (Brower, 2001, Colrain et al., 2014, Thakkar et al., 2015).

During adulthood, the sex differences in circadian entrainment and activity levels discussed above remained, while the majority of changes due to AIE exposure were no longer evident. However, the two AIE-related effects on circadian rhythmicity are compelling in their specificity and sex dependency. First, we observed a ~15 min delay in activity offset in adult female mice with prior AIE exposure compared to control females (Fig. 5D). This may reflect an increase in sleep latency resulting from chronic alcohol use or a circadian influence on delaying sleep timing, which is interesting in light of the strong evidence for eveningness in humans as a predictor of alcohol and other drug use (Kanerva et al., 2012, Kervran et al., 2015, Lemoine et al., 2013, Robinson et al., 2013, Tavernier and Willoughby, 2014, Kloss et al., 2011, Urban et al., 2011, Watson et al., 2013, Whittier et al., 2014). Relatedly, females in our study consumed more alcohol, as has been documented by many others (Melon et al., 2013, Moore et al., 2010, Spear, 2018, Strong et al., 2010), and they

consistently initiated the rest phase later than males. This late activity offset appears to preexist ethanol exposure, as it was observed in adolescent female control mice (Fig. 3D), but also seemed to be further exacerbated by ethanol availability in adolescent mice regardless of sex (Fig. 4C). The circadian and homeostatic sleep components of these observations remain to be disentangled experimentally.

Our data for male mice confirm the hypothesis that adolescent-like insensitivity to moderate ethanol-induced inhibition of photic phase-resetting is retained in adult mice previously exposed to AIE. As ethanol blocks glutamate-mediated photic phase-resetting responses in the SCN (Prosser et al., 2008), a possible mechanism for our current observations may involve lasting disruptions of AIE on glutamatergic neurotransmission. There is much evidence that excitatory glutamatergic signaling is increased in adolescents compared to adults and that adolescent and adult glutamatergic systems are affected differentially by both acute and chronic ethanol (Carpenter-Hyland and Chandler, 2007, Spear, 2018). For example, Carrara-Nascimento et al. (2011) report opposing regulation of extracellular glutamate levels in the nucleus accumbens (NAc) of Swiss mice after repeated ethanol (1.8 g/kg) exposure, with increases noted in adolescents and reductions in adults (Carrara-Nascimento et al., 2011). A timecourse study in adolescent rats showed biphasic effects of ethanol (1.0 g/kg) on spontaneous glutamate release in the prefrontal cortex, with an initial drop followed by a rebound increase at 3 hours post-injection (Mishra et al., 2015). The ethanol-sensitive NR2B subunit of the NMDA receptor, which mediates photic signaling cascades in the SCN (Wang et al., 2008), appears to be differentially affected by chronic ethanol exposure and withdrawal in adolescent versus adult rodents, with effects dependent upon brain region of interest (Pian et al., 2010). In the hippocampus, two binge-like ethanol exposures (3.0 g/kg) administered with a 9 h interval in adolescent rats were shown to upregulate NR2B-mediated signaling after 48-hour abstinence (Silvestre de Ferron et al., 2015), and numerous NR2B-related signaling proteins were altered by AIE in the adult (Swartzwelder et al., 2016). Thus, it is reasonable to speculate that glutamate release is higher and/or that NR2B levels in the SCN may be persistently upregulated by AIE in males, allowing normal photic phase-delays to occur in the presence of ethanol challenge. The dearth of information on developmental changes in glutamatergic and other neurotransmitter systems (e.g. serotonin, neuropeptide Y; (Glass et al., 2010) in the adolescent SCN represents an important and exciting direction for future study.

In contrast to males, female mice showed normal photic phase-resetting responses after ethanol challenge regardless of whether they were exposed to AIE or not, suggesting they are simply not sensitive to the challenge dose of ethanol we used (1.5 g/kg). These data also suggest that the main underlying mechanism by which acute ethanol attenuates photic phase-resetting in ethanol-naïve males (inhibition of glutamate signaling), may be regulated differentially between the sexes (though no studies to date have examined such a possibility). Several other studies suggest that females and males have different patterns of sensitivity to ethanol and that AIE affects the sexes differently. Sexually dimorphic behaviors include ethanol conditioned taste aversion (Morales et al., 2014, Schramm-Sapota et al., 2014), social preference (Varlinskaya and Spear, 2015, Varlinskaya et al., 2015a, Varlinskaya et al., 2015b), anxiolysis (Tanchuck-Nipper et al., 2015), exercise-induced modulation of ethanol drinking in adolescent mice (Gallego et al., 2015), acquisition and

reinstatement of conditioned place preference in adolescent mice (Roger-Sanchez et al., 2012), and in humans, ethanol elimination rates (Dettling et al., 2008, Dettling et al., 2009) and the degree to which sensitivity to intoxication can predict later alcohol use (Schuckit et al., 2012). Although sex differences in phase-resetting were not analyzed *per se*, it is clear when comparing saline-treated groups that the magnitude of phase-delay was similar between males and females (~1.5 h). This is consistent with another recent study showing no difference in photic phase-shifting between wildtype male and female mice at ZT14 (Royston et al., 2016). However, these and other authors (Blattner and Mahoney, 2013) also report evidence that estrogen modulates phase-resetting and other circadian measures (discussed above). Estrogen appears to enhance phase-responsiveness to light at ZT16, as estrogen receptor 1 KO (ERKO) and nonclassical estrogen receptor knock-in (NERKI) mice that lack ERE-mediated transcription showed smaller phase-delays (Blattner and Mahoney, 2013). However, variability was similar in males and females in our study, suggesting estrous stage may not have had much impact on phase-shifts. We are currently examining acute ethanol sensitivity to chronodisruption in adolescent *versus* adult female mice to determine whether there is a rightward shift of ethanol dose-response and/or age-related differences in females compared to males (Ruby et al., 2017), and plan to monitor estrous cyclicity.

Although it is somewhat premature to speculate in depth about how our data relates to the breadth of literature on the long-term effects of AIE, we hypothesize that the few AIE effects we observed on circadian timing may contribute to motivation to consume alcohol as a function of age and sex. Circadian rhythm and/or sleep disruption, either pre-existing or arising from chronic alcohol intake, has been typically conceptualized to drive drinking and relapse through negative reinforcement (e.g. self-medication of insomnia with a sedative dose of alcohol). AIE-exposed female mice in our study did show a delay in activity offset, perhaps reflecting increased sleep latency, which could ostensibly fit with that hypothesis. It has also been suggested that women, particularly young women, may drink largely for negative reinforcement (e.g. to overcome stress, anxiety, etc.; (Dir et al., 2017). However, it is difficult to reconcile chronodisruption-driven alcohol drinking with the evidence suggesting the opposite may be true for adolescents and AIE-exposed adults. Adolescent mice and adult mice with prior AIE exposure drink more ethanol (Holstein et al., 2011, Melendez, 2011, Moore et al., 2010, Quoilin and Boehm, 2016), yet AIE-exposed males in our study actually showed *less* circadian rhythm disruption in adulthood when challenged with acute ethanol compared to their ethanol-naïve counterparts, retaining an adolescent-like phenotype (Ruby et al., 2017). Likewise, female mice, who generally outdrink males, were unaffected by the ethanol challenge dose regardless of whether or not they had prior AIE exposure. Thus, it is possible that disruption of the ability to adjust internal biological time to environmental time may be interpreted as an aversive alcohol effect, and in that sense, a protective factor against excessive, repeated drinking. Therefore, the absence of “consequences” of acute ethanol in AIE-exposed males (and in all females) may in part underlie propensity to overconsume alcohol. In line with this idea, adolescent rodents and humans are markedly less sensitive than adults to other aversive effects of ethanol, including ataxia and sedation, and they retain these phenotypes into adulthood after AIE (Spear, 2018; White et al., 2002). Sensitivity to ethanol-induced ataxia and sedation vary diurnally in adult

mice, both with a nadir at the beginning of rest phase (Perreau-Lenz et al., 2009, Ruby et al., 2016); rhythmicity in ethanol-induced sedation is absent in mice lacking the circadian clock gene *mPer2* (Perreau-Lenz et al., 2009). In humans, the stimulatory (rewarding) effects of alcohol appear to be governed by the circadian clock, peaking in the early evening when drinking is also most likely to occur (Van Reen et al., 2013). Future studies are necessary to determine the extent to which the circadian system regulates factors underlying motivation to consume alcohol and whether or not these mechanisms vary as a function of age and/or sex.

In conclusion, we show for the first time lasting effects of adolescent alcohol exposure on circadian activity rhythms in adult mice that are sex-specific in nature. We also show novel evidence that the subtle alcohol-related changes in circadian activity during AIE differ markedly from the impact of chronic ethanol on adult male rodents. Our study represents an important first step in understanding how alcohol interacts with biological sex to alter circadian rhythmicity, and how this in turn may influence myriad physiological and molecular processes. Interfering with the lasting effects of adolescent drinking holds great promise in the treatment and prevention of alcohol use disorders.

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Fig. 1.

Experimental procedure. **(A)** Timeline showing the experimental procedure relative to the ages of mice (AIE = adolescent intermittent ethanol, LD = light-dark photocycle, P = postnatal day, Inj = injection, DD = constant darkness). **(B)** Design for the phase-resetting experiments showing the duration of time in each photocycle used in the analyses, the timing of injection (INJ), and timing of the light-pulse (star; control mice received injections at the same time, but no light pulse). Also shown are the periods of time represented by the data in subsequent figures.

Fig. 2. Sex differences in ethanol consumption during AIE. **(A)** On average, females ($n = 18$) consumed more ethanol than males ($n = 19$) during AIE, but preference did not differ **(B)**. Data are mean $\pm$ SEM; * $p < 0.05$.

Fig. 3.

Sex differences in photocycle entrainment among adolescent mice during AIE. (A) Circadian rhythm amplitude was greater in female mice ($n = 32$) compared to males ($n = 36$) regardless of exposure. (B) Active-phase duration (alpha) was longer in female mice compared to males. (C) Activity onsets were earlier in females than in males, while (D) activity offsets tended ($p = 0.0753$) to be later in females than in males. Compared to their male counterparts, female mice had (E) fewer nightly bouts of activity, (F) more time spent active at night, and (G) more counts of nighttime activity. Compared to males, female mice had (H) more bouts of daytime activity, (I) more time spent active during the day, and (J) more counts of daily activity. No differences between AIE-exposed and control mice were noted when data were averaged over the entire 24-day exposure period. Data are mean $\pm$ SEM; * $p < 0.05$.

Fig. 4.

Changes in photocycle entrainment during ethanol availability in AIE-exposed mice. Pairwise analyses of circadian entrainment measures in AIE-exposed mice on days when ethanol was available (On EtOH) versus those when ethanol was not available (Off EtOH) revealed that (A) both males ($n = 18$) and females ($n = 17$) were active for longer (alpha) when ethanol was available, that (B) females became active earlier when ethanol was available, and that (C) activity offsets were delayed during ethanol availability in both sexes. Female mice had (D) more bouts during ethanol availability, while (E) both males and

females spent less time active at night when ethanol was available, and (**F**) adolescent mice were less active at night when drinking ethanol. Compared to males, female mice had (**G**) more bouts of daytime activity, (**H**) more time spent active during the day, and (**I**) more counts of daily activity, whether ethanol was available or not. Data are mean $\pm$ SEM; * $p < 0.05$.

Fig. 5.

Sex differences in photocycle entrainment during Washout in adult mice. (A) Circadian rhythm amplitude remained greater in adult female mice ($n = 32$) compared to males ($n = 36$) regardless of whether or not they had prior AIE exposure. (B) Active-phase duration (alpha) remained longer in adult female mice compared to males, and (C) activity onsets continued to occur earlier in females than in males during adulthood, with neither measure affected by prior AIE exposure. (D) Activity offsets were later in adult females than in males and prior exposure to AIE delayed offsets even further in females only. Compared to their adult male counterparts, female mice continued to have (E) fewer nightly bouts of activity, (F) more time spent active at night, and (G) more counts of nighttime activity. Compared to males, female mice still showed (H) more bouts of daytime activity, (I) more time spent active during the day, and (J) more counts of daily activity during adulthood regardless of AIE exposure. No differences in nightly or daily activity were seen due to previous AIE exposure. Data are mean $\pm$ SEM; * $p < 0.05$.

Fig. 6. Lasting insensitivity to ethanol-induced attenuation of photic phase-resetting in male, but not female, adult mice with prior AIE exposure. **(A)** Only ethanol-naïve (control) male mice ($n = 5$) showed attenuated photic phase-shifts when given an ethanol challenge dose (1.5 g/kg, i.p.), while AIE-exposed males ($n = 6$) had normal phase-shifts after ethanol challenge compared to saline-treated groups ($n = 7$ -8/exposure). **(B)** Female mice showed robust phase-shifts to light regardless of prior AIE exposure or ethanol challenge. **(C)** Neither AIE

nor ethanol challenge had phase-resetting effects on their own (or in combination) in the absence of a light pulse. Data are mean $\pm$ SEM; * $p < 0.05$.

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Fig. 7.

Representative, double-plotted actograms of male mice in each exposure X treatment group. Actograms of two mice from each group are shown, with Water X Saline at the top left, AIE X Saline at the top right, Water X EtOH Challenge at the bottom left, and AIE X EtOH Challenge at the bottom right. Lightly shaded portions at the top of each actogram shows the 24-day AIE (or control) exposure period. Unshaded middle regions of the actograms show the 24-day washout period; mice received a challenge injection and light pulse 2.5 hours into

the dark phase on the final day of this period. Darkly shaded portions at the bottom of each actogram show the time of constant darkness (DD) after the light pulse.

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Fig. 8. Representative, double-plotted actograms of female mice in each exposure X treatment group. Actograms of two mice from each group are shown, with Water X Saline at the top left, AIE X Saline at the top right, Water X EtOH Challenge at the bottom left, and AIE X EtOH Challenge at the bottom right. Lightly shaded portions at the top of each actogram shows the 24-day AIE (or control) exposure period. Unshaded middle regions of the actograms show the 24-day washout period; mice received a challenge injection and light

pulse 2.5 hours into the dark phase on the final day of this period. Darkly shaded portions at the bottom of each actogram show the time of constant darkness (DD) after the light pulse.

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Table 1.

Circadian activity during adolescence. Data are expressed as mean $\pm$ SEM. Statistics are summarized for significant effects and trends.

	AIE		Control		Sex	Exposure	S $\times$ E
	Males (n = 17)	Females (n = 16)	Males (n = 19)	Females (n = 16)			
Amplitude	2441.15 $\pm$ 164.87	3408.01 $\pm$ 233.74	2759.37 $\pm$ 204.26	3841.47 $\pm$ 353.20	$F_{(1,64)} = 17.56$	ns	ns
	$p < 0.001$						
Alpha (h)	11.45 $\pm$ 0.11	11.78 $\pm$ 0.14	11.56 $\pm$ 0.08	11.83 $\pm$ 0.13	$F_{(1,64)} = 6.898$	ns	ns
	$p = 0.0105$						
Onset (h)	-0.47 $\pm$ 0.06	-0.33 $\pm$ 0.05	-0.39 $\pm$ 0.04	-0.31 $\pm$ 0.05	$F_{(1,64)} = 4.820$	ns	ns
	$p = 0.0318$						
Offset (h)	0.08 $\pm$ 0.10	-0.11 $\pm$ 0.12	0.06 $\pm$ 0.07	-0.14 $\pm$ 0.14	$F_{(1,64)} = 3.270$	ns	ns
	$p = 0.0753$						
Night Bout (#)	6.80 $\pm$ 0.22	5.69 $\pm$ 0.25	6.63 $\pm$ 0.19	5.50 $\pm$ 0.18	$F_{(1,62)} = 27.63$	ns	ns
	$p < 0.0001$						
Night Activity (min)	274.40 $\pm$ 16.91	372.38 $\pm$ 12.92	269.68 $\pm$ 17.54	382.25 $\pm$ 16.26	$F_{(1,62)} = 41.85$	ns	ns
	$p < 0.0001$						
Night Activity (counts)	945.73 $\pm$ 97.85	1347.94 $\pm$ 85.72	882.68 $\pm$ 83.82	1510.63 $\pm$ 175.07	$F_{(1,62)} = 19.90$	ns	ns
	$p < 0.0001$						
Day Bout (#)	2.40 $\pm$ 0.13	3.25 $\pm$ 0.17	1.90 $\pm$ 0.24	3.50 $\pm$ 0.32	$F_{(1,62)} = 19.90$	ns	ns
	$p < 0.0001$						
Day Activity (min)	13.40 $\pm$ 2.01	27.63 $\pm$ 3.19	10.21 $\pm$ 1.93	33.94 $\pm$ 5.23	$F_{(1,62)} = 32.89$	ns	ns
	$p < 0.0001$						
Day Activity (counts)	50.33 $\pm$ 6.44	86.56 $\pm$ 9.67	37.63 $\pm$ 6.75	114.63 $\pm$ 21.4	$F_{(1,62)} = 20.76$	ns	$F_{(1,62)} = 2.691$
	$p < 0.0001$						$p = 0.1060$

Table 2.

Circadian activity on vs. off ethanol. Data are expressed as mean $\pm$ SEM. Statistics are summarized for significant effects and trends.

	Males (n = 17)		Females (n = 16)		Sex	Availability	S $\times$ A
	On EtOH	Off EtOH	On EtOH	Off EtOH			
Alpha (h)	11.57 $\pm$ 0.12	11.33 $\pm$ 0.11	11.91 $\pm$ 0.15	11.64 $\pm$ 0.14	$F_{(1,31)} = 3.391$	$F_{(1,31)} = 32.82$	ns
					$p = 0.0751$	$p < 0.0001$	
Onset (h)	-0.47 $\pm$ 0.06	-0.46 $\pm$ 0.06	-0.30 $\pm$ 0.05	-0.35 $\pm$ 0.06	$F_{(1,31)} = 3.130$		$F_{(1,31)} = 5.133$
					$p = 0.0867$		$p = 0.0306$
Offset (h)	-0.04 $\pm$ 0.1	0.21 $\pm$ 0.10	-0.21 $\pm$ 0.13	0.01 $\pm$ 0.12	ns	$F_{(1,31)} = 27.88$	ns
						$p < 0.0001$	
Night Bout (#)	6.47 $\pm$ 0.31	6.94 $\pm$ 0.22	6.13 $\pm$ 0.24	5.31 $\pm$ 0.36	$F_{(1,31)} = 7.214$	ns	$F_{(1,31)} = 13.71$
					$p = 0.0115$		$p = 0.0008$
Night Activity (min)	228.24 $\pm$ 20.74	274.41 $\pm$ 24.68	334.38 $\pm$ 12.20	409.00 $\pm$ 15.21	$F_{(1,31)} = 20.92$	$F_{(1,31)} = 92.65$	$F_{(1,31)} = 5.138$
					$p < 0.0001$	$p < 0.0001$	$p = 0.0305$
Night Activity (counts)	786.71 $\pm$ 95.29	926.59 $\pm$ 124.18	1223.69 $\pm$ 73.80	1472.00 $\pm$ 103.05	$F_{(1,31)} = 12.47$	$F_{(1,31)} = 30.38$	ns
					$p = 0.0013$	$p < 0.0001$	
Day Bout (#)	2.29 $\pm$ 0.14	2.29 $\pm$ 0.17	3.19 $\pm$ 0.23	3.31 $\pm$ 0.18	$F_{(1,31)} = 17.26$	ns	ns
					$p = 0.0002$		
Day Activity (min)	12.47 $\pm$ 1.98	12.59 $\pm$ 1.97	27.75 $\pm$ 3.31	27.44 $\pm$ 3.25	$F_{(1,31)} = 17.30$	ns	ns
					$p = 0.0002$		
Day Activity (counts)	47.24 $\pm$ 6.35	46.65 $\pm$ 6.99	85.94 $\pm$ 10.42	86.75 $\pm$ 9.31	$F_{(1,31)} = 12.14$	ns	ns
					$p = 0.0015$		

Table 3.

Circadian activity during adulthood. Data are expressed as mean $\pm$ SEM. Statistics are summarized for significant effects.

	AIE		Control		Sex	Exposure	S $\times$ E
	Males (n = 17)	Females (n = 16)	Males (n = 17)	Females (n = 16)			
Amplitude	2986.83 $\pm$ 203.64	3524.05 $\pm$ 175.99	2979.01 $\pm$ 196.06	3634.22 $\pm$ 218.65	$F_{(1,64)} = 8.882$	ns	ns
					$p = 0.0041$		
Alpha (h)	11.93 $\pm$ 0.09	12.62 $\pm$ 0.09	12.07 $\pm$ 0.06	12.41 $\pm$ 0.07	$F_{(1,64)} = 44.65$	ns	$F_{(1,64)} = 5.087$
					$p < 0.0001$		$p = 0.0275$
Onset (h)	-0.38 $\pm$ 0.06	-0.17 $\pm$ 0.03	-0.31 $\pm$ 0.05	-0.13 $\pm$ 0.04	$F_{(1,64)} = 17.65$	ns	ns
					$p < 0.0001$		
Offset (h)	-0.31 $\pm$ 0.07	-0.79 $\pm$ 0.09	-0.38 $\pm$ 0.04	-0.54 $\pm$ 0.06	$F_{(1,64)} = 24.63$	ns	$F_{(1,64)} = 5.949$
					$p < 0.0001$		$p = 0.0175$
Night Bout (#)	6.77 $\pm$ 0.27	4.19 $\pm$ 0.25	6.11 $\pm$ 0.25	4.44 $\pm$ 0.35	$F_{(1,64)} = 57.23$	ns	ns
					$p < 0.0001$		
Night Activity (min)	334.59 $\pm$ 22.78	483.56 $\pm$ 20.44	348.63 $\pm$ 22.70	455.00 $\pm$ 20.55	$F_{(1,64)} = 34.00$	ns	ns
					$p < 0.0001$		
Night Activity (counts)	1414.71 $\pm$ 203.66	2327.75 $\pm$ 277.38	1537.53 $\pm$ 153.89	2202.50 $\pm$ 260.35	$F_{(1,64)} = 12.45$	ns	ns
					$p = 0.0008$		
Day Bout (#)	2.18 $\pm$ 0.30	3.50 $\pm$ 0.29	2.11 $\pm$ 0.25	3.63 $\pm$ 0.32	$F_{(1,64)} = 24.31$	ns	ns
					$p < 0.0001$		
Day Activity (min)	15.35 $\pm$ 3.73	41.13 $\pm$ 7.55	14.95 $\pm$ 2.77	42.81 $\pm$ 8.48	$F_{(1,64)} = 20.85$	ns	ns
					$p < 0.0001$		
Day Activity (counts)	60.24 $\pm$ 16.14	138.94 $\pm$ 29.11	54.90 $\pm$ 10.43	152.81 $\pm$ 34.69	$F_{(1,64)} = 14.07$	ns	ns
					$p = 0.0004$		

Table 4.

Photic phase-resetting in adulthood. Data are expressed as mean $\pm$ SEM. Statistics are summarized for all significant effects.

	AIE		Control		Exposure	Challenge	E $\times$ C
	EtOH Challenge	Saline	EtOH Challenge	Saline			
Males (n = 5-8)	-1.43 $\pm$ 0.08	-1.50 $\pm$ 0.04	-0.85 $\pm$ 0.16	-1.39 $\pm$ 0.11	$F_{(1,22)} = 11.45$	$F_{(1,22)} = 8.782$	$F_{(1,22)} = 5.388$
					p = 0.0027	p = 0.0072	p = 0.0299
Females (n = 4-7)	-1.37 $\pm$ 0.06	-1.41 $\pm$ 0.21	-1.32 $\pm$ 0.21	-1.35 $\pm$ 0.12	ns	ns	ns
No Light Pulse (n = 5-6)	-0.05 $\pm$ 0.05	-0.04 $\pm$ 0.05	0.03 $\pm$ 0.03	0.02 $\pm$ 0.05	ns	ns	ns