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SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU
Preddiplomski sveučilišni studij
"Biotehnologija i istraživanje lijekova"

Valentina Dukić

Utjecaj metamfetamina na redoks status kod *Drosophila melanogaster*

Završni rad

Rijeka, 2020.

Mentor rada: dr. sc. Rozi Andretić-Waldowski
Ko – mentor rada: dr. sc. Ana Filošević

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The thesis has 25 pages, 6 figures, 0 tables and 22 references.

Sažetak:

Metamfetamin (METH) je psihostimulans koji utječe na središnji živčani sustav povećavajući koncentraciju slobodnog dopamina, čija oksidacija uzrokuje trajna oštećenja strukture i funkcije neurona kao posljedicu povećane proizvodnje i akumulacije slobodnih kisikovih radikala (ROS-a).

Korištenjem *Drosophile melanogaster* kao modelnog organizma istražen je utjecaj akutne doze volatiliziranog METH-a (vMETH) na koncentraciju vodikovog peroksida (H_2O_2), u mozgu, kao jednog od predstavnika ROS-a i redoks statusa. U eksperimentu su korištene individualne mušice divljeg tipa *CantonS* (*wt*) izložene vMETH–u korištenjem FlyBong metode. Koncentracija H_2O_2 je mjerena u ekstraktima tkiva glava mušica 10 sati nakon administracije upotrebom dihidroetidij fluorescentne boje. Rezultati su pokazali da akutno izlaganje vMETH-u ne dovodi do promjene u koncentraciji H_2O_2 kod *wt* mušica u odnosu na kontrolu.

S obzirom na literaturno poznatu povezanost administracije METH-a s povećanom ekspresijom *period* (*per*) gena i aktivnosti enzima uključenog u sintezu dopamina, tirozin hidroksilaze, testirano je ima li razlike u koncentraciji H_2O_2 nakon akutne izloženosti vMETH-u između različitih cirkadialnih mutanata i *wt* mušica. *Za* eksperimente su korišteni cirkadialni mutanti u genima *per , timeless (tim), Clock (Clk) i cycle (cyc)* sa sljedećim alelima: per^{01}, tim^{01}, Clk^{Jrk} i cyc^{01}. per^{01} i tim^{01} mušice su pokazale smanjenu koncentraciju H_2O_2 u odnosu na svoje kontrole, dok su Clk^{Jrk} i cyc^{01} mušice imale veću koncentraciju H_2O_2 nakon akutnog izlaganja vMETH-u. Koncentracija H_2O_2 je značajno niža kod tim^{01} mušica u usporedbi s *wt* mušicama nakon izlaganja vMETH-u, dok cyc^{01} mušice imaju značajno veću koncentraciju nego *wt* mušice.

S obzirom na rezultate ovoga rada predlažemo utjecaj cirkadialnih gena na endogenu koncentraciju H_2O_2 koja je u odsustvu *per* ili *tim* povećana, a u odsustvu *Clk* ili *cyc* smanjena u odnosu na *wt*. Administracija vMETH-a potiče proizvodnju H_2O_2 u odsustvu *cyc* dok odsustvo *tim* gena sprječava taj efekt. Nedostatak razlike kod *wt* mušica je iznenađujući te je potrebno razmotriti moguće pogreške u metodi ili njezinom provođenju. Utvrđivanjem molekularnih putova koji uključuju H_2O_2 i cirkadialne gene mogle bi se razviti nove metode liječenja ovisnosti o METH-u.

Ključne riječi: *Drosophila melanogaster*, metamfetamin, cirkadijalni geni, vodikov peroksid

Summary:

Methamphetamine (METH) is a psychostimulant that affects central nervous system by increasing the concentration of the free dopamine, whose oxidation leads to the permanent damage in the structure and function of neurons, as the consequence of the increased production and accumulation of the radical oxygen species (ROS).

By using *Drosophila melanogaster* as a model organism we have tested impact of an acute dose of volatilized METH (vMETH) on the concentration of hydrogen peroxide (H_2O_2), in the brain, as one of the ROS and redox status representative. Individual flies of *wild type* strain *CantonS* (*wt*) used in the experiment were exposed to vMETH using the FlyBong method. The concentration of H_2O_2 was measured in the head tissue extracts, 10 hours after the administration, using dihydroetidium florescent dye. The results have shown that the acute exposure to vMETH does not lead to the change in the concentration of H_2O_2 in the *wt* flies compared to the control group.

Considering that research have shown connection between administration of the METH and increased expression of *period* (*per*) gene with enzyme activity of tyrosine hydroxylase, included in the synthesis of dopamine, I tested whether there is a difference in the concentration of H_2O_2 after the acute exposure to vMETH between different circadian mutants and *wt* flies. Circadian mutants of genes *per*, *timeless* (*tim*), *Clock* (*Clk*) and *cycle* (*cyc*) with alleles per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01} were used in the experiment. per^{01} and tim^{01} flies showed the decreased concentration of H_2O_2 compared to the controls while Clk^{Jrk} and cyc^{01} flies had higher concentration of H_2O_2 after the acute exposure to vMETH. The concentration of H_2O_2 is significantly lower in tim^{01} flies compared to *wt* flies after the exposure to vMETH while cyc^{01} flies had a significantly higher concentration than *wt* flies.

Based on the results of this thesis, we suggest the influence of circadian genes on the endogenous concentration of H_2O_2, which is in the absence of *per* or *tim* increased, and in the absence of *Clk* or *cyc* decreased compared to *wt*. The administration of vMETH potentiates the production of H_2O_2 in the absence of *cyc* while the absence of *tim* gene attenuates that effect. The lack of difference in *wt* flies is surprising and possible mistakes in the method or its use should be considered. By establishing the molecular pathways involving H_2O_2 and circadian genes new methods for treatment of addiction could be developed.

Key words: *Drosophila melanogaster*, methamphetamine, circadian genes, hydrogen peroxide

Content:

1.1 Introduction

Methamphetamine (METH) is one of the most popular synthetic psychostimulants. It is a derivative of amphetamine with a stronger pharmacological impact on the central nervous system.[1] METH and amphetamine are used to treat narcolepsies and hyperactivity but can lead to the development of addiction and death if the consumption is abused. The development of addiction to all classes of addictive drugs, including METH, is connected to the dopaminergic mesolimbic reward pathways in the brain. Abuse of addictive drugs can lead to a wide spectra of psychological, behavioural and social problems.[2]

Acute effects of METH administration include decreased appetite, hyperthermia and cerebrovascular problems.[1] Continuous usage may cause mental diseases such as paranoia, anxiety and psychosis.[2] Chronic abuse of METH leads to the neuropathological changes in the brain and neurodegenerative processes. This includes abnormalities in the molecular mechanisms such as decreased level of dopamine and serotonin transporters on the membranes of neurons, abnormal metabolism of glucose, activation of microglia and astroglia which leads to the inflammation, apoptosis of neurons and increased levels of radical oxygen species (ROS).[3,4] These pathological processes that underlay neurodegenerative effects of METH abuse are numerous and highly complex.

The formation of ROS is one of the most researched causes of METH induced neurodegeneration. Many studies were done on the topic of METH induced formation of ROS, especially in relation to the role of the dopaminergic neurons. METH induced neurodegeneration has similar patterns as neurodegenerative diseases like Parkinson's or Alzheimer's disease.[3] They are characterized by increased production of ROS in the brain of patients and decreased level of antioxidants, such as, glutathione.[5] Currently, many studies are based on curing the negative effects of METH by increasing the level of antioxidants in the body of patients. Antioxidants such as ascorbic acid, β – arestin and N-acetylcysteine amide are being tested for their abilities to attenuate ROS production. [3, 5]

Very little is known about genetic bases of METH - induced neuropathology and addiction in humans.[2] Most studies investigating the effect of METH are done on model organisms such as mice, rats, primates and lately *D. melanogaster*. The model organisms need to be chosen and experiments designed in a way that the outcome of the studies furthers the knowledge of molecular mechanisms in humans.[6]

1.2 METH and production of ROS

METH affects the brain by several mechanisms, mainly including the effects on the brain, specifically neurons. One of the major mechanisms is the effect of METH on the production of ROS as this can lead to the neuronal damage and neurodegeneration.[7]

After the exposure to METH the cytoplasmic and extracellular concentration of dopamine rises in the dopaminergic neurons. The excess of the free dopamine is oxidized in quinones by iron - catalysed dopamine metabolism and metabolism of dopamine by monoamine oxidase A. These consequences favour the production of superoxide anion, hydroxyl ion and hydrogen peroxide (H_2O_2), which will be the focus of this thesis.[7]

Another mechanism that leads to the METH induced oxidative stress is the increase in the activity of the nitric oxide synthase and increase in the concentration of reactive nitrogen species. This process is the result of the increased concentration of glutamate activated receptors. After the administration of METH, due to the large increase in ROS production, there is a decrease in the concentration of antioxidants which further contributes to oxidative stress.[7]

METH alters the energy metabolism by damaging the mitochondria respiratory chain (Figure 1). During the exposure to METH, mitochondria in the brain are more active and produce more energy. After the peak in energy production, there is a depletion in energy stores and metabolism which lead to the mitochondrial damage. Since the main location of the oxidative metabolism in mitochondria is damaged, the concentration of ROS is increased.[7]

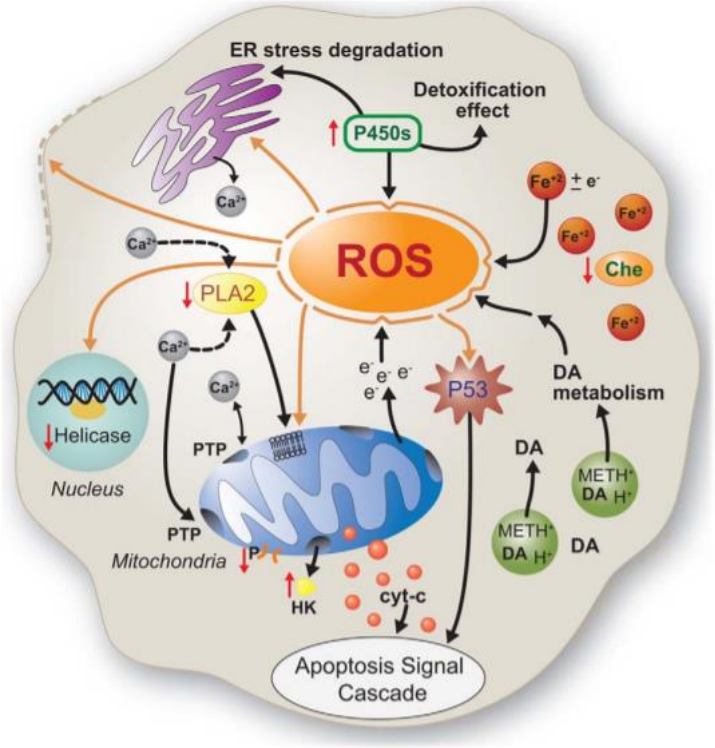

Figure 1. Mechanisms of ROS production pathways in the neurons after METH exposure. METH enters the dopaminergic neurons through dopamine transporters (DAT), P450 is upregulated and promotes the production of ROS. METH increases the concentration of free dopamine which is oxidized by Fe^{2+}. Mitochondria gets damaged and contributes to the production of ROS. ROS can cause further damage of mitochondria, apoptosis, ER stress and damage the DNA. Adapted from -[8]

1.3 *Drosophila melanogaster* as a model organism

Asides from the vertebrate animal model organisms, rats, mice and primates, *D. melanogaster* is a widely used model organism in the behavioural research including the studies of addictive drugs, like METH.[3] The genetic and environmental factors (such as dose of the drug) can be closely monitored. The research done on *D. melanogaster* have a major significance because 65% of their genes are orthologue to genes in humans.[2]

The changes in fly's behaviour after the exposure to psychostimulants are diverse and there are efficient ways to monitor them: reduced sleep, increased arousal, reduced consumption of food and an effect on locomotor activity. There are evidence that the mechanisms of METH effect on the behaviour and physiology are highly conserved in flies and vertebras.[2]

The advantages of using *D. melanogaster* compared to the other animal models are in the short life span and a large number of offspring. There are many genetic techniques that can be easily applied, the anatomy is well studied and various mutants are available. Vertebras have a more complex genome, so there may be some factors that are involved in the processes that do not exist in *D. melanogaster*.[9]

In *D. melanogaster* it is possible to efficiently track the consequences of ROS on the integrity of macromolecules and structures in the organism. As there are different mutants, including circadian mutants, available on the market it is also possible to research the role of different genes in production of ROS. All of this, and the highly conserved genes, make *D. melanogaster* a very useful model organism to study effects of addictive drugs on ROS production and behavioural and physiological consequences.

1.4 Circadian genes and METH

Drugs like METH have an effect on the molecular and behavioural rhythms. An experiment on mice showed that chronic METH treatment during the day leads to the higher activity of the mammalian circadian genes like *Per1*, *Per2*, Gene model 129 (*Gm129*) and D-Box Binding PAR BZIP transcription factor (*Dbp*). METH causes the locomotor activation in mice which are nocturnal animals. As the drug is administrated during the day, mice are forced to stay awake. This change in behaviour leads to the abnormal day cycle and alters the activity of circadian genes.[10]

The effect of the drug on the circadian genes and rhythm depends on the time of the cycle when the drug was administrated. It is highly possible that these differences are related to the different expression of circadian genes at various times of the cycle. *Per1* and *Per2* are two circadian genes altered in the chronic METH treatment, but also in the acute METH treatment. In the case of acute treatment, expression of *Per* genes is induced.[10]

1.5 Circadian genes in *Drosophila melanogaster*

Circadian rhythm is a daily occurring rhythm that repeats approximately every 24 hours. Rhythm can be influenced by environmental factors but it is also sustainable on its own. *D. melanogaster* represents a base of knowledge of molecular mechanisms and genetics of circadian rhythms.[11]

The first circadian gene that was discovered in *D. melanogaster* is *period* (*per*). Today it is known that *per*, together with *timeless* (*tim*), *Clock* (*Clk*) and *cycle* (*cyc*), creates the core transcriptional feedback loop that regulates circadian rhythms.[11] *per* RNA and protein express itself cyclically. The amount of protein rises as the levels of mRNA declines. This leads to a conclusion that PER protein regulates its own activity by negative feedback loop. Later studies of *tim* showed that *per* and *tim* mRNA cycle in a phase.[11]

Levels of *per* and *tim* mRNA rise during the day and reach their peak of concentration in the evening. Proteins start to accumulate in the cytoplasm and by the middle of the night are transported to the nucleus. PER is stabilized by TIM in the cytoplasm and gets transported to nucleus (Figure 2). To be able to transport, PER gets phosphorylated. The transport to nucleus is simultaneous with the reduction in the concentration of mRNA because of negative feedback loop.[11]

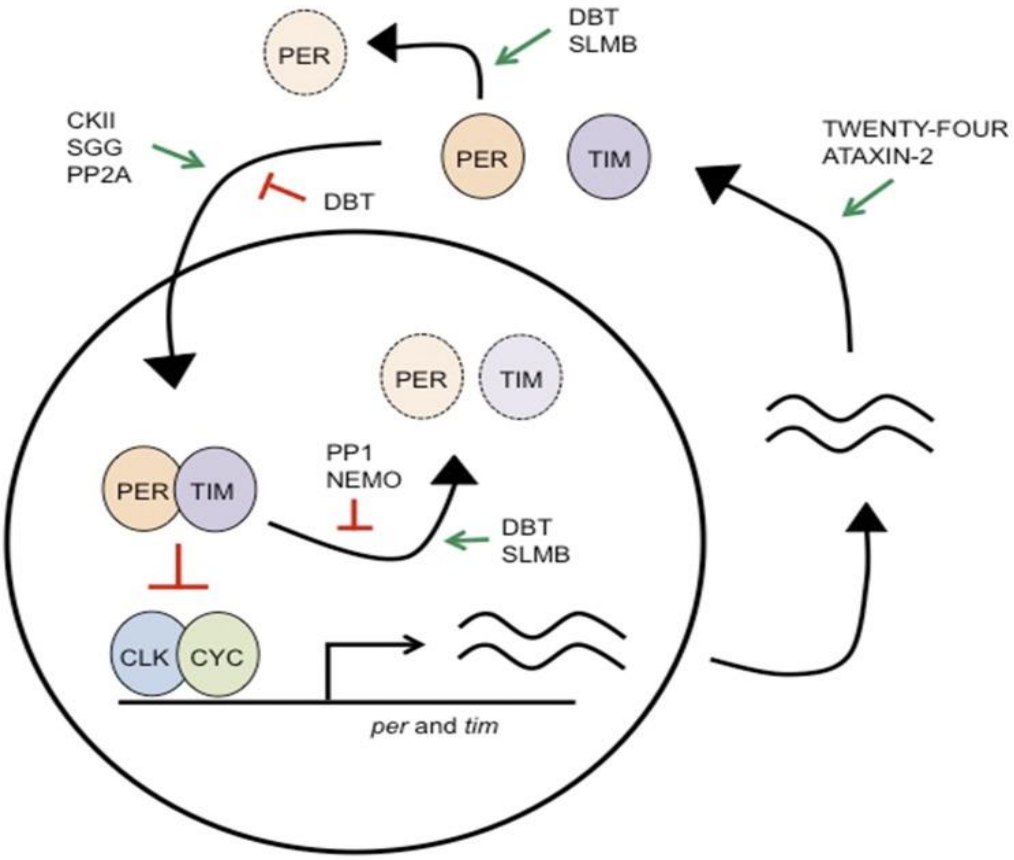

Figure 2. The molecular feedback loop of circadian rhythm in *D. melanogaster.* The main mechanism is the negative feedback of PER and TIM on their transcription. Once in nucleus, PER and TIM interact with transcriptional activators CLK and CYC. PER and TIM get degraded and the feedback loop repeats. This circadian rhythm repeats every 24 hours. Adapted from - [11]

PER and TIM regulate their own transcription by inhibiting the transcriptional activators *Clock* and *Cycle*. The mechanism of the negative feedback loop is unclear. PER may inhibit the CLK – CYC heterodimer by sequestrating it (Figure 2). The concentration of TIM drastically decreases in the morning, while PER expression is the lowest at the mid-afternoon.[11, 12] The *Clk* and *cyc* genes also have the loop of its own. The expression of *Clk* mRNA is maintained in a rhythmic pattern. It is unclear why the mRNA are cycling as the CLK protein does not show the cycling pattern. It is possible that the second loop stabilizes the system.[11]

The mechanism of a circadian negative feedback loop is highly conserved throughout many organisms. Invertebrate and mammals share the regulatory aspect of the loop and the functions of circadian genes. Some homologous genes found in humans are mutated or inactive in some neurological or sleeping disorders.[11]

1.6 Circadian genes and oxidative stress

Each core clock gene has functions aside from its role in the circadian rhythm that are crucial for the cellular processes. They regulate mechanisms of cellular metabolism and redox homeostasis, especially in the peripheral tissues. These effects are often connected to the aging process.[13]

The clock genes encode various transcriptional regulators whose daily fluctuations influence the rhythmic expression of their target genes. Those target genes are often included in the processes such as metabolism, energy balance, DNA – damage repairs and detoxification in *D. melanogaster*.[14] It has been shown that concentration of ROS and oxidative damaged proteins fluctuate during the day in the brains of *wt* flies. This is influenced by the circadian genes whose expressions cycles following the daily rhythm. In circadian mutants the rhythm is changed so the fluctuations in the concentrations of ROS are different.[13] mRNA of *per* and *tim* genes cycle in a phase and their transcription is regulated by negative feedback loop. TIM stabilises PER and allows it to transport into the nucleus. PER and TIM inhibit transcriptional activators *Clock* and *cycle*. CLK and CYC proteins form a heterodimer which is later inactivated by PER.[11]

In *D. melanogaster, per^{01}* mutants showed the increased susceptibility to neurodegenerative diseases and faster accumulation of the oxidative damage. Disruption of a positive limb of circadian clock in the brain leads to the oxidative stress. The circadian clock positive – limb transcriptional complex (Figure 3.) has an important role in the neuronal redox homeostasis in the mammalian brain.[14,13]

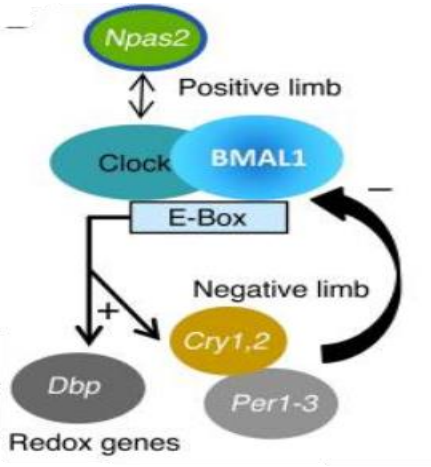

Figure 3. The mammalian circadian clock positive – limb transcriptional complex (BMAL1:CLOCK/NPAS2). The BMAL1:CLOCK/NPAS2 heterodimers regulate the transcription and activity of many circadian and non-circadian genes. The heterodimer binds to the E – Box and activates the redox genes like *Dbp* and circadian genes like *Per 1 – 3*. Adapted from - [13]

In mammals, *Per2* is a circadian gene that responds to the environmental signals, like light and temperature. The redox state is essential for the regulation of clock gene dependent transcription influenced by nicotinamide adenine dinucleotide (NAD). NAD modulates DNA binding factors Clock and NPAS2. Nicotinamide adenine dinucleotide hydride (NADH) positively influences the formation of BMAL1:CLOCK/NPAS2 heterodimer. The increased levels of NADH increase the oxidative protection abilities of the cell and lead to the increased survival. *Per2* mutation causes the increase in the NADH concentration and cells become more resistant to ROS.[15]

The circadian genes regulate the NAD^+ biosynthesis which is involved in the oxidative metabolism. Clock genes regulate the expression and transcription of the central antioxidant, glutathione. Glutathione is an important antioxidant involved in the thiol antioxidant system. The second antioxidant system, synergistic to the thiol one, is CAT/SOD (catalase/superoxide dismutase) based on the oxygen and, therefore, H_2O_2. The rhythm of glutathione is dysregulated in cyc^{01} and per^{01} mutants which negatively influences the mechanism of protection against oxidative stress.[14]

The mutation of nuclear transporter, *Bmal1* (mammalian homologue to *cyc*), in mice leads to the loss of behavioural and physiologic circadian rhythms and increased oxidative stress.[13] The loss of circadian genes activity and the increase of oxidative stress is also present during the aging process. With aging, the expression of circadian genes decreases, as has been reported for gene *Bmal1*, which affects genes involved in the redox defence.[13]

Higher levels of ROS have been noticed in the circadian mutants, not only in the brain, but in the whole body. It is suggested that the core clock genes are essential for the protective response to oxidative stress.[13] The *Clock* mutant mice have been shown to have higher endoplasmic reticulum (ER) stress and production of ROS. By different molecular mechanisms, primarily the positive – limb transcriptional complex, circadian genes influence the ROS production and ER stress.[16]

In this thesis the changes in ROS production after the acute exposure to METH will be analysed in four different circadian mutants by comparing H_2O_2 levels before and after volatilized METH (vMETH) exposure. The results will improve our understanding of the role that circadian genes have on the regulation of H_2O_2 and this can help in the development of more effective therapies in METH addiction.

2. Aims

The main aim of this thesis is to determine whether the exposure to an acute dose of vMETH has effect on the redox status of *D. melanogaster*. The main hypothesis is that if METH has an effect on the redox status of *D. melanogaster* there will be a change in the concentration of H_2O_2 after the exposure to vMETH. The indicator of H_2O_2 change is the measurement of change in the dihydroetidium (DHE) fluorescence in head homogenates.

First, the concentration of H_2O_2 will be measured in the head homogenates of five genotypes of *D. melanogaster*. These five genotypes are *wild type CantonS.* strain and four circadian mutants: *period*, *timeless*, *Clock* and *cycle* mutants (per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01}). The concentration of H_2O_2 will be measured in all genotypes that were previously exposed to vMETH and compared to the control group of flies that were not exposed to vMETH.

The second hypothesis is based on the potential different response after the exposure to vMETH of circadian mutants compared to *wt* flies. Using the measurements obtained in the first experiment it will be tested if the concentration of H_2O_2 varies among different circadian mutants after the exposure to vMETH.

3. Materials and methods

3.1 The genotypes of *Drosophila melanogaster*

The experiments were done using the males of species *D. melanogaster.* Five genotypes were used in the experiment: *wild type Canton S.* strain and circadian mutants per^{01}, tim^{01}, cyc^{01} and Clk^{Jrk}.

3.2 Chemicals and reagents

Methamphetamine hydrochloride (METH-HCl), quercetin dihydrate (QUE), hydrogen peroxide (H2O2), dihydroethidium (DHE), Triton X-100, N,N,N',N'-tetramethylethylenediamine (TEMED) and propionic acid were provided from Sigma Aldrich. All other chemicals used NaCl, KCl, Na_2HPO_4, KH_2PO_4, EDTA were of analytical grade.

3.3 Exposure to vMETH using FlyBong setup

First step was collecting the male flies under the microscope and CO_2 anaesthesia. Only males of *D. melanogaster* were used because females have shown to be more sensitive to the warm air so it would be unclear whether they reacted to the vMETH exposure itself or to the mixture of warm air and vMETH. FlyBong consists of commercially available activity monitoring system (DAMS) and vertical monitors with one infrared beam. DAMS monitor can house 32 individual flies housed in the polycarbonate tubes. Each tube has two holes on the one end and food based on dry yeast, molasses, glucose and agar wrapped with parafilm to prevent dehydration. Transferring flies from the cultivation vials to an individual tube was performed using an aspirator. Each tube was connected to the dispenser on the opposite side of the food, which was used to equally distribute the aerosol of METH (Figure 4). I connected the monitor, dispenser and tubes installation to the FlyBong. The preparation of experiment was done at 9 AM.

METH is volatilized in a three – neck glass flask situated in the heating cap. 75μL of 10 mg/mL METH – HCl ethanol solution is pipetted through the middle neck of the flask (Figure 5.). To eliminate the effect of ethanol on the production of H_2O_2 in the brains of *D. melanogaster* ethanol solution of METH was added 4 to 6 hours before the exposure, therefore, enabling ethanol to vaporize. Vaporization of METH was done by heating of the three neck flask for 8 minutes at the temperature of 100 – 120 °C to volatilize the METH. After 8 minutes the heating cap is turned off and the aerosol is pumped into the tubes through dispensers for 1 minute at the air flow rate of 150 L/h. The administration of vMETH to flies was done at 7 PM. All experiments were performed in the triplicate (3x32 flies).

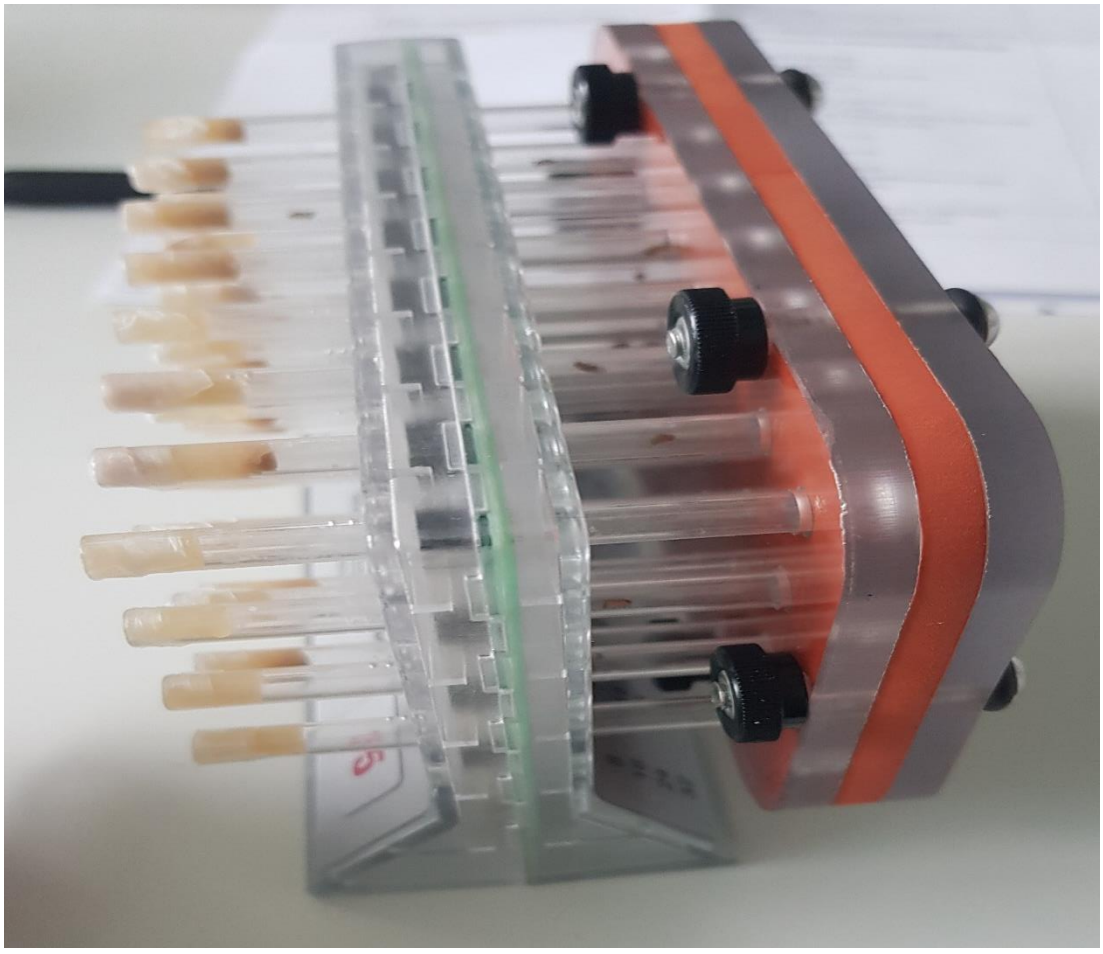

Figure 4. The DAMS monitor and dispenser. The photo shows how the tubes with individual flies are attached to the monitor and the dispenser.

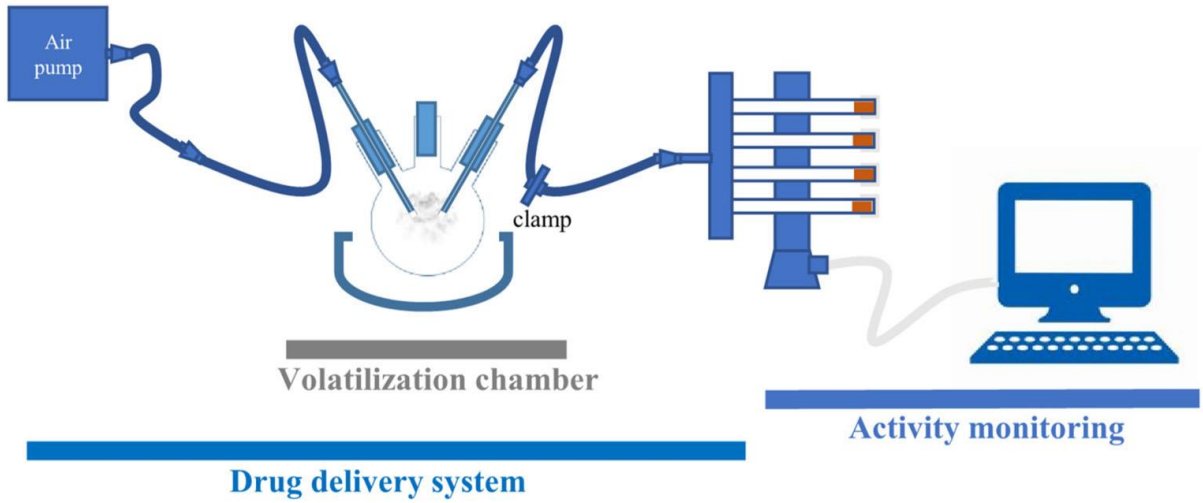

Figure 5. Fly Bong platform for administration of volatilized METH. METH and ethanol solvent solution are pipetted in the middle neck of the flask. The flask is connected on one side to air pump and on the other to DAMS monitor. The heating cap heats the flask for 8 minutes and causes the volatilization of METH which is then pumped into the tubes and to each individual fly. Adapted from - [17]

3.4 Sample preparation and H_2O_2 measurement

10 hours after the vMETH administration, at 9 AM of the next day, flies were frozen at the temperature of -20°C for 30 minutes. In the empty and weighed Eppendorf tube I put 32 heads which were detached from the body after the freezing, using microscope and dissection forceps. I homogenised the head tissue mechanically using a blender. Extraction buffer PBS x 1 + TritonX 0,1% was used to destroy the cell membrane. The volume of extraction buffer was determined on the proportion 300µL of buffer: 5mg of the tissue sample. The homogenates were centrifuged for 30 minutes on +4°C and 14.000 rpm. Supernatants were collected and used for the H_2O_2 measurements.

On the 96 well microplate I pipetted the 200 µL solution of 10µM DHE in PBS x 1. The DHE stock solution must be kept in the dark and +4°C in order to prevent dye photo oxidation and degradation. The 5 µL of each sample was pipetted in a triplicate on the microplate. The microplate with samples was incubated for 30 minutes at the temperature of 37°C in the dark. The amount of H_2O_2 in the head homogenates was measured using the microplate reader TecanInfinitePro200 at the $\lambda_{excitation}$ = 480 nm and $\lambda_{emmision}$ = 625 nm which is florescence maximum of oxidised DHE.

H_2O_2 concentration in the head homogenates was determined using the calibration curve for DHE with known H_2O_2 concentration. To eliminate DHE oxidation induced by environment, each measured sample relative fluorescence units (RFU) were corrected for dilution, and RFU of DHE was subtracted from the RFU of the sample. Formula for the calibration curve and sample preparation was adapted from Alma – Tihana Miletić's undergraduate thesis. All samples were prepared and measured in the triplicate (3x3)

3.5 Data processing and statistical analyses

The data processing was done using MS Excel program and further statistical analyses were done with GraphPad Prism 8.4.3. I analysed data for control (CTRL) group (Eva Mihelec's undergraduate thesis) and my experimental group in GraphPad Prism 8.4.3. program. I analysed the data for experimental group with One – way ANOVA test and with post hoc Bonferroni's correction for multiple comparison. I analysed the data for CTRL and experimental group with Two – way ANOVA (Mixed model) test and with post hoc Bonferroni's correction for multiple comparison.

4. Results

The first step in the experiment was to measure the H_2O_2 concentration in the head homogenates of five genotypes of *D. melanogaster*: *wt, per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01}*. The measurements of H_2O_2 before the exposure to vMETH were done by Eva Mihelec previously in her experiment. I exposed the flies to an acute dose of vMETH and measured the concentration of H_2O_2 in the heads.

METH was administrated to 3x32 fruit flies of each genotype at the concentration of 75 µg using the FlyBong method. The next day I homogenised the heads of fruit flies and measured the oxidation of the fluorescent colour DHE in the samples. I calculated the level of H_2O_2 in the head homogenates of fruit flies from the values of DHE oxidation. The level of H_2O_2 reflects the redox status of *D. melanogaster* and the effect of METH on the redox status of flies.

Here I present two comparisons: first, between mock – treated control group and vMETH exposed, experimental group (Figure 6. A.), and second comparison between different genotypes in the experimental group (Figure 6. B.).

4.1 Circadian mutants of *Drosophila melanogaster* show different response to vMETH

The first aim was to determine whether the exposure of *D. melanogaster* to an acute dose of vMETH has an effect on the concentration of H_2O_2 in the head homogenate of the flies. The experiment showed that the exposure to vMETH leads to a change in the concentration of H_2O_2 in the heads of *D. melanogaster* of all genotypes, except of the *wt* flies (Figure 6. A) when compared to control (CTRL).

There is a significant decrease in the concentration of H_2O_2 in the heads of *per^{01}* and *tim^{01}* flies after the exposure to an acute dose of vMETH ($p < 0,0001$) compared to their CTRL. The opposite effect was observed in the heads of *cyc^{01}* and *Clk^{Jrk}* flies where, vMETH induces a significant increase in the concentration of H_2O_2 ($p = 0,0008$). Based on this result we were interested in comparing levels of H_2O_2 among different genotypes.

The amount of H_2O_2 differs in the head homogenates of flies exposed to acute dose of vMETH between *wt* and circadian mutants. cyc^{01} circadian mutants show the highest concentration of H_2O_2 among all five genotypes (Figure 6. B; the data shown in the Figure 6. B. is the same as the data shown for the experimental group in the Figure 6. A.).

There is a significant increase in the concentration of H_2O_2 in cyc^{01} circadian mutants ($p < 0,0001$) and reduction in tim^{01} ($p = 0,05$) compared to *wt*. This shows that vMETH exposure influences H_2O_2 concentration in a different way in *wt* flies compared to circadian mutants, and also between different circadian mutants.

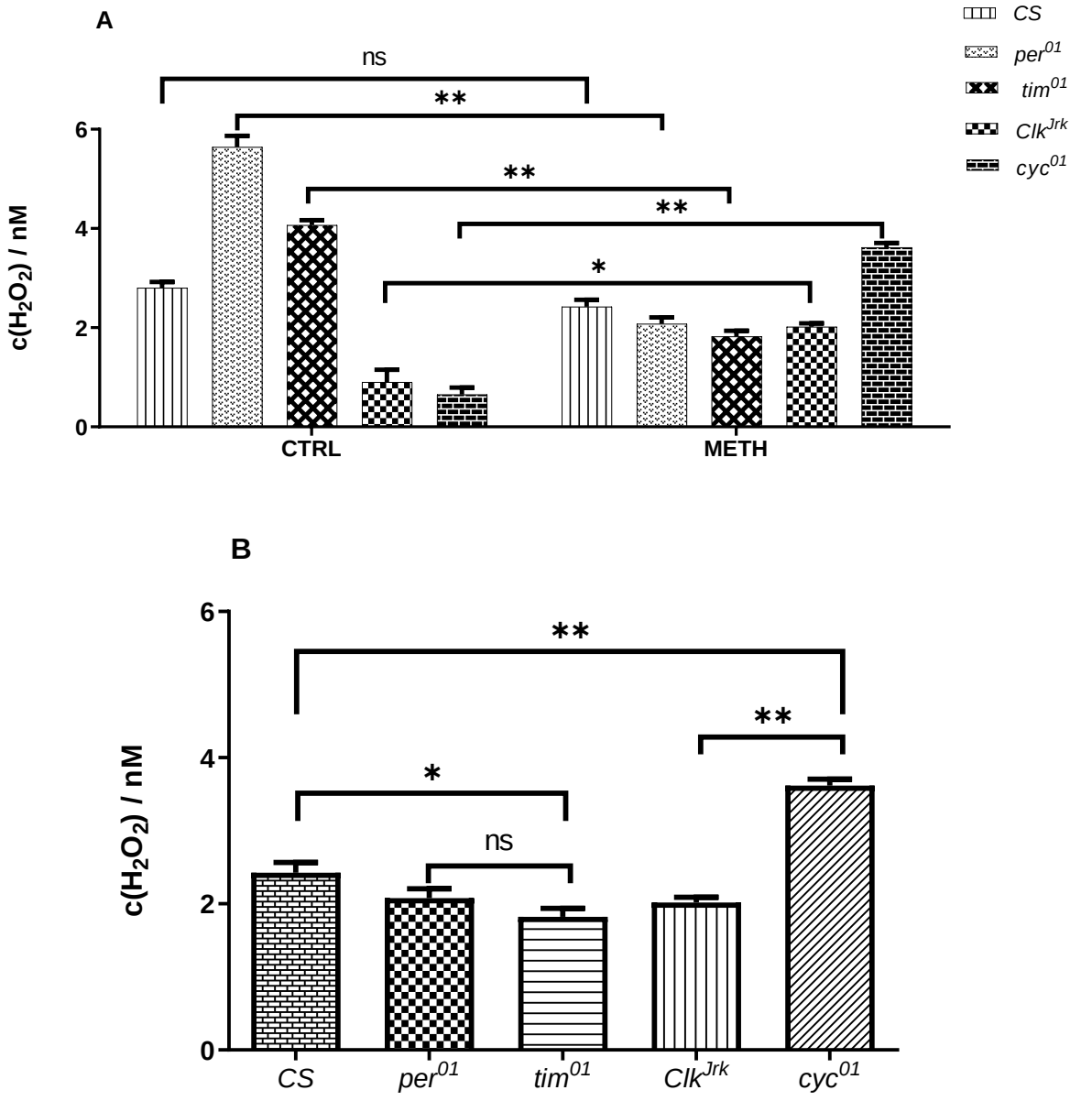

Figure 6. Changes in H_2O_2 concentration after vMETH exposure. A) The average concentration of H_2O_2, in nM, for control (CTRL) and the experimental (METH) group. The measurements for CTRL group were done by Eva Mihelec. The five genotypes that were used in the experiment are *wild type CS* strain (*Canton S*.) and mutants of four genes: per^{01} (*period*), tim^{01} (*timeless*), Clk^{Jrk} (*Clock*) and cyc^{01} (*cycle*). METH group was exposed to 75 µL vMETH using FlyBong while CTRL group was not exposed to vMETH. The concentration of H_2O_2 in heads of both groups was calculated from the values of fluorescent colour DHE RFU values. **B)** Differences in H_2O_2 concentration among *wt* and circadian mutant flies after the vMETH exposure based of the data shown in the experimental group of A). **ns** = no significance, * = significance p = 0,005 (figure 6. B) and p = 0,0008 (figure 6. A), ** = significance p < 0,0001. The statistical analyses were the Two – way ANOVA (Mixed model) test with post hoc Bonferroni's correction for multiple comparison in 6A and the One – way ANOVA test with post hoc Bonferroni's correction for multiple comparison in 6B.

5. Discussion

Circadian mutants per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01} have been selected for this study based on their behaviour response to repeated dose of METH.[17,18] The process of the sensitization is a form of neural plasticity that takes 10 hours to develop after the repeated exposure to METH and often develops in people addicted to METH.[19] There is sparse evidence for the connection of redox balance and the development of addiction, but there is evidence that redox balance is connected to the mechanism of neural plasticity.[20] Therefore, I have measured the concentration of H_2O_2 before and after the exposure of flies to vMETH.

The concentration of H_2O_2 in *wt* flies did not change significantly after the exposure to vMETH which is inconsistent with the previous research. Furthermore, there is a significantly smaller difference among five genotypes after the vMETH treatment than before the treatment. Before the exposure to vMETH there is a significantly higher concentration of H_2O_2 in per^{01} and tim^{01} flies compared to *wt* flies, and significantly lower in Clk^{Jrk} and cyc^{01} flies compared to *wt* flies. Exposure to vMETH significantly lowers the concentrations of H_2O_2 in per^{01} and tim^{01} circadian mutants, while the opposite effect is present in cyc^{01} and Clk^{Jrk} circadian mutants. After the vMETH treatment there are no significant differences in H_2O_2 concentration between tim^{01} and per^{01} flies, while cyc^{01} flies show significantly higher concentration of H_2O_2 than any other genotype of *D. melanogaster*.

Two mutants that showed the most promising results for the future research are cyc^{01} and tim^{01} but the other two cannot be excluded because of the complex molecular mechanisms of the circadian rhythm. tim^{01} and cyc^{01} mutants show the opposite effects after the exposure to vMETH. Lack of *tim* seems to be a protective factor, while lack of *cyc* seems to be a factor of susceptibility to the effect of METH.

The large line of evidence has established that the exposure to METH leads to the increased production of ROS in the neurons. Production of ROS causes numerous consequences on the neurons and brain such as neurodegeneration, disruption of brain blood barrier and the cell death of neurons, especially dopaminergic.[21] Research, like one identifying cytotoxic markers in the METH treated rats, shows that the increase in ROS leads to the shorter viability of cells.[1] *D. melanogaster* was used in a GWAS study with the aim to identify genes involved in the response to METH exposure, some of which turned out to be circadian genes.[2]

There is no significant difference in the concentration of H_2O_2 in the heads of *wt* flies after the exposure to vMETH (Figure 6. A). It would be expected to see a larger increase in the concentration of H_2O_2 after the vMETH exposure based on the previous research .[7] There are few of the possible reasons for the lack of significant difference: different concentrations of METH used in the research, different times of exposure, using acute or chronic regime of administration of METH or different mode of administration. In terms of effect on fly's behaviour, the dose of 75 μg used in this thesis is a moderate one, flies were exposed to an acute dose in the evening and METH was given in the form of aerosol. Thus, these conditions could explain no noticeable difference in the H_2O_2 production. Exposing flies to vMETH more than one time may show a significant difference. There are other ways of METH administration to flies, such as by injection or by food, that have shown different results in the production of H_2O_2.[6]

The research on the effects of METH on the production of ROS were done on different model organisms. Rats or mice could show different results concerning the production of ROS compared to *D. melanogaster*. This factor should be taken in the account because these animals have different metabolic and other molecular pathways that can influence the rate by which METH will be metabolised. In other model organisms H_2O_2 production may increase earlier or to a greater extent because of differences in the metabolism of METH. Furthermore, in most research with vertebras the effect of METH on the production of ROS was observed in the specific parts of the brain while in this thesis I used the homogenates of the whole brains of *D. melanogaster*.[6]

Another possible explanation for the lack of effect of a single moderate dose of vMETH in *wt* flies is that, in this genotype, all circadian genes are functional and that could result in the efficient removal or prevention of the increase of H_2O_2 production. In other genotypes, at least one of circadian genes is non - functional which has an effect on the whole circadian system. That is why, even without the vMETH exposure, *wt* flies show significantly different concentration of H_2O_2 compared to circadian mutants. Circadian genes interact and regulate each other's activity, so lack of one gene leads to the dysregulation of the whole system. Furthermore, *wt* flies could have shown more of the difference in the H_2O_2 concentration if the measuring was done earlier. In *wt* flies the redox balance returns to the normal state within 10 hours of exposure while in circadian mutants this process is dysregulated.

In this thesis I used the heads of flies because of the largest abundance of circadian genes in the brain.[22] Since H_2O_2 is an important molecule influencing neural plasticity and the redox balance, we were interested in the H_2O_2 concentrations in the heads of four circadian mutants of *D. melanogaster*. H_2O_2 can have effects on synaptic plasticity and influence the cellular mechanisms involved in learning and memory. The important correlation has been established between levels of H_2O_2 and the long term memory.[20] Furthermore, even a slight change in the concentration of the signalling molecules can have a significant impact on the synaptic plasticity. Reduced production or overproduction of H_2O_2 inhibits the formation of the long term memory.[20] The increased concentration of H_2O_2 caused a short – term depression of synaptic transmission which inhibited the formation of a long term memory. H_2O_2 can either potentiate or attenuate the long term memory through the effects on the concentration of intracellular Ca^{2+} levels.

ROS are modulators in the wide range of processes involved in the synaptic plasticity.[20] Those include activity of the protein kinases, phosphatases, transcription and translation factors, GTPase's and Ca^{2+} dependent enzymes. The NMDA (N-methyl-D-aspartate) receptor and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) inhibitor receptor – dependent signalling are one of the main processes included in the synaptic plasticity and are directly affected by ROS.

Four circadian mutants (*per*^{01}, *tim*^{01}, *Clk*^{Jrk} and *cyc*^{01}) showed a significant change in H_2O_2 concentration after the exposure to vMETH. There is a significantly smaller concentration of H_2O_2 in *tim*^{01} flies than in *wt* flies after the exposure to vMETH, while there is no significant difference between *wt* and *per*^{01} flies. This indicates that functional *tim* is more important for the regulation of H_2O_2 concentration after the exposure to METH than *per,* and emphasizes the importance of the molecular pathway in which *tim* is involved. As part of the circadian system TIM degrades quickly after the exposure to light and in this way transmits information about the environmental signals to the rest of molecular circadian timing system.[11] Furthermore, *tim* may be an important candidate gene when looking into the effects of METH on the ROS production, neurodegeneration and degradation of brain cells. Lack of *tim* and *per* shows, in this thesis, a protective effect attenuating the influence of METH on the molecular pathways involved in the production of ROS. Thus, inactivating *tim* may lead to decreased effects of ROS on neuronal plasticity. Detecting the molecular pathway in which *tim* is involved could help in the treatment of humans suffering from addiction to METH.

There is no significant difference in the concentrations of H_2O_2 between *wt* and *Clk*^{Jrk} flies after the exposure to vMETH, while there is a significant difference in H_2O_2 concentration between *wt* and *cyc*^{01} flies (Figure 6. B). *Clk*^{Jrk} flies show significantly lower concentration of H_2O_2 in the head homogenates compared to *cyc*^{01} flies. CLK and CYC proteins form heterodimers, so such difference in the H_2O_2 concentrations between *Clk*^{Jrk} and *cyc*^{01} flies is unexpected. It has been shown that CLK proteins do not cycle in the circadian fashion, while CYC do, which could be an influencing factor in my results.

cyc^{01} flies show the largest change in the concentration of H_2O_2 in the head homogenates after the exposure to vMETH, suggesting that in *wt* flies *cyc* has an important role in preventing the increase of the H_2O_2 concentration. It has been shown that when *cyc* is mutated the levels of glutathione are dysregulated which is consistent with our results where *cyc*^{01} shows the highest concentration of H_2O_2 after the exposure to vMETH. There is an evidence that *cyc* in flies, and its homologues gene *Bmal1* in mammals, have other significant interactions with proteins apart from those included in the regulation of circadian rhythm, so *cyc* could be an important candidate gene to investigate in relation to the molecules that regulate H_2O_2 and the processes influenced by H_2O_2.[12]

per^{01} and *tim*^{01} circadian null mutants showed a significant decrease in the H_2O_2 concentration after the exposure to vMETH. Before the vMETH treatment, *per*^{01} and *tim*^{01} had the highest concentrations of H_2O_2, while after the treatment they had the lowest concentrations compared to all other genotypes (Figure 6. A). It has been shown that the METH exposure increases the activity of *per* gene that is highly sensitive to the changes in the redox status. With the lack of *tim* or *per*, there is no increase in its activity so the concentration of H_2O_2 does not increase.[10] However, there is no significant difference in the H_2O_2 concentration between *per*^{01} and *tim*^{01} flies after the exposure to vMETH (Figure 6. B). This finding can be explained by the fact that those genes regulate their own transcription and TIM protein stabilises PER in the cytoplasm and enables its transport to the nucleus.[11] These two proteins interact and regulate each other's activity, so in the absence of one protein the function of the other is disrupted as well. It is then expected that their activity and the effect on H_2O_2 production is similar.

There is a significant increase in the concentration of H_2O_2 in the heads of *cyc*^{01} and *Clk*^{Jrk} flies after the exposure to vMETH. (Figure 6. A). The increase of H_2O_2 in the case of *Clk*^{Jrk} and *cyc*^{01} flies follows the prediction that METH increases the production of H_2O_2 in the heads of flies, because of disrupted mechanism of ROS removal. The data from this thesis shows that the biggest difference in the H_2O_2 production after the exposure to vMETH, is in *cyc*^{01} flies. Analysing the activity of this genes and its molecular pathways may help in the understanding of these changes. Different circadian mutants show different influence on the redox regulation, before and after, METH exposure. Better understanding of the mechanisms through which the circadian genes influence redox related genes may lead to the better understanding of their effect on the overall redox balance.

6. Conclusion

The *wt* genotype of *D. melanogaster* showed no difference in the H_2O_2 concentration after the exposure to vMETH, which comes as a surprise because of the research showing that METH increases the production of H_2O_2. Further research with the corrections of the existing FlyBong method should be considered. The circadian mutants showed changes in the H_2O_2 concentrations after the exposure to vMETH. per^{01} and tim^{01} flies showed a decrease in the concentration of H_2O_2 after the exposure to METH in comparison to *wt* flies. On the opposite, Clk^{Jrk} and cyc^{01} flies showed an increase in the H_2O_2 concentration after the exposure to vMETH compared to *wt* flies. After the exposure to vMETH tim^{01} mutants showed significantly lower concentrations of H_2O_2 compared to *wt* genotype, while cyc^{01} mutants showed a significantly higher concentrations of H_2O_2 compared to all other genotypes.

This experiment gives an insight into how the circadian genes regulate H_2O_2 balance after the exposure to vMETH. Based on our results, we propose that *tim* gene potentiates the effect of METH, while the circadian *cyc* gene may have an attenuating effect on the H_2O_2 production. Further research is needed to understand the mechanisms by which circadian genes regulate the H_2O_2 production after the METH exposure. Once the molecular pathways are better understood in *D. melanogaster* the knowledge can be used to develop new ways of treatments in humans because of significant genetic homology.

7. Literature

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