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SVEUČILIŠTE U RIJECI

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Laura Fućak

Utjecaj cirkadijalnih gena i metamfetamina na oksidativni status *Drosophile melanogaster*

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Abstract

Methamphetamine (METH) is a potent psychostimulant that causes relapsing substance addiction. Negative consequences of substance abuse are numerous and challenging to study in humans. Fortunately, effects of addictive drugs can be studied in model organisms, such as *Drosophila melanogaster* or fruit fly. Fruit flies are ideal for this research because their genome is sequenced and extensively studied. Methamphetamine causes elevated reactive oxygen species (ROS) production and disrupts their elimination, leading to oxidative stress. Circadian genes are partially responsible for ROS cycling and in *Drosophila* four core circadian genes are: *period* (*per*), *timeless* (*tim*), *cycle* (*cyc*) and *clock* (*Clk*). In this paper hydrogen peroxide (H_2O_2) concentration is followed after two exposures to volatilized METH in heads of five fly genotypes: *wild type Canton S.*, per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01}. Hydrogen peroxide concentration was measured with dihydroethidium (DHE) stain. Concentration of H_2O_2 is expected to increase with higher METH exposure, and differences in H_2O_2 amount should also be noticed between the genotypes. Results show different H_2O_2 levels among genotypes and this provides insight into the role of circadian genes in the regulation of the oxidative status of *Drosophila*. The obtained data shows that *wild type*, per^{01} and tim^{01} flies show decrease with first vMETH dose, followed by increase in H_2O_2 concentration after the second dose. Clk^{Jrk} and cyc^{01} mutants show steady increase in H_2O_2 concentration with each dose. This shows that irrespective of the mutation METH leads to the increase in the oxidative species and poses questions about the molecular mechanism involved in the regulation of redox state after psychostimulant exposure.

Keywords: *Drosophila melanogaster*, methamphetamine, circadian genes, oxidation state, hydrogen peroxide

Sažetak

Metamfetamin (METH) je snažan psihostimulans koji uzrokuje relapsirajuću ovisnost. Negativne posljedice ovisnosti o drogama brojne su i izazovno ih je izučavati na ljudima. No, učinci droga mogu se vrlo jednostavno inducirati u modelnim organizmima kao što je *Drosophila melanogaster* ili vinska mušica. Vinske mušice pogodni su organizam za izučavanje u ovakvim istraživanjima jer su njihovi geni sekvencirani i dobro proučeni. Metamfetamin uzrokuje povišenu proizvodnju reaktivnih kisikovih vrsta (ROS) i remeti njihovo uklanjanje, što dovodi do oksidativnog stresa. Cirkadijalni geni su djelomično odgovorni za kruženje ROS-a, a četiri ključna cirkadijalna gena u *Drosophile* su: *period* (*per*), *timeless* (*tim*), *cycle* (*cyc*) i *clock* (*Clk*). Ovaj rad prati koncentraciju vodikovog peroksida (H_2O_2) u glavama mušica nakon drugog izlaganja volatiliziranom METH-u. Oscilaciju koncentracija pratilo se u pet genotipa mušica (*wild type Canton S.*, per^{01}, tim^{01}, Clk^{Jrk} i cyc^{01}) pomoću dihidroetidija (DHE). U rezultatima se očekuju razlike u koncentraciji među genotipovima i porast koncentracije vodikova peroksida sa porastom broja izlaganja METH-u. Rezultati ukazuju na različite razine vodikova peroksida u različitim genotipovima i to pruža uvid u ulogu cirkadijalnih gena u oksidativnom statusu *Drosophile*. Nadalje, rezultati prikazuju da u *wild type*, per^{01} i tim^{01} mušica, koncentracija H_2O_2 pada nakon prve, a raste nakon druge vMETH doze, dok u Clk^{Jrk} and cyc^{01} mutanata koncentracija H_2O_2 raste nakon svake doze vMETH-a. To ukazuje da bez obzira na mutaciju, METH uzrokuje porast oksidativnih vrsta i postavlja pitanja o molekularnim mehanizmima uključenim u regulaciju redoks stanja nakon izlaganja psihostimulansima.

Ključne riječi: *Drosophila melanogaster*, metamfetamin, crikadijalni geni, oksidativni status, vodikov peroksid

Content

1. Introduction

Drug addiction is a relapsing and complex mental illness with negative consequences for the abuser (1). Substance abuse is compulsive and creates a short-term euphoric feeling which causes dependance. According to 2021 world drug report (United Nations Office on Drugs and Crime) amphetamines are one of the most often abused illegal psychostimulants with 27 million users in the world, and their leading derivate is methamphetamine (METH). After the METH intake user feels a burst of energy and euphoria which lasts for several hours (half-life of METH is 10-12 hours), which is followed by agitation, aggression, hypertension, and other symptoms. High doses of METH can lead to hyperthermia (over 41℃), liver failure, heart attacks, seizures, and cerebrovascular hemorrhages (2) METH is clinically used to treat attention deficit hyperactivity disorder and narcolepsy (3), but its chronical abuse causes neurotoxicity and neuroplasticity.

Chronic METH consummation causes neurodegeneration by changing brain metabolism which is often region specific (4). METH increases dopamine (DA) and serotonin release by decreasing the levels of DA and serotonin transporters. Because of structure similarity between METH and DA, the drug easily enters neurons through dopaminergic transporters causing DA release by reverse transport through vesicular monoaminergic transporters into the cytoplasm (2).

METH in the cell interferes with numerous cellular processes that lead to decreased cell functioning and potentially the cell death. METH can induce oxidative stress, which then causes long-term damage to dopaminergic and serotonergic axon terminals. METH causes excitotoxicity by creating reactive nitrogen species which are partially responsible for glutamate overproduction. Process of excitotoxicity consists of excessive glutamate release, followed by intracellular calcium accumulation and proteolytic

enzyme activation which activates apoptosis and creates free radicals which damage the cell (2,5). Glutamate neurotransmission is increased by microglial activation, especially near the damaged dopaminergic and serotonergic axons. Activated microglia releases inflammatory cytokines, prostaglandins, reactive oxygen (ROS) and nitrogen (NOS) species that induce neuron damage (5). In cortical and subcortical brain areas the metabolism of glucose is abnormal (2). In addition, METH induces grey matter loss, hypertrophy of white matter and shrinkage of hippocampal volume (2). It also leads to apoptosis by creating caspase-3 molecules and activating Fas/FasL pathway (2,5).

1.1. Oxidative stress in the context of METH

Reactive oxygen species (ROS) cause oxidative stress, and the most common consequences are DNA, protein, and lipid damage and cell apoptosis. They are produced as part of essential metabolic processes or when cells are under stress. Hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), hydroxyl radicals (OH^-), alkoxyl radicals, and peroxyl radicals are just some of the ROS species. Because of their reactivity and negative effects, organisms have developed systems for their rapid elimination. For example, antioxidants counteract negative ROS effects, and they can be endogenous, such as enzymes superoxide dismutase (SOD) and catalase (CAT), or they can be obtained from food, like vitamins C and E, flavonoids, and carotenoids (6). While these defense mechanisms are mostly sufficient, they can be disrupted by exogenous toxins and stresses, such as METH abuse.

METH causes oxidative and nitrosative stress and disrupts antioxidant defense system (4). As mentioned above, METH abuse increases intra- and extracellular DA, and consequently damages DA axons due to dopaminergic catabolism that disrupts redox balance (5). DA gets oxidized followed by DA quinones formation. This leads to superoxide and hydroxyl peroxide

formation. There is evidence that METH induced extracellular DA release could generate oxidative stress in non-DA neurons trans-synaptically by overpowering extracellular SOD and CAT (7).

The role of METH in oxidative stress is further confirmed by the fact that it reduces the levels of protective cell antioxidant enzymes. By affecting ROS production and antioxidants enzyme scavenging system, METH enables excessive ROS circulation and formation of protein carbonyls and lipid peroxidation. Oxidation stress is further emphasized by discovering intracellular abnormalities such as: mitochondrial membrane potential decrease and microtubule degradation which leads to cytoskeletal collapse (2,5,7).

1.2. Why *Drosophila*?

Drug addiction and the neurobiological mechanisms associated with the development of addiction, such as neural plasticity and neurotoxicity, are commonly studied in laboratory animals (8) such as monkeys, rats, mice (2) and invertebrates, *Drosophila melanogaster*. In our research we used *Drosophila* because the molecular and cellular features of vertebrate and invertebrate nervous systems are similar, and the several genes involved in drug abuse are conserved (1). The entire genome of the fruit fly has been sequenced so mutations can be simply tracked and analyzed. Moreover, a large population is easily obtained because the cost of fruit flies upkeep is low, and they do not require a lot of space. Lastly, because fruit flies are invertebrates, the research using fruit flies does not require permits and is in agreement with the 3R principle (Reduce, Replace, Refine).

1.3. Circadian genes in *Drosophila*

Circadian rhythms are present in most organisms as an adaptation to the daily light: dark cycle. These 24-hour rhythms are controlled by cells named the circadian clocks in animals, plants, fungi, and prokaryotes. The

name circadian is derived from Latin "*circa diem*" which means about a day. One common feature in all circadian cells is that they use proteins to construct the circadian oscillators. Besides light, circadian cells use other environmental signals, such as temperature, food, and social interactions, to synchronize circadian oscillations that regulate metabolic, behavioral, and psychological response. Homologous circadian proteins have been described in eukaryotes indicating a common origin for eukaryotic circadian clocks (9,10).

In *Drosophila melanogaster* brain there are approximately 100,000 neurons and about 150 of them are circadian cells essential for overt circadian rhythmicity. In a genetic screen in the 1970s Konopka has identified the first *Drosophila* mutants with altered circadian rhythm. The mutation was in the gene that was named *period (per)*. Following this other circadian genes have been identified, in the 1990s, Shengal et al. have identified *timeless* (*tim*), Allada et al. *Clock* (*Clk*) and Rutila et al. *cycle* (*cyc*) genes (11). Together with *cryptochrome (cry), doubletime* (*dbt*), *shaggy* (*sgg*) and other regulation components, they make the base of negative transcriptional-translational feedback loop (12). Transcription-translation negative feedback loop means that the rhythmic gene transcription is regulated by the produced proteins. PER and TIM proteins form a dimer and accumulate in the cytoplasm during the day. Besides stabilizing PER in cytoplasm, TIM protein is also required for PER transport to nucleus. In the nucleus CLK and CYC proteins dimerize and bind to E-box elements of *per* and *tim* promotor genes and to activate their transcription. However, when the PER-TIM complexes enter the nucleus, they bind to CYC-CLK dimers and repress their own transcription through phosphorylation. The cycle starts again when there is enough CLK protein in the nucleus, which triggers CLK-CYC heterodimerization (10–13).

1.4. Circadian genes and oxidative stress relationship

Circadian rhythms, controlled by their genes, have an impact over various cellular functions. In *Drosophila* some of those functions are eclosion, locomotor activity, sleep, and metabolism (14). The disruption of the circadian rhythm, therefore, creates a cascade of changes in the biochemical metabolism of a cell.

Current information suggests that circadian genes regulate expression of proteins involved in cellular oxidation stress response. For example, the circadian rhythm directs ROS protective cell response by controlling antioxidant enzymes activity (SOD, Glutathione S-transferase, CAT). Krishnan et al. found that elevated carbonylation of CAT, a H_2O_2 scavenging enzyme, in per^{01} mutants leads to reduced activity of CAT. They also demonstrated that fruit flies are least susceptible to oxidative stress during the late-night phase because high PER levels protect them from oxidative protein damage, and they are the most susceptible during the late-light phase because of the low levels of PER proteins. Furthermore, they noted faster H_2O_2 accumulation in per^{01} flies and as a result, higher oxidative damage. This suggests that *period* gene is extremely important for maintaining oxidative balance in *Drosophila* and supports the connection between ROS, circadian gene and health and lifespan (15).

In this thesis we have analyzed the oxidative status in *Drosophila melanogaster* after second volatilized METH (vMETH) administration by measuring H_2O_2 levels. The H_2O_2 was measured after single and double vMETH exposure in *wild type (wt)* flies and four genotypes carrying null mutations in circadian genes *per*, *tim*, *Clk* and *cyc*.

2. Aims

The main aim of this thesis is to determine if different *Drosophila melanogaster* genotypes influence the oxidation state in flies that were exposed to repeated volatilized METH dose. If the genotype of *Drosophila melanogaster* influences the redox regulation, then the concentration of hydrogen peroxide will vary depending on the genotype.

Additionally, we wanted to compare if there is a significant difference in the baseline levels of H_2O_2 in different genotypes relative to levels after the second administration of vMETH. We expected that relative to the baseline levels vMETH exposed flies will have more H_2O_2 in their head tissue.

Similarly, we compared if there is a significant difference in H_2O_2 concentration between first and second administration of vMETH. Again, our expectation was that relative to the baseline and the first exposure the higher H_2O_2 level will be present in flies exposed to the second dose of vMETH.

3. Materials and methods

3.1. Fly strains

Drosophila melanogaster fly stock consisted of *wild type Canton S.* strain and circadian mutants per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01}. The assays were performed on male flies raised in 12-hour day: night intervals, at 25℃ and 70% humidity. The fly food was agar based and consisted of agar, sugar, tap water, molasses, dry yeast, propionic acid and nipagin.

3.2. Drug administration

The FlyBong is a drug administration setup that was used to distribute volatilized methamphetamine in this experiment. The platform consists of drug delivery part and activity monitoring part. The drug delivery part consists of a volatilization chamber, an air pump and a Gas Distribution Manifold, while the activity monitoring part consists of commercially available monitoring system and air delivery manifold (16).

Under CO_2 induced anesthesia male flies were separated using a light microscope. Males housed in cultivation tubes were individually collected with an aspirator and transferred to polycarbonate tubes with two small holes for air flow. On one end the polycarbonate tubes were filled with food mixture and sealed with parafilm to prevent starvation and dehydration and on the other end they were connected to the dispenser. Dispenser has equalized methamphetamine aerosol distribution among 32 individually housed flies (maximum dispenser housing). Besides the dispenser, polycarbonate tubes were also connected to the *Drosophila* activity monitoring system (DAMS). DAM system measured the locomotor activity of the flies with a single vertical infrared light beam placed in the middle of polycarbonate tubes. The assembled apparatus was then connected to the drug delivery part of FlyBong via clamped rubber tubes.

75µL of 10 mg/mL methamphetamine hydrochloride (METH-HCl) ethanol solution was pipetted through the central neck of the three-neck flask that served as volatilization chamber. The solution was then left for four to six hours to allow ethanol vaporization. This way the ethanol induced ROS production factor was eliminated as possible cause for H_2O_2 levels in collected samples. The flask was situated in a heating cap and after the flask was corked, the caps were turned on for eight minutes and warmed to 120℃ to allow METH vaporization. Afterwards the caps were turned off, the tube clasps were opened, and air pumps were turned on to pump METH aerosol to the flies through the dispenser for one minute. The first vMETH dose was administered at 9 AM and the second at 7 PM. All performed assays were done in triplicates of *Canton S.* strain and Clk^{Jrk} mutants and in duplicates of per^{01}, tim^{01} and cyc^{01} mutants.

3.3. Sample collection and hydrogen peroxide measurement

The sample tissue consisted of fly heads that were collected 14 hours after the second vMETH dose administration. The flies were collected and frozen at -20℃ for 30 minutes. Next, using a light microscope and scissors the heads were detached from the bodies and put in empty, weighted Eppendorf tubes. The full Eppendorf tubes were weighted again so that extraction buffer volume could be calculated. The extraction buffer used was PBSx1 (Phosphate Buffered Saline) with 0.1% TritonX-100 detergent for cell permeabilization. The buffer volume was calculated by formula:

$$5\ \mathrm{mg} : 300\ \mu\mathrm{L} = \mathrm{m} : \mathrm{x}$$

where m is the mass of fly head sample and x is buffer volume.
The sample-buffer homogenization was done with an electric blender. After homogenates centrifugation (30 minutes at 1400 rpm and +4℃) the supernatants were collected and used for H_2O_2 measurement.

To determine ROS amount in the samples dihydroethidium (DHE) stain was used. When DHE is added to the samples that contain hydrogen peroxide, it gets oxidized and forms ethidium (E^+). Ethidium is non-specific red fluorescent that can be detected by fluorescent-based techniques (17). To prepare 10 µL DHE solution, 50 µL of DHE in dimethyl sulfoxide solution (DMSO) and 5 mL of PBSx1 were pipetted into an aluminum foil wrapped Falcon tube. Wrapping was done to prevent DHE solution from photo oxidizing. 200 µL od DHE solution and 5 µL of each sample supernatant were pipetted in triplicates onto a 96 well black polystyrene microplate. One row was a control blank, meaning just DHE solution was pipetted to eliminate DHE error possibility while measuring. Immediately after pipetting, the microplate was covered in aluminum foil and incubated for 30 minutes at 37℃. Tecan Infinite pro 200 was used to measure fluorescence intensity at excitation wavelength of 480 nm and emission wavelength of 625 nm.

To calculate the exact amount of hydrogen peroxide in fly head samples, a calibration curve was needed. The calibration curve was made using DHE solution and known H_2O_2 concentrations and relative fluorescence unit (RFU) correction. The calibration curve used in this experiment was calculated in Eva Mihelec's undergraduate thesis.

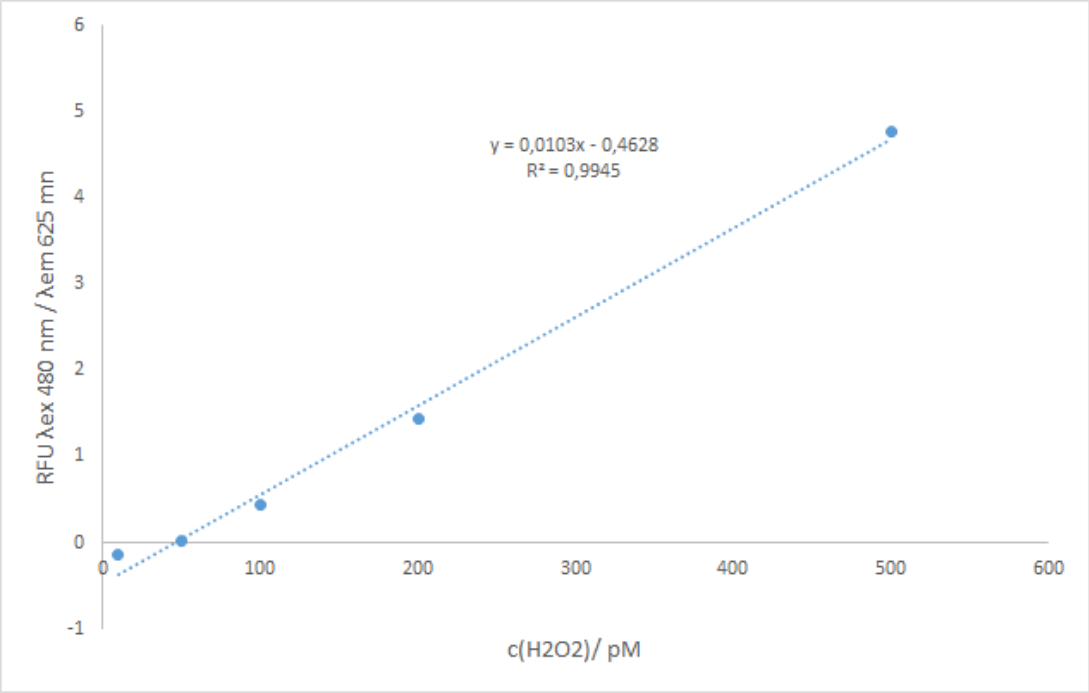

Figure 1. Calibration curve used for calculating H_2O_2 concentration levels in fly samples, created by Eva Mihelec in "*The Measurement of Redox States in Drosophila melanogaster Circadian Rhythm Mutants*".

3.4. Data analysis

Raw data analysis was done using MS Excel program. Additional statistical analysis and figure creating were done in GraphPad Prism 9.1.2. The measured experimental data was analyzed using Ordinary One-way ANOVA test and Bonferroni`s multiple comparisons test. Mixed-effects Two-way ANOVA analysis with Bonferroni`s multiple comparisons test was used for baseline, first and second dose comparison analysis in GraphPad Prism.

4. Results

To determine whether the genotype of *Drosophila melanogaster* influences the oxidation state in flies exposed to repeated vMETH dose, hydrogen peroxide levels were measured. There were five *Drosophila* genotypes used: *wt Canton S.*, per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01}. The flies were exposed to 75 µL of volatilized METH via FlyBong platform.

After the second vMETH administration, fly heads were homogenized and centrifugated to collect the supernatant for H_2O_2 level measurement. The H_2O_2 concentration was determined by oxidizing DHE stain that creates a red fluorescent dye whose amount corresponds to the H_2O_2 concentration.

Differences in measured H_2O_2 concentration among the tested genotypes can be seen in Figure 2. After the second vMETH dose administration, circadian mutant cyc^{01} had similar amount of H_2O_2 production as the *wt Canton S.* flies. per^{01} mutants had significant decrease of H_2O_2 concentration compared to both *wt* and cyc^{01} mutants (p= 0,0020 and p= 0,0030, respectively). Further H_2O_2 decrease relative to per^{01} mutants was observed in tim^{01} and Clk^{Jrk} (p=0.0311) mutants.

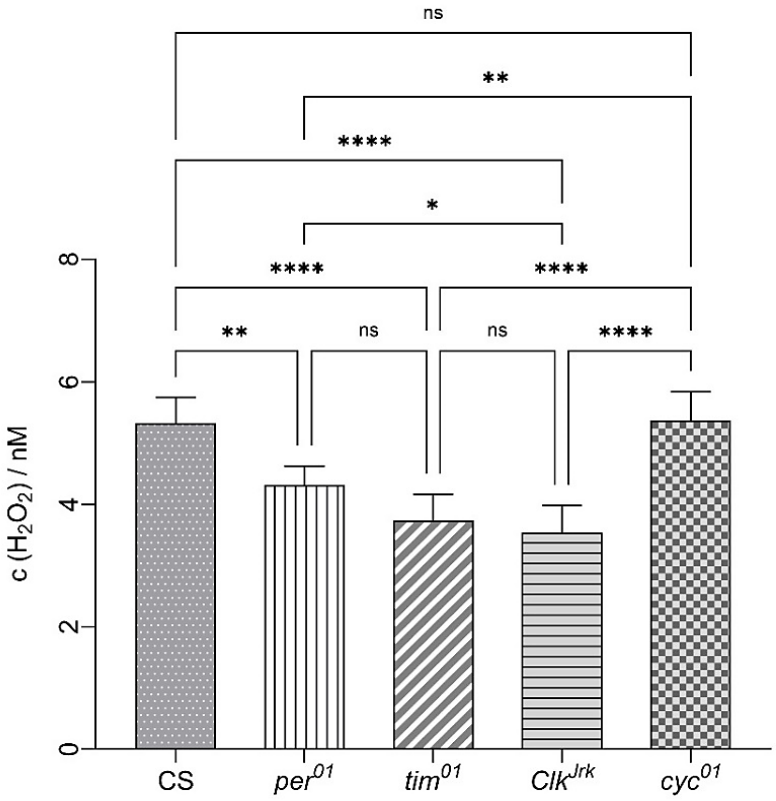

Figure 2. *Drosophila melanogaster* genotypes show varying H_2O_2 concentrations after two vMETH administration. 14 hours after second administration of 75µL of volatilized METH, the amount of H_2O_2 was measured in supernatants derived from fly heads. The amount of oxidized DHE corresponded to H_2O_2 concentration and was measured at 480 nm excitation wavelength and 625 nm emission wavelength. One-way ANOVA statistical analysis with Bonferroni's multiple comparisons test showed multiple significances: **ns**= no significance, ***** significance p=0.0311, ****** p<0.0020 (CS vs. *per*^{01}) and p<0.0030 (*per*^{01} vs. *cyc*^{01}), ******** p<0.0001.

Following this, I compared the difference between baseline, first and second dose of vMETH. The aim was to observe how H_2O_2 concentration changes over time in different genotypes. The first administration consisted of one 75 µL vMETH dose administered via FlyBong. Sample collecting and data processing of the baseline condition and after the first dose was done in the same way as described above. The data from the baseline and first dose

groups was provided by Eva Mihelec and Valentina Dukić, respectively, in their bachelor thesis.

In the *wt* flies first dose of vMETH led to the decrease of H_2O_2 ($p<0.0001$), while the second dose increased the concentration to the levels observed in the baseline condition (Figure 3). per^{01} and tim^{01} mutants displayed the similar dynamic of changes, decreasing the H_2O_2 concentration after the first dose ($p<0.0001$), and increasing after the second dose ($p<0.0001$), but with some difference from the *wt*. Amount of H_2O_2 concentration in per^{01} and tim^{01} mutants was similar as in *wt* flies at the baseline, but was lower relative to *wt* after the second dose ($p= 0.0004$ for per^{01}).

However, Clk^{Jrk} and cyc^{01} mutants showed a distinct pattern of change after the exposure to the vMETH. At the baseline Clk^{Jrk} and cyc^{01} flies had significantly lower amount of H_2O_2 then *wt* flies or per^{01} and tim^{01} mutants. The first dose of vMETH increased the amount of H_2O_2 ($p<0.0001$ for cyc^{01} and $p=0.0015$ for Clk^{Jrk}), unlike in other genotypes. We measured further elevation of H_2O_2 concentration after the second dose of vMETH ($p<0.0001$). Ultimately this resulted in similar amount of H_2O_2 in cyc^{01} and *wt* flies, while Clk^{Jrk} still showed lower amount of H_2O_2.

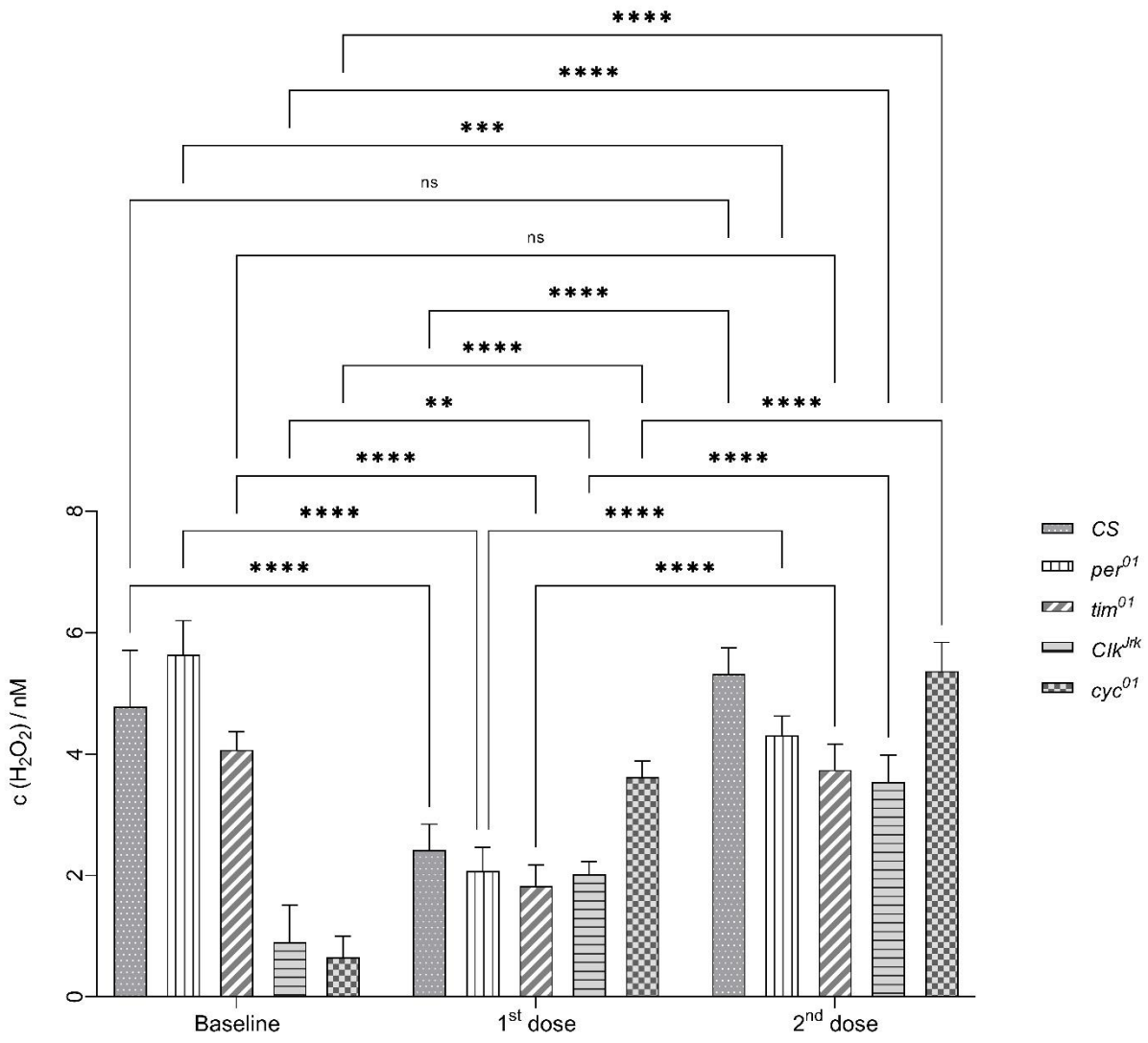

Figure 3. H_2O_2 concentrations in *Drosophila melanogaster* genotypes before and after one and two vMETH administrations. The first dose fly group has been exposed to one dose of 75µL volatilized METH, while the second dose (repeated) group has been exposed to two doses of 75µL volatilized METH via FlyBong. Time difference between first and second dose administration was 10 hours. 14 hours after drug administration, the flies were decapitated, and the heads were homogenized. The collected supernatants were used for H_2O_2 measurement based on the oxidation of DHE measured at 480 nm excitation wavelength and 625 nm emission wavelength. Data was analyzed with Mixed-effects Two-way ANOVA

with Bonferroni`s multiple comparisons test. **ns**= no significance, **** p= 0.0015, *** p= 0.0004, **** p<0.0001.

5. Discussion

The main aim in this thesis is to determine if different circadian genotypes of *Drosophila melanogaster* affect the redox state after second vMETH exposure. To determine this, we measured H_2O_2 concentration in the heads of *wt* strain and null mutants per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01} (16,18,19). We used heads, because clock genes are located in the fly brain and brain is predominantly affected by DA alterations after vMETH exposure (20). Our data indicates that oxidative state differs between different genotypes after second vMETH exposure, further emphasizing the connection between circadian genes and the regulation of the oxidative state.

Throughout the work, the measured indicator molecule for oxidative stress was hydrogen peroxide. H_2O_2 is a non-radical oxidant which controls signaling pathways in cells by oxidative modulation of sensitive proteins called redox switches. Many enzymatic pathways produce H_2O_2 directly or by producing superoxide radicals which get dismutated to a less harmful H_2O_2 (21). This poses a question if H_2O_2 it is the best ROS indicator for this experiment, or perhaps the circadian genes have a larger impact on regulation of other antioxidant enzymes. It is very probable that the obtained results would be vastly different if the focus was on another antioxidant pathway.

Methamphetamine disrupts oxidative state of organisms by ROS overproduction and antioxidant mechanism dysregulation. Oxidative stress affects numerous functions in different organisms; from muscle physiology disruption and actin reorganization in *Drosophila* to erythrocyte hexokinase inhibition in rabbits and cell death in plant species *Arabidopsis thaliana* and

Nicotiana benthamiana (22). The line of evidence about altered oxidative state due to oxidative stress persists among various model organisms (20). Previous research noted that METH interferes with other ROS linked pathways, like the Ca^{2+} homeostasis. Treating *Drosophila* flies with METH leads to increase in cytochrome P450 and downstream ROS formation (22). Increase in cytoplasmic Ca^{2+} causes mitochondrial membrane depolarization and disruption which generates large ROS amounts and leads to cell death. In METH treated fruit flies there is also a tenfold increase in glyceraldehyde-3-phosphate dehydrogenase (GAPHD) which supports the fact about increased glucose metabolism in fly brains (22).

Aforementioned consequences linked to ROS production have enticed numerous studies based on predicting and determining ROS amount, and hence, predicting disruption of organism's redox state. Using drug as a stressor also enables studying its toxic effects. In this thesis it is noticed that second exposure to vMETH causes significant difference in hydrogen peroxide levels among *wt*, per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01} genotypes. These results compared to the baseline and first exposure group give information about the dynamic of change in H_2O_2 amount that vMETH exposure causes in the fly brains. When discussing change of H_2O_2 concentration over time and dose increase, we observe two dynamics among the genotypes. H_2O_2 concentration in *wild type Canton S.*, per^{01}, and tim^{01} mutants decreases after the first dose and increases after the second, while in cyc^{01} and Clk^{Jrk} the increase is steady over time.

It is possible that the significant decrease in H_2O_2 concentration in *wt,* per^{01}, and tim^{01} is due to activation of antioxidant pathways. A possible explanation is that in *wt* flies all genes work properly, while in per^{01} and tim^{01} mutants ROS elimination pathways are not affected by gene mutation. So, when METH causes increase in ROS, cells respond with increase in CAT and SOD, which successfully eliminate ROS and other radicals. SOD

transforms superoxide ($O_2^{\cdot -}$) to H_2O_2 and CAT neutralizes it to water and oxygen. Additionally, in antioxidants pretreated flies vMETH does not affect CAT or SOD activity, which indicates that CAT activity is induced by antioxidants degradation (23). However, after the second METH dose, levels of H_2O_2 drastically increase which suggests that the activated enzymes are ineffective in eliminating produced H_2O_2.

per^{01} and *tim*^{01} flies show similar change in H_2O_2 concentration over time, which is expected considering that their proteins cycle in phase and that their lack impacts similarly on the cell. The H_2O_2 difference in *tim*^{01} baseline and second exposure mutants is statistically insignificant, which suggests that *tim* gene is not crucial for H_2O_2 creation or elimination.

Interestingly, when comparing baseline and vMETH treated *per*^{01} flies it is noted that treated flies have lower peroxide concentration. This implicates that lack of cycling *per* mRNA is a protective factor and that vMETH stimulates activity of *per*. This finding is inconsistent with previous research that suggests high increase in protein carbonylation, lipid oxidation and inability to neutralize ROS, but the difference could be that they used hyperoxia and H_2O_2 to create oxidative stress, while we used vMETH. Higher susceptibility to ROS damage is noted in *per*^{01} mutants due to carbonylation damage of CAT and its diminished activity (15,24). However, low *per* transcripts are present in *per*^{01} mutants, which implies partial *per* activation by low levels of CLK-CYC dimer, and this could explain decrease in H_2O_2 (25). Therefore, involvement of *per* genes in oxidation state of *Drosophila* should be further discussed and additional research is encouraged.

Regarding *cyc*^{01} and *Clk*^{Jrk} dynamics, they have lower H_2O_2 concentration at the baseline than other genotypes, and it increases with each vMETH dose. The similar H_2O_2 increase pattern is expected, since CLK and CYC proteins cycle in phase and their genes form a dimer in

transcriptional-translational feedback loop. Their lack appears to be a susceptibility factor, possibly because their lack causes antioxidant pathway inactivation or the antioxidant enzymes simply cannot remove the excessively accumulated ROS. Another research shows that vMETH administration causes increased transcription of circadian genes as well as increased CAT, but lowered SOD activity, which solidifies the explanation for their high ROS level (23). However, while Clk^{Jrk} mutants have a steady increase in H_2O_2 over time, even after the second vMETH dose they still have lowest H_2O_2 concentration among the second dose genotypes. This also leaves room for further research of the protective properties of mutation in Jrk allele in *Clock* gene. In contrast, cyc^{01} mutants show highest susceptibility to vMETH induced H_2O_2 production. Ultimately, after the second vMETH administration, the H_2O_2 concentration in cyc^{01} mutants and *wt* flies is similar. Their higher susceptibility indicates that the mutation in cyc^{01} either has no impact on ROS elimination or that the mutation is insignificant in terms of elevated H_2O_2 production. The later however, is inconsistent with the fact that the concentration significantly rises with each METH dose (18,19).

Observing H_2O_2 amount in flies after first and second vMETH dose clearly shows an increase in accumulation of H_2O_2 in all five genotypes, indicating that circadian mutants are similarly prone to the accumulation of H_2O_2. These results support current predictions that second METH administration causes severe consequences in *Drosophila* at the level of redox regulation.

6. Conclusion

This work supports the prediction that circadian genes regulate vMETH induced change in the oxidative state in *Drosophila melanogaster*. The baseline levels of H_2O_2 differ between *wt* flies and four genotypes with mutations in the circadian genes. The research shows that *Drosophila* mutants with a different gene disruption react differently to two dose vMETH exposure. per^{01} and tim^{01} mutants have similar dynamics as *wt Canton S.* flies. After the first METH dose they show decrease of H_2O_2, while its concentration increases after the second dose. This may indicate that vMETH stimulates strong antioxidative response, which leaves space for further research. The dynamics of cyc^{01} and Clk^{Jrk} mutants are somewhat different. Over time, cyc^{01} and Clk^{Jrk} show steady increase in H_2O_2 concentration which could indicate that their disruption inhibits H_2O_2 removal, possibly by antioxidant enzymes inactivation. Our findings still leave many unanswered questions about the molecular mechanisms involved in the regulation of redox state after psychostimulant exposure.

This data can be used to expand knowledge about redox regulation in the development of addiction using *Drosophila melanogaster* as model organism. Because of ethical questions studying addiction on humans is difficult, therefore results gathered in this work can be used to study METH caused oxidative damage in human metabolism. Furthermore, it can be used for assessing neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, that that are linked with ROS generation.

7. Literature

1. Wolf FW, Heberlein U. Invertebrate models of drug abuse. J Neurobiol. 2003;54(1):161–78.

2. Krasnova IN, Cadet JL. Methamphetamine toxicity and messengers of death. Brain Res Rev [Internet]. 2009;60(2):379–407. Available from: http://dx.doi.org/10.1016/j.brainresrev.2009.03.002

3. Highfill CA, Baker BM, Stevens SD, Anholt RRH, Mackay TFC. Genetics of cocaine and methamphetamine consumption and preference in Drosophila melanogaster. PLoS Genet. 2019;15(5):1–24.

4. Downey LA, Loftis JM. Altered energy production, lowered antioxidant potential, and inflammatory processes mediate CNS damage associated with abuse of the psychostimulants MDMA and methamphetamine. Eur J Pharmacol [Internet]. 2014;727(1):125–9. Available from: http://dx.doi.org/10.1016/j.ejphar.2014.01.032

5. Halpin LE, Collins SA, Yamamoto BK. Neurotoxicity of methamphetamine and 3,4-methylenedioxymethamphetamine. Life Sci [Internet]. 2014;97(1):37–44. Available from: http://dx.doi.org/10.1016/j.lfs.2013.07.014

6. Fanjul-Moles ML, López-Riquelme GO. Relationship between oxidative stress, circadian rhythms, and AMD. Oxid Med Cell Longev. 2016;2016.

7. Cubells JF, Rayport S, Rajendran G, Sulzer D. Methamphetamine Neurotoxicity Involves Vacuolation of Endocytic Organelies and Dopamine-Dependent Intracellular Oxidative Stress. J Neurosci April 1994, 74(4) 2260-2271. 1967;74(April):2260–71.

8. Nestler EJ, Hope BT, Widnell KL. Drug addiction: A model for the molecular basis of neural plasticity. Neuron. 1993;11(6):995–1006.

9. Yang Z, Sehgal A. Role of molecular oscillations in generating behavioral rhythms in Drosophila. Neuron. 2001;29(2):453–67.

10. Hardin PE. The circadian timekeeping system of Drosophila. Curr Biol. 2005;15(17):714–22.

11. Dubowy C, Sehgal A. Circadian rhythms and sleep in Drosophila melanogaster. Genetics. 2017;205(4):1373–97.

12. Panda S, Hogenesch JB, Kay SA. Circadian rhythms from flies to human. Nature. 2002;417(6886):329–35.

13. Blau J. The Drosophila circadian clock: What we know and what we don't know. Semin Cell Dev Biol. 2001;12(4):287–93.

14. Wijnen H, Young MW. Interplay of circadian clocks and metabolic rhythms. Annu Rev Genet. 2006;40:409–48.

15. Krishnan N, Davis AJ, Giebultowicz JM. Circadian regulation of response to oxidative stress in Drosophila melanogaster. Biochem Biophys Res Commun. 2008;374(2):299–303.

16. Filošević A, Al-Samarai S, Andretić Waldowski R. High throughput measurement of locomotor sensitization to volatilized cocaine in drosophila melanogaster. Front Mol Neurosci. 2018;11(February):1–12.

17. Zhang Y, Dai M, Yuan Z. Methods for the detection of reactive oxygen species. Anal Methods. 2018;10(38):4625–38.

18. Dukić V. The effect of methamphetamine on the redox status of Drosophila melanogaster. 2020;21(1):1–9.

19. Mihelec E. Mjerenje redoks statusa cirkadijalnih mutanata Drosophile melanogaster Eva Mihelec The Measurement of Redox States in Drosophila melanogaster Circadian Rhythm Mutants. 2020;

20. Mcdonnell-Dowling K, Kelly JP. The Role of Oxidative Stress in Methamphetamine-induced Toxicity and Sources of Variation in the Design of Animal Studies. Curr Neuropharmacol. 2017;(January):300–14.

21. Antunes F, Brito PM. Quantitative biology of hydrogen peroxide signaling. Redox Biol [Internet]. 2017;13(April):1–7. Available from: http://dx.doi.org/10.1016/j.redox.2017.04.039

22. Sun L, Li HM, Seufferheld MJ, Walters KR, Margam VM, Jannasch A, et al. Systems-scale analysis reveals pathways involved in cellular response to methamphetamine. PLoS One. 2011;6(4).

23. Filošević A. BEHAVIORAL AND GENETIC CHARACTERISTICS OF PSYCHOSTIMULANT-INDUCED NEURONAL PLASTICITY IN DROSOPHILA MELANOGASTER. 2018; Available from: https://urn.nsk.hr/urn:nbn:hr:193:851086

24. Krishnan N, Kretzschmar D, Rakshit K, Chow E, Giebultowicz JM. The circadian clock gene *period* extends healthspan in aging *Drosophila melanogaster*. Aging (Albany NY) [Internet]. 2009;1(11):937–48. Available from: https://doi.org/10.18632/aging.100103

25. Glossop NRJ, Lyons LC, Hardin PE. Interlocked feedback loops within the Drosophila circadian oscillator. Science (80-). 1999;286(5440):766–8.