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Ana Klasan

Utjecaj cirkadijalne modulacije na oksidativni status *Drosophile melanogaster*

Rijeka, 2021.

Mentor: dr.sc. Rozi Andretić-Waldowski

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In front of the committee:

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This thesis contains: 29 pages, 7 pictures and 26 references.

Abstract

Circadian rhythm is present in almost all living organisms due to the rotation of the Earth and regular light and dark changes. Its role is evident in 24 hour modulation of biological processes such as the sleep/wake cycle. Disrupted circadian rhythm correlates with oxidative stress, which is caused by increased production of Reactive Oxygen Species (ROS). In this thesis, we explored the connection between the circadian rhythm and oxidative state in *D. melanogaster*. Based on similarity in the responsiveness to volatilized cocaine between flies with mutation in *timeless* gene and *wild type* flies, we were interested to see whether these similarities will be paralleled in their redox regulation. An indicator of redox state was the concentration of the oxidative metabolism byproduct H_2O_2 in head homogenates. For detection of H_2O_2 we used the dihydroethidium (DHE) staining. Furthermore, we wanted to determine if H_2O_2 production is regulated by circadian rhythm. Measurements were conducted in flies raised in conditions of 12 hours of light-12 hours of dark (LD) and in conditions of constant dark (DD). We determined that H_2O_2 production was regulated by circadian rhythm for *wt* flies. However, H_2O_2 production in DD conditions was increased overall. In *tim* mutants there was an indication of circadian regulation of H_2O_2 production in LD conditions but the rhythm was abolished in DD conditions. *wt* and *tim* flies differed significantly in the 24 hour modulation of H_2O_2 in DD, but less so in LD. Further studies are needed to elucidate the role of *tim* in the regulation of H_2O_2 amount and the connection with cocaine responsiveness. Taken together, our data suggest that redox status is regulated in a circadian fashion and that a functional *tim* gene is essential for maintaining circadian rhythmicity.

Keywords: *Drosophila melanogaster*, oxidative stress, circadian rhythm, reactive oxygen species, hydrogen peroxide

Sažetak

Cirkadijalni ritam je prisutan u skoro svim organizmima. Nastao je kao posljedica rotacije Zemlje i izmjene dana i noći. Važan je za regulaciju brojnih bioloških procesa kao što je i ciklus spavanja. Narušeni cirkadijalni ritam je često povezivan s pojavom oksidativnog stresa koji nastaje kao posljedica povišene produkcije reaktivnih kisikovih vrsta (ROS). U ovome radu, istražili smo povezanost cirkadijalnog ritma i oksidativnog statusa u *D.melanogaster*. S obzirom da su *wild type* i *timeless* mušice pokazale sličnost u odgovoru na izlaganje volatiliziranom kokainu, zanimalo nas je hoće li sličnost biti prisutna i kod regulacije redoksa. Indikator redoks statusa je bila koncentracija H_2O_2 u homogenatima glava mušica. H_2O_2 je nusprodukt oksidativnog metabolizma, a za njegovu detekciju korištena je DHE metoda bojanja. Nadalje, ispitali smo je li proizvodnja H_2O_2 regulirana cirkadijalnim ritmom. Mjerenja su provedena na mušicama koje su držane u LD ili DD uvjetima. Pokazali smo da je kod *wt* mušica H_2O_2 proizvodnja regulirana cirkadijalnim ritmom. Međutim, proizvodnja H_2O_2 je bila sveukupno povišena u DD uvjetima. Kod *tim* mutanata, indikacije cirkadijalne modulacije u proizvodnji H_2O_2 prisutne u LD uvjetima nisu uočene i u DD uvjetima. *wt* i *tim* mušice se značajno razlikuju u modulaciji H_2O_2 koncentracija u DD uvjetima dok je u LD uvjetima razlika značajno manja. Potrebno je još studija koje će odrediti točnu ulogu *tim* gena u regulaciji H_2O_2 i koje će definirati njegovu ulogu u procesu odgovora na izlaganje kokainu. Sveukupno, dobiveni podatci sugeriraju da je metabolizam ROS-a povezan s cirkadijalnim ritmom te da je funkcionalni gen *tim* esencijalan za održavanje cirkadijalne ritmičnosti.

Ključne riječi: *Drosophila melanogaster, oksidativni stres, cirkadijalni ritam, reaktivne kisikove vrste, vodikov peroksid*

Table of contents

1. Introduction

Almost all life on Earth exhibits a 24 hours variation in physiological and behavioral performance [1]. That is due to the rotation of the Earth around its axis and around the Sun. To adapt to environmental daily changes such as changes in light, dark and food availability, organisms have developed a circadian rhythm [2]. This rhythm controls various biological processes such as the sleep/wake cycle and levels of various enzymes and hormones. The name ”circadian“ originates from the Latin ”*circa diem*“ or in a translation ”about a day“ [3].

Circadian rhythms are driven by an endogenous timekeeping system called the circadian clock [1]. The circadian clock is self-sustaining, which means that its rhythmicity will be maintained even in the absence of environmental cues. In contrast, changes such as a shift in the temperature or in the light and dark can entrain or synchronize the circadian clock [4]. The circadian clock consists of a network of neurons that express circadian genes with a circadian period in transcription and translation [5]. The molecular mechanism that controls the 24 hour expression of circadian genes is a transcriptional-translational feedback loop [6].

Mutations in circadian genes lead to disrupted rhythmic expression of other genes that control behavior and physiology, and in some cases protein accumulation and degradation. Consequently, this affects the functioning of the circadian clock, as well as the physiology and behavior of an organism [1]. It is suggested that circadian organization of rest/activity cycles is connected with the metabolism through metabolic intermediates such as Reactive Oxidative Species (ROS). Disruptions in the metabolism that lead to increases in ROS can have harmful effects on cellular structure [5]. Based on these findings we were interested in exploring the connection between circadian rhythm and oxidative state.

1.1. Circadian genes in *D. melanogaster*

D. melanogaster has approximately 150 000 neurons of which 150 are clock neurons. The clock neurons express circadian genes (*period (per), timeless (tim), Clock (Clk), and cycle (cyc)*), which are important in circadian timekeeping [7]. The molecular mechanism of circadian rhythm is based on a negative transcriptional-translational feedback loop. The *Clk* and *cyc* genes encode proteins that activate transcription of the *per* and *tim* genes. Then, the PER and TIM proteins repress CLK/CYC activators, thereby repressing transcription of *per* and *tim* [5] (Fig. 1).

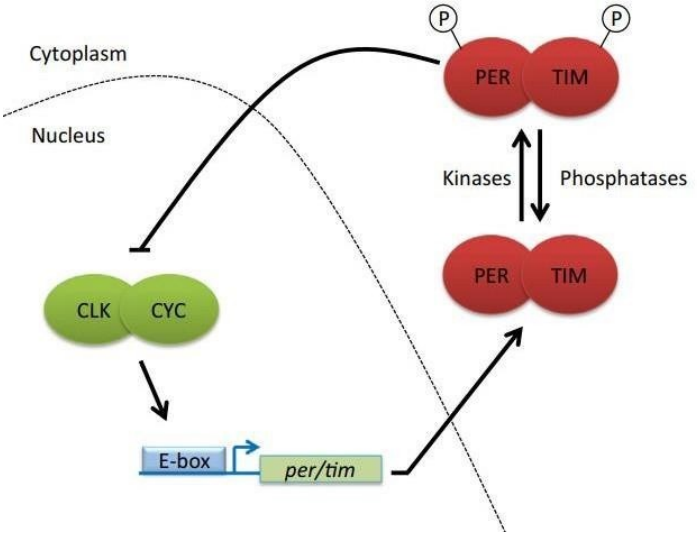

Figure 1. The transcriptional feedback loop of the circadian clock in *D. melanogaster.* CLK and CYC initiate transcription of the *per* and *tim* genes. PER and TIM then form a heterodimer and localize in the nucleus. They release CLK and CYC from repression. PER and TIM are phosphorylated and degraded, and a new cycle begins. Adapted from [4].

Flies with mutations in the circadian genes *per, tim, Clk* and *cyc*, were previously examined for their responsiveness to volatilized cocaine [8,9]. Flies were exposed to 75μg of free-base volatilized cocaine in 6 hour intervals. Repeated exposures to volatilized cocaine leads to locomotor sensitization in the *wt* flies. In humans, sensitization is a process associated

with enhanced craving and psychoses that can occur after repeated drug administration. *Clk, per* and *cyc* mutant flies did not develop locomotor sensitization to cocaine, but surprisingly the *tim* mutant behaved like *wt* flies and developed locomotor sensitization.

Because all four circadian genes are part of a negative feedback loop where they dimerize and interact with each other, it was a surprising finding that *tim* mutants behaved like *wt* flies. There are two possible explanations for this. Firstly, the process that controls drug responsiveness is not connected with the molecular mechanism of the negative feedback loop. Secondly, drug responsiveness is regulated by genes expressed in cells that do not belong to the circadian system [8,9].

1.2. Circadian modulation in *D. melanogaster*

Environmental cues, also called Zeitgebers, can entrain or synchronize the circadian clock. Entrainment is a process in which the phase of the circadian clock is affected by Zeitgebers. The intensity of a Zeitgeber's impact on the circadian clock depends on the strength of the stimulus and on the circadian phase in which the stimulus is applied. The most dominant and pervasive Zeitgeber for circadian clock is light [1]. *D. melanogaster* has a blue light receptor termed cryptochrome (CRY), whose role is to convey light signals to the circadian clock. It has been shown that CRY is responsible for the light-dependent modulation of the circadian rhythm [10].

A study has show how circadian rhythm, modulated by light, can affect locomotor activity in *D. melanogaster* [11]. The activity of the flies was measured with the Drosophila Activity Monitoring (DAM) System in 12 hours light-12 hours dark (LD) and 12 hours dark-12 hours dark (DD) conditions. The system registered activity as the number of times that each fly crossed an infrared beam inside a glass tube. Measurements were performed on

wild type flies and per^{01} and pdf^{01} mutants. *pdf* is a gene involved in conveying rhythmic information from the circadian clock to the rest of the brain, which controls behavioral rhythms. *wt* flies were entrained in LD conditions over six days and their activity showed two daily peaks in the morning and the evening. When *wt* flies were transferred in the DD conditions, they kept their rhythmicity. In LD conditions, per^{01} mutants had activity evenly distributed during the day, while pdf^{01} mutants had morning and evening activity peaks. Interestingly, when mutants were transferred to DD conditions, these peaks disappeared and flies became arrhythmic. It was concluded that in LD conditions light acts as a temporal reference and that it is a reason why all genotypes were entrained in LD. In DD conditions, light as a temporal reference is lost and a functional endogenous circadian clock is needed for over rhythmic behavior. per^{01} and pdf^{01} mutants do not have functional circadian clock and that was the reason why their behavior in DD conditions was arrhythmic [11].

1.3. Reactive oxygen species and oxidative stress

Reactive Oxygen Species (ROS) are metabolic by-products generated by endogenous or exogenous sources. They include superoxide radicals (O_2^-), hydroxyl radicals (OH^-) and peroxides (ROOR)[8]. Plenty of processes in the organism, such as immunity, apoptosis, phosphorylation and cell differentiation, depend on regulated control over production of ROS. Increased ROS production is termed oxidative stress and it can lead to harmful effects on cellular structures. More specifically, excess of hydroxyl radicals can cause lipid peroxidation, proteins can lose their enzymatic activity due to oxidative stress and DNA can have oxidative stress-related lesions [12]. However, moderate concentrations of ROS have beneficial roles for the organism. They are involved in the defense against pathogens and cellular signaling pathways [12].

To control the excessive amount of ROS, cells have developed an antioxidant defense system. This includes enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) [12]. SOD and CAT play a role in enzymatic decomposition of superoxide. Firstly, SOD converts O_2^- into O_2 and H_2O_2. Then, CAT and peroxiredoxins (Prx) catalyze the reaction of decomposing H_2O_2 into H_2O and O_2 [13] (Fig. 2).

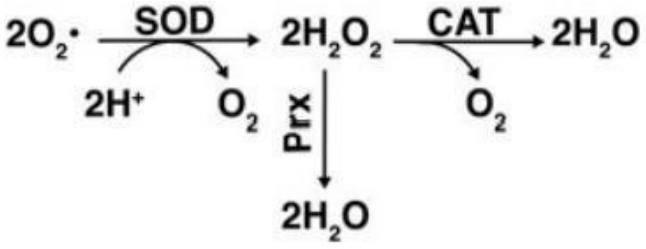

Figure 2. Decomposition of superoxide radicals by antioxidant defensive enzymes. Superoxide is converted into O_2 and H_2O_2 by SOD, then CAT and Prx decompose H_2O_2 to O_2 and H_2O. Adapted from [13].

H_2O_2 is a non-radical oxidant and a byproduct of oxidative metabolism. Its production is controlled and balanced by SOD and CAT. An increased concentration of H_2O_2 in the cell implies the occurrence of oxidative stress. However, in moderate concentrations H_2O_2 has the important role in cellular signaling [14]. The signaling pathway is controlled by the reaction of H_2O_2 and proteins with redox-sensitive parts. The redox-sensitive parts are metal centers and cysteine residues. These proteins are called redox switches, and their activity is controlled by oxidation. An example of redox-controlled switches are thiol proteins [14]. They contain cysteine residues, which form thiolates at physiological pH. Thiolates have a high affinity towards H_2O_2. When there is a change in the concentration of H_2O_2 in the cell, this causes a change of the protein's oxidation state. With these two changes, a downstream signaling cascade is activated in which H_2O_2 is the main

signaling molecule [14]. The role of H_2O_2 as a signaling molecule is proven in insulin signaling, and signaling cascades induced by growth factors such as epidermal growth factor (EFG), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). Furthermore, processes such as inflammation, tissue repair and aging also use H_2O_2 as a signaling molecule [15].

1.4. The connection between oxidative stress and circadian rhythms

In a similar way to physiological and behavioral rhythmicity, antioxidant production is regulated in a circadian fashion. One group of antioxidant enzymes that show circadian oscillation are SODs [13]. Furthermore, it was shown that there are differences in DNA damage, protein oxidation and lipid peroxidation at different times of the day. This implies a connection between circadian clock and ROS production during the day [13].

There is a contucted study which showed that responsiveness to oxidative stress during 24 hours is under circadian regulation [5]. In the experiment, *D. melanogaster* was exposed to oxidative stress induced by H_2O_2 during the day. Flies that were exposed to stress during the day, had significantly higher mortality rates compared to those that were treated during the night. Furthermore, the authors compared stress susceptibility between flies entrained to LD cycle to those whose circadian rhythm was disrupted due to exposure to constant light (LL) [5]. Interestingly, flies kept in LL conditions had similar mortality rates during the night and the day. These findings reinforce the idea that circadian rhythm is important in the protection of an organism from increased ROS levels. To show the connection between these effects and circadian genes, the experiment was repeated on flies that have a null mutation in the *per* gene [5]. per^0 mutants have shown increased susceptibility to H_2O_2 compared to *wt* flies. This

implies the importance of the circadian gene *period* in antioxidative defense [5].

The connection between circadian genes and redox regulation affects the biological process of aging. Reduced motor activity, disrupted sleep pattern, reduced ability for memory and learning, a decline in neurotransmitters and deregulation of metabolism are all processes associated with aging [16]. All these processes are connected with oxidative stress and disrupted generation or elimination of ROS in mitochondria. The connection between circadian genes and ROS production was shown in the experiment with per^{01} flies. Flies with this mutation tend to age faster and their lifespan is decreased [16]. Their aging is manifested in a faster decline in the ability to climb vertical surfaces and in increased neuronal degeneration [17]. This also coincides with elevated ROS accumulation in tissue samples of per^{01}. Together, this data suggests that dysfunction in circadian genes can contribute to the impaired antioxidant defense and thereby accelerate aging [17].

Another biological process connected to circadian rhythm is sleep. It has been shown that circadian rhythms regulate the timing of sleep [18]. Data suggest that sleep deprivation makes flies more sensitive to oxidative stress and leads to increases in their ROS levels. In contrast, prolonged sleep time promotes resistance to oxidative stress and reduces ROS levels in the brain [18]. This reinforces the idea that maintaining a normal circadian regulation of sleep/wake cycle is important for the proper generation and elimination of ROS [18].

To study how H_2O_2 affects behavior and locomotor activity in *D. melanogaster*, flies were fed or injected with H_2O_2 [19]. It was found that H_2O_2 increased speed movement in flies. A similar response was observed in flies with overexpressed SOD enzyme [19]. When they were repeatedly fed with H_2O_2, their movements were faster and circadian rhythmicity in daily locomotor activity was abolished. These findings suggest that there is

a bideractional relationship between circadian regulation and redox homeostasis [19].

To show the connection between circadian rhythm and oxidative state, flies were exposed to chronic circadian misalignments such as aberrant sleeping and eating schedules, whilst their locomotor activity, sleep and longevity were monitored [20]. Results showed that flies exibited periods of activity and that their lifespan was reduced. After RNA sequencing, it was shown that the expression of oxidative stress genes was significantly disrupted. These findings show the importance of maintaining a regular circadian rhythm for health [20].

There are connections between circadian rhythm and oxidative stress in other species too. In mice, respiratory chain components such as complex I, which generates ROS, shows a circadian expression pattern [21].

In summary, the circadian clock has the role of maintaining physiological homeostasis. Any kind of circadian rhythm disruption results in the dysregulation of this homeostasis. This leads to disruption in ROS metabolism and the occurrence of oxidative stress that can be harmful for the organism [16].

2. Aims

In previously conducted experiments, it was shown that *wt* flies and *tim* mutants have a similar response to repeated cocaine exposure [8]. Based on these findings, we formed our hypothesis that if *tim* and *wt* flies have the same responsiveness to repeated cocaine exposure, then their redox regulation will also be similar. The main aim of this thesis was to determine if H_2O_2 production, as an indicator of redox regulation, is similar in *wt* and *tim* flies.

Our study had three sub-aims. The first one was to determine if the production of H_2O_2 during a 24 hours period is regulated by the circadian clock.

The second sub-aim was to determine if there is a difference in amount of H_2O_2 between flies exposed to entrained conditions (12hours light:12 hours dark (LD)) or those in free running conditions (constant dark, DD).

The third sub-aim was to determine if there is a difference in the amount of H_2O_2 between circadian mutant tim^{01} compared to *wt* flies in LD and DD conditions.

3. Materials and methods

3.1. Preparation of experiment

We used flies with a *null* mutation in the *timeless* gene *(tim^{01})* and *wild type (wt)* flies. They were reared on a standard cornmeal/agar medium at 25°C, with 70% humidity. Each genotype of flies was divided into two groups. One group was raised in conditions of 12 hours dark/ 12 dark (DD) cycle, while another at 12 hours light/ 12 hours dark (LD) cycle.

3.2. Sample preparation

Flies were frozen at 20° C for 30 minutes and beheaded with dissection forceps under a microscope. I put 30 heads in each of six 1.5 ml Eppendorf tubes, that were previously weighed while empty. After transferring heads, Eppendorf tubes were weighed again to determine the weight of the head tissue. The tissue was homogenized with a blender. To calculate the required volume of extraction buffer, I used the equation: 300 µl / 5 mg = x µl / (sample mass) mg. The buffer contained detergent for the destruction of cell membranes. Then, the calculated volume of extraction buffer (PBS/0.1% + Triton X-100) was added to the tube. The mixture was homogenized one more time with a blender. The Eppendorf tubes containing samples were centifuged for 30 minutes at 14 000 rpm and 4°C, and the supernatants were collected.

The dihydroethidium (DHE) staining method was used for determining the concentration of H_2O_2 in the sample. DHE is a cell-permeable dye used for detection of superoxide radical anions [22]. I used the solution of 10 µM DHE in PBS. The dye is sensitive to light, so the solution was prepared with minimum exposure to light and kept in a tube covered with aluminum foil. In the wells of a Black Polyethylene Terephthalate 96-well microplate, I pipetted 200 µl of DHE solution. Then I pipetted 5 µl of each supernatant sample. All samples and dye solutions were pipetted in triplicate. DHE

solution without a sample was used as a control. The microplate was left to incubate for 30 minutes at 37 °C, covered with aluminum foil.

After incubation, the fluorescence signal from samples was measured with a Tecan Infinite 200 Pro microplate reader. The excitation wavelength was 480 nm, and the emission wavelength was 625 nm. The measurements were repeated at four different time points: 9:00 AM (one hour after light were turned on), 3:00 PM, 9:00 PM (one hour after light were turned off), and 3:00 AM.

3.3. Data analyses

The data from the microplate reader was first processed in MS Excel. The data was corrected for the amount of dye that oxidized during the experiment and for the dilution. To determine the concentration of H_2O_2 in each sample, I used a formula ($y=0.0103x-0.4628$, $R^2=0.9945$) for calibration curve from Alma – Tihana Miletić's undergraduate thesis. The program GraphPad Prism 9.1.2 was used for all graphs and statistical analyses. We analyzed data for each group of flies with one-way ANOVA test and Bonferroni's multiple comparison test. Two–way ANOVA test and Bonferroni's multiple comparisons test were used to compare results between two different groups of flies.

4. Results

To determine if the oxidative status in heads of flies changes over 24 hours, we measured the amount of H_2O_2 present in head homogenates at: 9:00 AM one hour after light were turned on), 3:00 PM, 9:00 PM (one hour after light were turned off), and 3:00 AM. Flies were raised either in the LD cycle to determine the effect of entrainment to the light/dark cycle or in the DD cycle to determine if there is an endogenous regulation of the amount of H_2O_2.

In *wild type* flies raised in the LD cycle there were significantly elevated levels of H_2O_2 at 3:00 PM (Fig. 3A). In *wild type* flies raised in the DD condition we observed elevated levels of H_2O_2 at 9:00 PM and 3:00 PM (Fig. 3B).

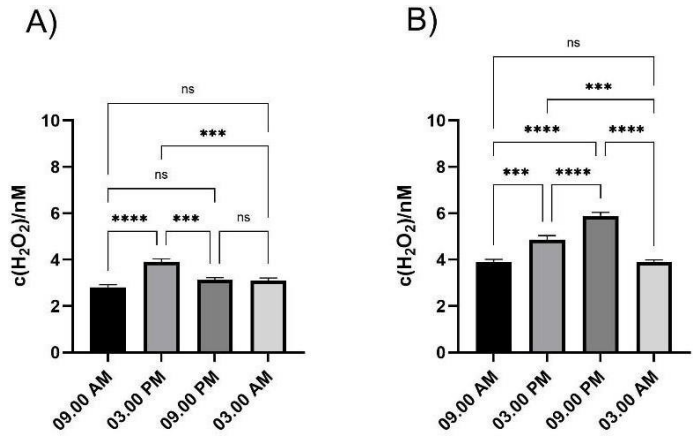

Figure 3. In *wt* flies H_2O_2 levels are regulated in a circadian fashion in LD and DD conditions (A) Concentration of H_2O_2/nM in head homogenates, at four time points for *wild type* flies raised in LD cycle (n= 30 for each time point) and **(B)** *wild type* flies raised in DD cycle (n= 30 for each time point). The peroxide concentration was measured as fluorescence intensity at an excitation wavelength of 480 nm and emission wavelenght of 625 nm. **ns**: no significance, ***: p= 0.0002, ****: p<0.0001, one-way ANOVA with post-hoc Bonferonni's test for multiple comparison correction.

To investigate if LD versus DD condition affects the concentration of H_2O_2 in fly's heads, we statistically compared the values from corresponding LD and DD time points. Flies raised in the DD cycle had significantly higher production of H_2O_2 at all time points, compared to flies raised in the LD cycle (Fig. 4).

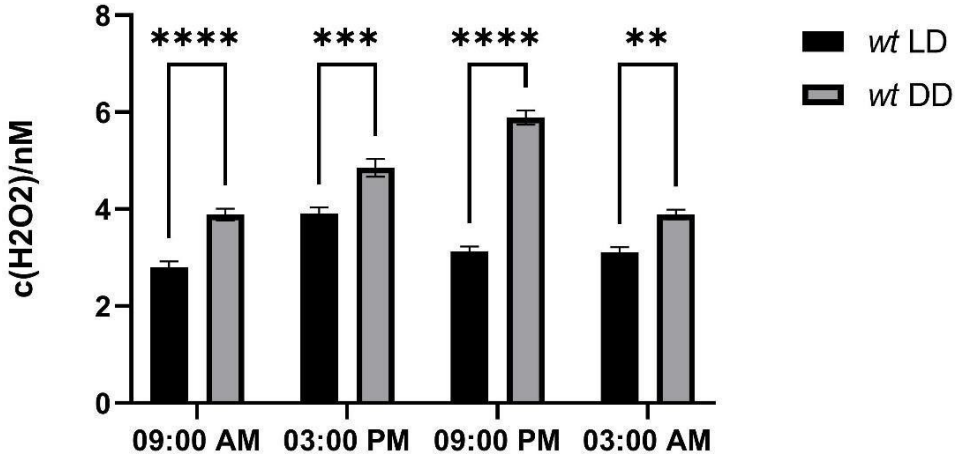

Figure 4. *wt* flies raised in DD conditions have higher levels of H_2O_2 than flies raised in LD conditions. Comparison of H_2O_2 concentrations for LD and DD conditions, based of the data shown in Figure 3 A) and B). **: p=0.0021, ***: p=0.0001, ****: p<0.0001, two-way ANOVA with post-hoc Bonferonni's test for multiple comparison correction.

To determine if the 24 hour change in the level of H_2O_2 differs between *wt* flies and those mutant for the circadian gene *tim*, we repeated the same procedure with tim^{01} flies. When raised in the LD cycle tim^{01} flies had similar levels of H_2O_2 at

all times except at 3:00 PM when the values were significantly lower (Fig. 5A).

When tim^{01} flies were raised in the DD cycle they showed a gradual decrease in the amount of H_2O_2 (Fig. 5B). The highest levels were present at 9:00

AM (the start of the subjective day), and the lowest at 3:00AM (in the middle of the subjective night) (Fig. 5B).

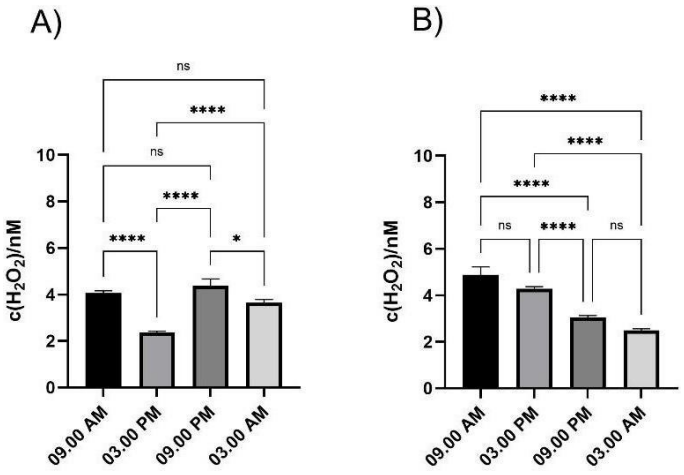

Figure 5. Different amounts of H_2O_2 in the heads *tim*^{01} flies raised in LD and DD
(A) Concentration of H_2O_2/nM in head homogenates, at four time points for *tim*^{01} flies raised in LD cycle (n= 30 for each time point) and **(B)** *tim*^{01} flies raised in DD cycle (n= 30 for each time point). The peroxide concentration was measured as fluorescence intensity at an excitation wavelength of 480 nm and emission wavelength of 625 nm. **ns**: no significance, *****: p=0.272, ********: p<0.0001, one-way ANOVA with post-hoc Bonferonni's test for multiple comparison correction.

To see if LD versus DD condition significantly affects the levels of H_2O_2 in *tim*^{01} flies, we compared LD and DD H_2O_2 levels at all four time points (Fig. 6). Flies raised in the LD cycle showed change in the H_2O_2 amount as a function of the time of day, while this rhythm was abolished in DD conditions, where *tim*^{01} flies showed gradual decrease in the amount of H_2O_2 (Fig. 6). This resulted in higher levels of H_2O_2 in DD in the first half of the day and lower levels in the second part of the day.

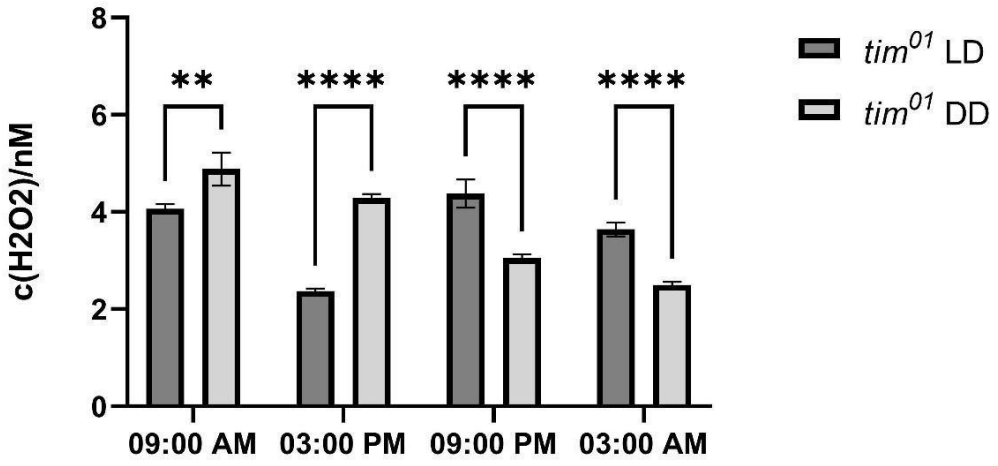

Figure 6. Amount of H_2O_2 in *tim*^{01} flies kept in DD conditions versus LD conditions. Comparison of H_2O_2 concentrations for LD and DD conditions, based of the data shown in Figure 5A and B. **: p=0.0017, ****: p<0.0001, two-way ANOVA with post-hoc Bonferonni's test for multiple comparison correction.

To determine if there is a significantly different regulation of the amount of H_2O_2 between *wt* an *tim*^{01} flies we statistically compared the values obtained for LD and DD conditions. In LD condition, both genotypes show diurnal modulation of H_2O_2 amount (Fig. 7A). However, the modulation of H_2O_2 is reversed between *wt* and *tim*^{01} flies, in that relative to *wt* flies *tim*^{01} flies have significantly lower amount of H_2O_2 at 3:00 PM.

In the DD cycle there were apparent differences in the profile of changes of H_2O_2 amount during 24 hours between *wt* and *tim*^{01} flies (Fig. 7B). *wt* flies appeared to maintain circadian modulation of H_2O_2 amount with the peak at 9:00 PM, with similar values at 3:00 AM and 9:00 AM. In contrast, in *tim*^{01} flies, the concentration of H_2O_2 showed steady decrease over four time points, suggesting a loss of circadian modulation.

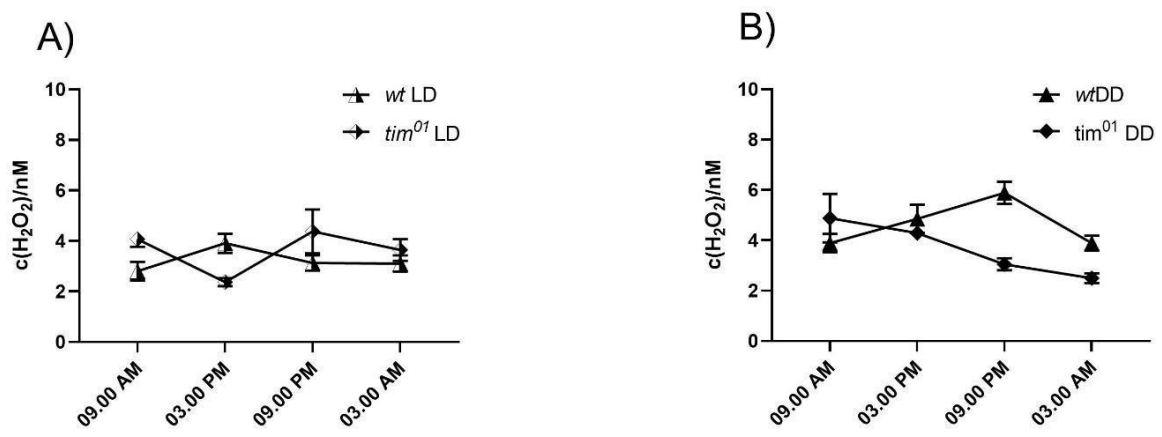

Figure 7. Different profile of H_2O_2 modulation in LD and DD conditions between *tim^{01}* and *wt* flies. (A) Comparison of H_2O_2/nM concentration in head homogenates, at four time points for *tim^{01}* and *wt* raised in the LD cycle (n= 30 for each time point) and for **(B)** *tim^{01}* and *wt* raised in the DD cycle (n= 30 for each time point)

5. Discussion

Circadian rhythmicity and oxidative state are essential to maintain homeostasis in an organism. Their disruption has harmful effects on cellular structures [12]. Data suggests that increased ROS levels and dysregulated oxidative state are often connected to disrupted circadian rhythm [5]. Based on these findings we were interested to see how circadian modulation would affect the oxidative state in *tim*^{01} and *wt* flies. The results of this thesis will aid in a better understanding of the connection between circadian rhythm and oxidative state.

Therefore, to examine if H_2O_2 production is regulated by circadian rhythm, we first measured H_2O_2 concentrations in head homogenates of *wt* flies during 24 hours. Data suggest that H_2O_2 production in LD conditions for *wt* flies has circadian rhythmicity. The same rhythmicity was also observed in DD conditions for *wt* flies. These results reinforce the idea that redox regulation is connected to circadian rhythm. There are other studies that support this statement. Circadian regulation of locomotor activity for *wt* flies was observed in LD conditions [11]. Furthermore, when flies were transferred to DD conditions, circadian regulation was still present [11]. Research has also shown that metabolites such as riboflavin oscillate with circadian periodicity in LD and DD conditions for *wt* flies [23].

Although circadian regulation of H_2O_2 production was observed in both the LD and DD conditions, production of H_2O_2 is overall higher for flies in DD conditions. That could be due to the fact that the presence of light is important for the proper function of circadian genes or genes that participate in redox regulation.

We also wanted to compare if circadian regulation of oxidative state was present in *tim*^{01} mutants as, it was in *wt* flies. We observed indications of circadian regulation of H_2O_2 production in LD conditions. However, circadian rhythmicity of H_2O_2 production was abolished in DD conditions. These changes in DD conditions imply impaired functioning of the circadian clock

because of the absence of a functional *tim* gene. In the molecular mechanism of the circadian clock, genes *Clk* and *cyc* encode the CLK and CYC proteins, which initiate *per* and *tim* transcription [24,25]. PER and TIM proteins start to accumulate in the cytoplasm, but later they localize to the nucleus. When they are in the nucleus, negative autoregulatory feedback is activated. They also phosphorylate CLK and CYC proteins, which results in repression of *per* and *tim* transcription [24,25]. When the *tim* gene is absent, the whole process of circadian regulation can not be carried out properly. This could be considered as one of the reasons why *tim* mutants have disrupted DD cycle. Indications of circadian regulation we observed in LD conditions for *tim* mutants could be the consequence of a passive response to changes in the light/dark schedule. Other experiments that tried to connect daily locomotor activity to circadian regulation had similar results. Mutants have shown circadian regulation of locomotor activity in LD conditions. However, in DD conditions rhythmicity was also abolished [11].

In previous experiments, mutantion of the *tim* gene have shown similar responses to repeated cocaine exposures as *wt* flies [8,9]. Based on this, we expected to see a similarity in redox regulation between *wt* and tim^{01}, however our hypothesis was not fully confirmed. tim^{01} flies show modulation of H_2O_2 and the exposure to volatilized cocaine is always done during the day. However, there is a differenece at 3:00 PM in H_2O_2 between *wt* and *tim* mutant flies, so we will need to conduct further studies to confirm if this difference is relevant or not in the regulation of locomotor sensitization to cocaine. One interpretation is that the role of *tim* in the regulation of H_2O_2 is not essential to the regulation of drug responsiveness.

In this experiment, we measured the concentration of H_2O_2 as an indicator of of redox balance. The effect of H_2O_2 is dual: significantlly elevated levels can be toxic while moderate and regulated levels participate in signaling [26]. Superoxide is a reactive free oxygen radical, which in the cell is rapidly converted into H_2O_2. Because of this, elevated concentrations of H_2O_2 could be connected to oxidative stress. However, increased H_2O_2 concentrations

may also be due to the fact that H_2O_2 is a signaling molecule. We cannot be sure whether elevated H_2O_2 concentrations reflected signaling or oxidative stress unless we knew the exact subcellular source, location and duration of it within the cell [26].

6. Conclusion

We measured the amount of H_2O_2 in head homogenates of wt and tim^{01} flies, in both LD and DD conditions. The aim was to determine if *wt* and tim^{01} flies have similar regulation of H_2O_2, which would correlate to their similar behavioral responses to volatilized cocaine. The daily H_2O_2 production in *wt* flies is regulated by circadian rhythm in both LD and DD conditions. However, production in DD conditions is overall higher. In *tim* mutants, circadian regulation of H_2O_2 production is only shown in LD conditions, whilst in DD conditions circadian regulation is lost and production has a gradual decrease. We propose that rhythmicity was abolished because of the dysfunctional *tim* gene. Our experiments showed that regulation of H_2O_2 is different between *wt* and tim^{01} flies, suggesting that in *tim* mutant flies, H_2O_2 is not an essential molecule for the development of locomotor sensitization to volatilized cocaine. These results will aid in the better understanding of the circadian regulation of the oxidative state and the role of the circadian gene *tim*.

7. Literature

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USBRI

In the project "Student mentor"