Behavioural Brain Research 302 (2016) 213–219

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Mutations in the circadian gene *period* alter behavioral and biochemical responses to ethanol in *Drosophila*

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HIGHLIGHTS

- *Drosophila* show faster ethanol-induced sedation and slower recovery with age.
- *perL* flies have longer recovery time from ethanol sedation compared to wild-type.
- *perL* flies have higher residual ethanol post-sedation compared to wild-type.
- Alcohol dehydrogenase activity appears to fluctuate in a circadian pattern and influence ethanol response.

ARTICLE INFO

Article history:
Received 24 September 2015
Received in revised form 12 January 2016
Accepted 17 January 2016
Available online 20 January 2016

Keywords:
Alcoholism
Circadian rhythm
Drosophila
Ethanol
period

ABSTRACT

Clock genes, such as *period*, which maintain an organism's circadian rhythm, can have profound effects on metabolic activity, including ethanol metabolism. In turn, ethanol exposure has been shown in *Drosophila* and mammals to cause disruptions of the circadian rhythm. Previous studies from our labs have shown that larval ethanol exposure disrupted the free-running period and *period* expression of *Drosophila*. In addition, a recent study has shown that arrhythmic flies show no tolerance to ethanol exposure. As such, *Drosophila period* mutants, which have either a shorter than wild-type free-running period (*perS*) or a longer one (*perL*), may also exhibit altered responses to ethanol due to their intrinsic circadian differences. In this study, we tested the initial sensitivity and tolerance of ethanol exposure on *Canton-S*, *perS*, and *perL*, and then measured their Alcohol Dehydrogenase (ADH) and body ethanol levels. We showed that *perL* flies had slower sedation rate, longer recovery from ethanol sedation, and generated higher tolerance for sedation upon repeated ethanol exposure compared to *Canton-S* wild-type flies. Furthermore, *perL* flies had lower ADH activity and had a slower ethanol clearance compared to wild-type flies. The findings of this study suggest that *period* mutations influence ethanol induced behavior and ethanol metabolism in *Drosophila* and that flies with longer circadian periods are more sensitive to ethanol exposure.

1. Introduction

Alcohol is one of the most commonly abused drugs in the world. Consequently, alcohol use disorders (AUD) are extremely common. Therefore, it is important to understand the biological effects of ethanol and mechanisms behind ethanol metabolism. Ethanol intoxication exhibits a biphasic effect, inducing initial hyperactivity at lower doses followed by depressed activity resulting in sedation at higher doses. Alcoholism and alcohol consumption in humans are known to affect a multitude of molecules and pathways including biological rhythms e.g. melatonin [1], temperature rhythms [2], and cycling of circadian genes [3]. Interestingly, human genetics studies have linked mutations to the *per* genes and increased alcohol consumption [4].

Furthermore, numerous mammal studies have shown that alcohol exposure can affect the period [5–7] and phase [7–10] of the free-running locomotor activity rhythm. Additionally, alcohol can affect transcript and protein levels of core clock genes (including *period* (*per*)) [11,12]. Alternatively, previous studies have suggested that ethanol intake and drug addictions are strongly associated with mutations in clock genes, including *per*. Mice with mutated *per* genes, including *per1* and *per2*, exhibit increased alcohol consumption and alcohol seeking behavior [13,14]. In turn, animals

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http://dx.doi.org/10.1016/j.bbr.2016.01.041
0166-4328/

selectively bred for high or low alcohol consumption have also shown aberrations in their circadian clock function [15–17]. Taken together, these studies demonstrate clear connections between circadian mutations and alcohol exposure by showing profound effects of alcohol on the molecular rhythms and behavioral and physiological outputs of the circadian clock.

The focus of this study was to explore sedation and tolerance to high-dose acute ethanol exposure in adult *Drosophila per* mutants, in order to investigate how ethanol affects animals with rhythmic, but atypical circadian periods. *Drosophila* can be a powerful tool to investigate the physiological and behavioral effects of alcohol intoxication for the following reasons. First, *Drosophila* is amenable to efficient genetic and behavioral analysis. Second, the ethanol-induced biphasic behavior in humans is mirrored in *Drosophila*; during initial stages of exposure, hyperactivity and increased locomotion was observed, but after greater amounts of ethanol had accumulated, the flies were sedated [18]. Third, there exists a high degree of homology between the *Drosophila* and mammalian clock rhythm [19]. Therefore, fruit flies have been extensively used to investigate molecular and behavioral aspects of the circadian rhythm.

Work on the effects of ethanol on *Drosophila* has demonstrated that, similar to mammals, ethanol can disrupt the biological clock and has the capability to modulate the expression of circadian genes [20,21]. In addition, a recent study showed that *Drosophila* arrhythmic mutants (including *per* null (*per*$^{\circ}$)) do not exhibit ethanol tolerance, indicating that a circadian clock is necessary for alcohol tolerance [22]. Although such studies have found strong correlations between circadian rhythm defects and ethanol exposure, few have directly investigated the effect of acute ethanol exposure on *Drosophila* with altered free-running circadian periods—*period Short (perS)* and *period Long (perL)* mutants. The *perS* and *perL* flies have a stable entrainment to a LD cycle, but they have free-running circadian periods of approximately 19.5 h and 28.5 h, respectively [23]. As alcohol consumption may have differing affects on the circadian rhythm depending upon free-running rhythm length [6,20], there may be a connection between period length and alcohol related behaviors. Here we show that the *perS* and *perL* mutations have altered sedation rates, recovery from sedation, and ethanol tolerance and demonstrate that these effects may be the result of interplay between circadian rhythms and ethanol metabolism.

2. Materials and methods

2.1. Drosophila *stocks*

Drosophila fly stocks *Canton-S* (*CS*, stock number 1), and *adh*^{null} (stock number 3976), were obtained from the Bloomington *Drosophila* Stock Center. *perS* and *perL* mutants were generated in *CS* flies [23] (generously provided by Dr. Michael Rosbash, Brandeis University). To rule out effects of genetic background differences, similar behavioral effects were observed in stocks independently maintained at Colby College and Bridgewater State University. The flies were raised on standard Nutri-Fly^{TM} Bloomington Formulation (Genesee Scientific) at 25 °C under 12-h light/12-h dark conditions, unless otherwise stated.

2.2. Ethanol sedation, recovery, and tolerance assays

To determine the effect of *period* mutations on *Drosophila* behavioral responses to ethanol, a sedation assay was carried out according to previous assays outlined by Maples and Rothenfluh, with minor modifications [24]. This study used male flies due to gender-related differences in ethanol-induced behavior and their stronger tolerance to ethanol exposure [18]. *CS*, *perS*, and *perL* male flies of different ages (one-day, one-week, and two-week-old—typical experiments with adult flies are done on 3–7-day old flies) were collected and sorted into standard food vials of 8 flies each approximately 18 h before sedation in order to prevent confounding effects of the CO_2 sedation on ethanol-induced sedation. Flies were transferred to new empty vials at ZT4 on the day of sedation and allowed to acclimate to the new environment for 15 min. Sedation was carried out by dispensing 500 μL ethanol (200-proof) (Sigma–Aldrich) onto a new cotton plug, and then immediately replacing the original plug with the ethanol infused one, forming an ethanol chamber. Vials were deliberately tapped 5 times after each minute to determine the number of sedated flies. Sedation is defined as the inability of the flies to move, right themselves, or uncontrollable beating of the wings after a ten-second observational period. ST50 is defined as the time until 50% of the flies have been sedated. The flies were left in the ethanol chamber for a total of 15 min regardless of sedation time.

Recovery began after the flies have been removed from the ethanol chamber. The flies were returned to their original food vials with the vial laid horizontally to prevent non-sedation-related mortality. Every 30 min, the vials were gently rotated and observed to determine if the flies had recovered, which is indicated by the regaining of their ability to right themselves. RT50 is defined as the time required for 50% of the sedated flies to recover. Flies that have not recovered at the beginning of tolerance were excluded from the total number of flies for RT50 calculations. Tolerance after repeated exposure was tested 24 h after the initial sedation at ZT4. The flies were once again transferred into a new empty food vial and the sedation process was repeated to determine the ST50 for tolerance measurement. For each genotype, tolerance was calculated by determining the percent change in sedation times on consecutive days (%T—to normalize tolerance between genotypes).

Statistical analyses on independent biological replicates (n = 3–5) for sedation, recovery, and tolerance were performed in STATA using ANOVA; post hoc Scheffe pair-wise comparison test.

2.3. Alcohol dehydrogenase enzyme activity assay

Alcohol dehydrogenase (ADH) enzyme activity was measured for both fluctuations throughout the circadian cycle and age-dependent differences according to a previously described protocol [25,26]. Twenty-four-hour ADH cycling differences between *CS, perS and perL* were measured at time points ZT0, ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24, with *adh*^{null} as the control. One to three-day old flies were kept at 25 °C under 12-h light/12-h dark standard conditions until the appropriate time point. To determine age-dependent differences, 1-day, 1-week, and 2-week-old flies of each genotype were collected at ZT4.

Five flies were homogenized from each genotype at each time point and age in 150 μL of 20 mM phosphate buffer. 100 mM potassium phosphate buffer was made with 19.2: 90.8, monobasic potassium phosphate (100 mM): dibasic potassium phosphate (100 mM) (Sigma–Aldrich). The homogenate was centrifuged at 12500 rpm in 4°C for 5 min, and then the supernatant collected and stored at −20 °C until all extracts had been collected. ADH assay solution, 940 μL, was prepared for each sample, composed of 500 μL potassium phosphate buffer (100 mM), 40 μL NAD^+ (50 mM), 100 μL absolute ethanol (200-proof), and 300 μL dH_2O. Directly before absorbance measurements were made, 60 μL of the sample extract was added to the assay solution to initiate the reaction. The absorbance over time of each sample produced by colorimetric changes was measured at room temperature and a wavelength of 340 nm for 10 min (Beckman Coulter DU530 UV/Vis Spectrophotometer). Measurements were made every 2 min for a total of 6 optical density readings. The data points were plotted