

Differentiation of acute and chronic exposure to METH on FAGEs accumulation and behavioral phenotypes

Klasan, Ana

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UNIVERSITY OF RIJEKA
DEPARTMENT OF BIOTECHNOLOGY
University Master's program
"Drug Research and Development"

Ana Klasan

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Mentor: dr.sc. Rozi Andretić-Waldowski

Co – mentor: dr.sc. Ana Filošević Vujnović

SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU
Diplomski sveučilišni studij
"Istraživanje i razvoj lijekova"

Ana Klasan

Diferencijacija akutnog i kroničnog izlaganja METH-u na akumulaciju
fAGEs te bihevioralni fenotip

Diplomski rad

Rijeka, 2023.

Mentor: dr.sc. Rozi Andretić-Waldowski

Ko – mentor: dr.sc. Ana Filošević Vujnović

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In front of the Committee:

1. prof. dr. sc. Miranda Mladinić Pejatović
2. prof. dr. sc. Mladen Merćep
3. dr.sc. Ana Filošević Vujnović

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Abstract

Methamphetamine (METH) and cocaine (COC) are often used recreational drugs that target dopaminergic synaptic transmission. Repeated exposure to a specific drug that causes increased motor-stimulant response is termed behavioral sensitization. In *D. melanogaster*, behavioral sensitization can be measured and induced with METH and COC. Dopamine has a role in changes included in neural plasticity after drug intake, such as behavioral sensitization. Furthermore, it determines various behaviors, including amount of sleep and locomotor activity. It increases levels of oxidative stress and affects accumulation of fluorescent advanced glycation end products (fAGEs). Based on these findings, we were interested to explore how different duration of feeding with METH affects motor activating effects of volatilized METH (vMETH) and volatilized cocaine (vCOC), amount of locomotor activity and sleep, and biochemical indicators of oxidative stress such as fAGEs and hydrogen peroxide (H_2O_2). Feeding with METH did not influence changes in locomotor activity after vCOC and vMETH administration using FlyBong. However, we did observe that locomotor activity and response to psychostimulants decreases with age. Furthermore, results suggest that neural changes caused by METH are governed by different mechanisms to those that control neural changes influenced by COC. Flies pretreated with METH exhibited decreased activity and increased sleep through the period of fourteen days. Furthermore, we measured levels of fAGEs and H_2O_2 in the head of flies. Measurements showed decrease of H_2O_2 and increase of fAGEs through the period of fourteen days. This indicates possible activation of antioxidative defense which decreased H_2O_2 levels. Increase of fAGEs through fourteen days confirmed its role as indicators of ageing in *D. melanogaster*. Further studies are needed to elucidate the exact effect of oral METH feeding on behavioral and biochemical factors in *D. melanogaster*.

Keywords: *Drosophila Melanogaster*, methamphetamine, cocaine, behavioral sensitization, locomotor activity, Reactive oxygen species, oxidative stress, fAGEs

Sažetak

Metamfetamin (METH) i kokain (COC) su često konzumirane rekreativne droge, čiji mehanizam utječe na sinaptičku transmisiju dopamina. Uzastopno izlaganje specifičnoj drogi uzrokuje povećani motorički stimulirani odgovor kojeg se naziva bihevioralna senzitacija. METH i COC mogu inducirati bihevioralnu senzitaciju kod *D. melanogaster*. Dopamin ima ulogu u promjenama vezane uz neuralnu plastičnost, kao što je bihevioralna senzitacija, koje nastupaju nakon uzimanja droge. Također, ima ulogu u regulaciji količine spavanja, lokomotorne aktivnosti te utječe na akumulaciju fAGEs-a i količinu oksidativnog stresa. Uzevši u obzir prethodne činjenice, u ovome radu smo željeli istražiti kako različita dužina oralnog hranjenja METH-om utječe na motorno-aktivirajuće efekte volatiliziranog METH-a (vMETH) i volatiliziranog kokaina (vCOC), količinu lokomotorne aktivnosti i spavanja, te biokemijske indikatore oksidativnog stresa fAGEs i vodikovog peroksida (H_2O_2). Hranjenje METH-om nije utjecalo na promjene u lokomotornoj aktivnosti nakon administracije vCOC i vMETH putem FlyBong platforme. Međutim, uočili smo da lokomotorna aktivnost i odgovor mušica na psihostimulante opadaju sa starosti. Rezultati sugeriraju da su neurološke promjene koje uzrokuje METH regulirane mehanizmom koji nije povezan s mehanizmom koji kontrolira neurološke promjene nakon administracije COC-a. Nadalje, mušice koje su hranjenje METH hranom pokazale su smanjenu aktivnost i povećano spavanje kroz četrnaest dana. Nadalje, mjerili smo koncentraciju fAGEs-a i H_2O_2 u glavama mušica. Mjerenja su pokazala opadanje koncentracije H_2O_2 i porast koncentracije fAGEs-a kroz četrnaest dana. Pad u koncentraciji H_2O_2 možemo opravdati aktivacijom antioksidativne obrane organizma nakon izlaganja METH-u. Povećanje koncentracije fAGEs-a daje potvrdu prethodnim istraživanjima koja sugeriraju da je fAGEs indikator starosti mušica. Potrebno je još studija da bi se saznao točan efekt oralnog hranjenja METH-om na bihevioralne i biokemijske faktore kod *D. melanogaster*.

Ključne riječi: *Drosophila Melanogaster*, metamfetamin, kokain, bihevioralna senzitacija, lokomotorna aktivnost, reaktivne kisikove vrste, oksidativni stres, fAGEs

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1. Introduction

Methamphetamine (METH) is a highly addictive and powerful central nervous system (CNS) stimulant (1). In clinical practice, it is used as treatment for attention deficit hyperactivity disorder and narcolepsy (2). However, it is mostly known as a recreational drug. METH enhances the release of the monoamine neurotransmitters, such as serotonin, dopamine, and norepinephrine (1). Similarly, another CNS stimulant cocaine (COC) has a mechanism that also targets dopaminergic synaptic transmission. It inhibits the reuptake of neurotransmitters at serotonergic, dopaminergic, and noradrenergic synapses (2).

Compulsive use of the drugs such as METH and COC, despite adverse consequences, is termed addiction (3,4). Addiction and the underlying mechanisms associated with the development of addiction are often studied in laboratory animals, which also include *Drosophila melanogaster* (2). Similar genetic background with humans and control over environmental factors enables precise administration of COC or METH to *D. melanogaster* and makes it a great model for studying various effects of psychostimulants (2). Flies are also a great example of the 3R (Replacement, Reduction and Refinement) principle. Additionally, their neurotransmitters are the same as in mammals and they have similar mechanisms of neurotransmitter storage, release, and recycling (3). We used *D. melanogaster* to explore how different duration of oral METH administration will affect the motor activating effect of volatilized METH (vMETH) and volatilized cocaine (vCOC), locomotor activity and sleep, and biochemical indicators of oxidative stress such as fluorescent advanced glycation ends products (fAGEs) and hydrogen peroxide (H₂O₂).

1.1. Behavioral sensitization to psychostimulants

Sensitization is an enhanced response to stimulus, after repeated exposure to that same stimulus (5). Behavioral sensitization is defined as the increased motor-stimulant response that occurs with repeated exposure to a specific drug (5). Behavioral sensitization is often studied in laboratory animals as behavioral endophenotype commonly focused on the motor-activating effects of COC and METH and as an expression of neuronal plasticity (6). Behavioral sensitization can also be induced and measured in *D. melanogaster* providing the means to study the genetic basis of this phenotype and its relation to human addiction(6).

It was shown that flies develop locomotor sensitization (LS) to vCOC (6) and vMETH (7). Flies increase the amount of locomotor activity upon single psychostimulant administration (sensitivity(SENS)), indicating the action of psychostimulants on the brain areas that control locomotor activity. Activity is measured as the number of times that an individual fly crosses the midline of the glass tube in which it is housed in one min (6). LS is defined as an increase in locomotor activity of an individual fly, after the first administration (compared to baseline) combined with a further increase following the second administration (compared to the first) (6).

In *D. melanogaster*, behavioral sensitization is regulated by the circadian genes *period (per)*, *Clock (Clk)*, *cycle (cyc)*, and *doubletime (dbt)*. These genes were shown to modulate drug-induced plasticity (8). The mutants for these circadian genes were exposed to vCOC and their locomotor activity was measured (6). The results show that for flies to develop LS to vCOC, functional *per*, *Clk* and *cyc* genes are required. That suggests that cocaine induced neuronal plasticity is modulated by the circadian clock (6). It is suggested that dopamine (DA) has a role in long-lasting changes included in neuronal plasticity after drug intake (9). Other than its role in neuronal plasticity, DA signaling controls various behaviors in *D. melanogaster*, as in humans(10).

1.2. Dopamine signaling in *D. melanogaster*

Behaviors determined by DA signaling in *D. melanogaster* include olfactory learning, memory, sleep amount, baseline locomotor activity and behavioral responsiveness to stimulus (10). The sleep and wake cycle are regulated by DA and genes involved in dopaminergic signaling. In flies DA levels have direct link to the amount of sleep; higher DA levels are associated with less sleep, and lower DA with more sleep. These results suggest that DA has a role in arousal in the brain of a fly. Arousal is evident as an increase or change in behavior. When flies are exposed to psychostimulants, such as METH and COC, their DA signaling is increased leading to decreased levels of sleep (10). DA undergoes modulation after administration of psychostimulants (11). Disruption in DA transport and signaling is responsible for various neuropathological conditions including addiction. The reason for that is that DA affects neuronal plasticity. After administration of drugs, which affect DA metabolism, DA leads to behavioral changes that characterize addictive behavior. Furthermore, the change in DA signaling influences accumulation of fAGEs (11).

1.3. Advanced glycation end products

Advanced glycation end products (AGEs) are heterogenous group of molecules (12). These diverse molecules have a common characteristic which is the presence of lysine residue in their structure. Over twenty different types of AGEs have been identified. Altogether they can be divided into two groups: fAGEs and nonfluorescent AGEs (13). fAGEs formation in fluids and tissues can be measured as fluorescence in the area around an excitation wavelength of 379 nm and an emission of 440 nm (14).

AGEs are formed in the Maillard reaction (MR). Electrophilic carbonyl sugar groups react with amino acids free groups and create Schiff base. The rearrangement of Schiff base leads to the formation of Amadori product.

Schiff base and Amadori product can irreversibly act with peptides and form compounds that can undergo further reactions of oxidation, dehydration, and polymerization to form various types of AGEs (12,13).

Increased AGEs production is involved in aging (12), diabetes (14), some types of cancer and development and progression of degenerative, cardiovascular, and neurological diseases (15). Other than endogenous production, AGEs can be exogenously ingested through food and their formation can be affected by environmental factors such as smoking. Studies with twins have shown that AGEs formation was 26% affected by environmental factors, but genetic effect was accounted for 74% of variance in AGEs levels (15).

1.4. Influence of psychostimulants on fAGEs

The formation of fAGEs in *D. melanogaster* is influenced by dopaminergic signaling (DA) (11). It was shown that DA signaling increases fAGEs levels *in vivo*, after the administration of psychostimulants METH or COC. METH enters the presynaptic neuron and monoaminergic vesicle, causing their release. Furthermore, COC prevents the uptake of DA into the presynaptic neuron by blocking the monoaminergic transporters. However, METH causes more intracellular disruption of the DA metabolism compared to COC (11). That was shown by the fact that there is a higher increase in the amount of fAGEs formation after METH feeding, compared to COC feeding. These changes in fAGEs formation under the influence of the dopaminergic signaling are likely connected with disrupted oxidative balance (11).

Disrupted oxidative balance indicates state of elevated oxidative stress which promotes formation of fAGEs (16). After the Maillard reaction, glycated proteins can undergo glycooxidation reactions that include reactive oxygen species (ROS) and form various types of AGEs. When formed, they exert oxidative stress through interaction with receptors for the advanced glycation end products (RAGE). RAGE are transmembrane multifunctional

immunoglobulin superfamily of proteins. When AGEs bind to RAGE extracellular domain, they activate a cascade of intracellular signaling events which leads to the production of ROS and inflammatory cytokines. That results in further upregulation of RAGE, cellular proliferation, increased oxidative stress and cell apoptosis. AGEs alone are considered inflammatory agents and oxidative stress promoters. Their interaction with RAGE initiates a harmful cycle of oxidative stress (16).

1.5. Oxidative stress and Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) is a term for family of oxidants derived from molecular oxygen (17). They include both oxygen radicals, such as superoxide ($O_2^{\cdot-}$), hydroxyl (OH^{\cdot}), peroxy (RO_2^{\cdot}), and hydroperoxyl (HO_2^{\cdot}) radicals, and some nonradical oxidizing agents, such as H_2O_2 , hypochlorous acid (HOCl), and ozone (O_3), which can convert easily to into radicals (18). They have the ability to undergo reactions of reduction and oxidation, form oxidative macromolecules and influence redox signaling and biological function. When oxidative stress is not present, ROS are present in low concentrations, and they react with specific targets thereby regulating a physiological redox signaling (17). They are included in enzymatic reactions, mitochondrial electron transport, signal transduction, activation of nuclear transcription factors, gene expression, and the antimicrobial action of neutrophils and macrophages (18). In the presence of oxidative stress, increased levels of ROS react with unspecific targets and cause disruption in redox signaling (17), and oxidative damage on numerous cellular components, including DNA, lipids, and proteins (18).

H_2O_2 is the most stable ROS important in signaling pathways. Signaling pathways are regulated by reaction of H_2O_2 with proteins called redox switches (18). They are proteins harboring redox sensitive moieties, such as cysteine residues or metal centers. The oxidation controls their activity, while the oxidation state is controlled by the concentration of H_2O_2 in the

cell. One example of redox switches are thiol proteins. They have cysteine residues with low pKa, which thiolate at physiological pH. Thiolates are more reactive towards H₂O₂. Only the most reactive switches will sense H₂O₂ in low concentrations, but at higher concentrations less reactive switches will also react (18).

1.6. The effects of METH on DA metabolism and ROS creation

METH enhances the release of DA and induces oxidative stress in DA terminals (19). This effect is achieved by interaction of METH with three molecular targets: the synaptic vesicles and vesicular monoamine transporter type-2 (VMAT-2), the plasma membrane DA transporter (DAT), and the monoamine oxidase A (MAO-A) enzyme. METH inhibits VMAT2 which regulates cytosolic DA homeostasis and its release. It impairs the activity of DAT, which selectively takes up extracellular DA within DA terminals. Additionally, METH inhibits the function of MAO-A that has a role in the oxidative deamination of DA. Due to high pKa of METH, the acidic environment changes towards basic. That enhances the free diffusible release of nonpolar DA (19).

All these influences of METH lead to increased levels of DA in extravesicular space in DA axons (19). Those DA molecules, due to inhibition of MAO-A, cannot be deaminated. Then DA autooxidates and large amounts of reactive aldehyde 3,4-dihydroxyphenylacetaldehyde (DOPAL) are created. DOPAL has high oxidative potential and quickly reacts with proteins, especially targeting those with oxidation-prone domains. Additionally, oxidation of DA leads to the creation of ROS species such as H₂O₂ and superoxide radical. They cause damage on proteins, nucleic acids and lipids and change intracellular signaling (19).

1.7. The effect of COC on DA metabolism and ROS creation

Like METH, COC effects are mediated via enhancement of extracellular DA by inhibition of DAT (20). Additionally, COC oxidative metabolites that have a direct role in the development of toxicity and addiction are: norcocaine, its derivatives and formaldehyde. They directly influence the creation of ROS and electron transport (21).

The effect of COC on ROS was confirmed in the study where male rats were injected intraperitoneally with COC (22). The injection was done either once or in repeated doses for 10 days. The levels of H₂O₂ were increased in mice brains after COC administration. In addition to increased H₂O₂ levels, enlarged lipid peroxidase levels were observed (22).

The effect of COC on accumulation of ROS, antioxidative enzymes and fAGEs was also measured in *D. melanogaster* (23). Flies were exposed to one or two doses of vCOC in six hours intervals. Single exposure to vCOC resulted in increased levels of ROS and catalase (CAT). However, concentration of H₂O₂ and superoxide dismutase (SOD) did not change. After two doses of vCOC, levels were significantly increased for all four parameters. This shows that even one short exposure to vCOC is sufficient to disturb oxidative balance and to activate antioxidative defense. Additionally, these changes in redox regulation correlate with the development of LS. Based on this, it is suggested that oxidative events could have a role in events included in the drug induced neuronal plasticity (23).

Furthermore, in flies orally fed with COC for 24 or 48h increased CAT activity and fAGEs accumulation were observed (23). The levels of CAT after 24h were like those measured after 48h. However, increased fAGEs concentration is observed just after 48h indicating that fAGEs are latter indicators of increased oxidative stress (23).

2.Aims

Mechanisms of psychostimulants such as METH and COC directly target dopaminergic synaptic transmission, which consequently influences psychostimulants motor activating effects, locomotor activity, sleep and ROS and fAGEs formation (1,2,6,7). Based on these findings, the main aim of this thesis was to determine how different duration of METH oral exposure influences behavioral and biochemical indicators.

Our study had four sub-aims. The first one was to determine how different duration of METH oral exposure influences motor activating effects of vMETH.

The second sub-aim was to determine if different duration of orally administrated METH will cause changes in the nervous system that will influence the response to vCOC.

Since METH affects dopaminergic signaling which is directly linked to the regulation of sleep-wake cycle (10), our third sub-aim was to examine how the different duration of oral administration of METH affect the amount of activity and sleep.

The fourth sub-aim was to determine how different duration of oral feeding with METH influence the formation of fAGEs and H₂O as biochemical indicators of increased oxidative stress in the head of *D. melanogaster*.

3. Materials and methods

3.1. Fly strains

We used *wild type (wt) Canton S.* strain of *D. melanogaster* grown in bottles containing standard cornmeal/agar medium (sugar (7,9%), dry yeast (5.0%), agar (0,8%), cornmeal (7.2%), 15 mL of 10% anti-fungal agent methyl parahydroxybenzoate ($\geq 98\%$, Carl Roth) dissolved in 96% ethanol (GramMol) and 8 mL of propionic acid ($\geq 99.5\%$, Sigma Aldrich)) at 25°C, with 70% humidity on a 12h light/ 12h dark cycle.

3.2. Experimental design

CO₂ anesthesia and a microscope were used for transferring groups of 40 males from bottles into the plastic vials containing food. Half of the vials with flies contained food mixed with methamphetamine hydrochloride ($\geq 97.5\%$, Sigma Aldrich) in the concentration 0,5 mg/ml and other half was on regular food. Vials with flies were held in the incubator for either one, seven or fourteen days.

3.3. Sample preparation for H₂O₂ and fAGEs quantification

Flies were frozen at -20° C for 30 minutes and decapitated with dissection forceps under a microscope. Twenty heads were placed in Eppendorf tube in triplicate. To determine the weight of the sample tubes were weighed before and after the sample was added. Samples were mechanically homogenized and placed in an ice bath. Based on the samples weight, we calculated the required volume of extraction buffer (phosphate buffered saline (PBS) $\times 1$ + Triton X 0,1% (Sigma Aldrich)) using the equation $300 \mu\text{l} / 5 \text{ mg} = x \mu\text{l} / (\text{sample mass}) \text{ mg}$. NaCl, KCl, Na₂HPO₄, and KH₂PO₄, used for preparation of PBS $\times 1$ were of analytical grade. The calculated volume of extraction buffer was added to the tubes and samples were mechanically homogenized. The homogenates were centrifuged for 40

minutes at +4°C and 14 000 rpm. Supernatants were collected and used for further measurements.

3.4. fAGES and H₂O₂ quantification

For fAGES quantification, 4 µl of sample and 196 µl of Na₂HPO₄ buffer (0,2 M; pH 7.4) were pipetted in triplicate in a Thermo Fischer Scientific Nunclon 96 Flat Black microplate. Control samples consisted of 196 µl of Na₂HPO₄ buffer alone and were also pipetted in triplicate. Quantification was done on Tecan Infinite 200 PRO microplate reader with an excitation wavelength of 360 nm and an emission wavelength of 440 nm. Concentration of fAGES in the samples was calculated using a fAGES-BSA calibration curve.

To determine hydrogen peroxide (H₂O₂) concentrations, 5 µl of sample and 200 µl of 10 µM dihydroethidium (DHE, ≥95%, Sigma Aldrich) dissolved in PBS × 1 were pipetted in triplicate in a Thermo Fischer Scientific Nunclon 96 Flat Black microplate. DHE solution in PBS × 1 was used as control sample pipetted in triplicate since DHE is sensitive to light. The microplate was left to incubate for 30 minutes at 37 °C, covered with aluminum foil. Quantification was done on Tecan Infinite 200 PRO microplate reader with an excitation wavelength of 480 nm and an emission wavelength of 625 nm. The concentration of H₂O₂ in samples was determined using the calibration curve for DHE with known H₂O₂ (Sigma Aldrich) concentration.

3.5. Drug administration- FlyBong

The FlyBong is an assay used for the administration of volatile substances originally develop for cocaine hydrochloride and then adopt for methamphetamine hydrochloride (METH) (6). It has two main parts: a drug delivery and activity monitoring part [Fig 1]. The drug delivery part consists of volatilization chamber which is a three-neck flask connected to an air

pump and gas distribution manifold. The activity monitoring part consists of a manifold that delivers air to the monitoring system.

Flies were individually collected from vials and transferred into polycarbonate tubes with two small holes for air flow. One end of the tube was filled with food and sealed with parafilm to prevent starvation or dehydration of flies during the experiment. On the other end, tubes were connected to the dispenser for delivery of volatilized METH (vMETH) or volatilized cocaine (vCOC). Dispenser was connected to *Drosophila* activity monitoring system (DAMS), which measured the locomotor activity of individual flies based on the break of the infrared beam in the middle of tube for one minute. The activity monitoring part was connected to the drug delivery part via clamped rubber tubes.

COC and METH were volatilized in a three-neck flask positioned in a heating cap. The central neck was used for pipetting 75 μ l of 10 mg/ml ethanol solution of COC or METH. The solutions were pipetted four to six hours before administration so that ethanol would evaporate. Then, the flask was corked with glass plugs and heating caps were turned on for eight minutes. This gives time for drugs to vaporize. Afterward, heating caps were turned off, tube clasps were opened, and air pump were turned on so that drug aerosol could be delivered to flies through dispenser. After one minute air pumps were turned off. Drug administration was conducted at two time points during the day. vMETH was administered at 9:00 and 19:00, while vCOC at 9:00 and 15:00. The control group of flies was administered with warm air.

Data was analyzed on a population level. For a population data analysis, data were collected 10 minutes before and 10 minutes after the drug administration. The heating and drug delivery time were excluded from the analysis. The result was a calculation of average counts per minute for 32 flies in 10 minutes interval.

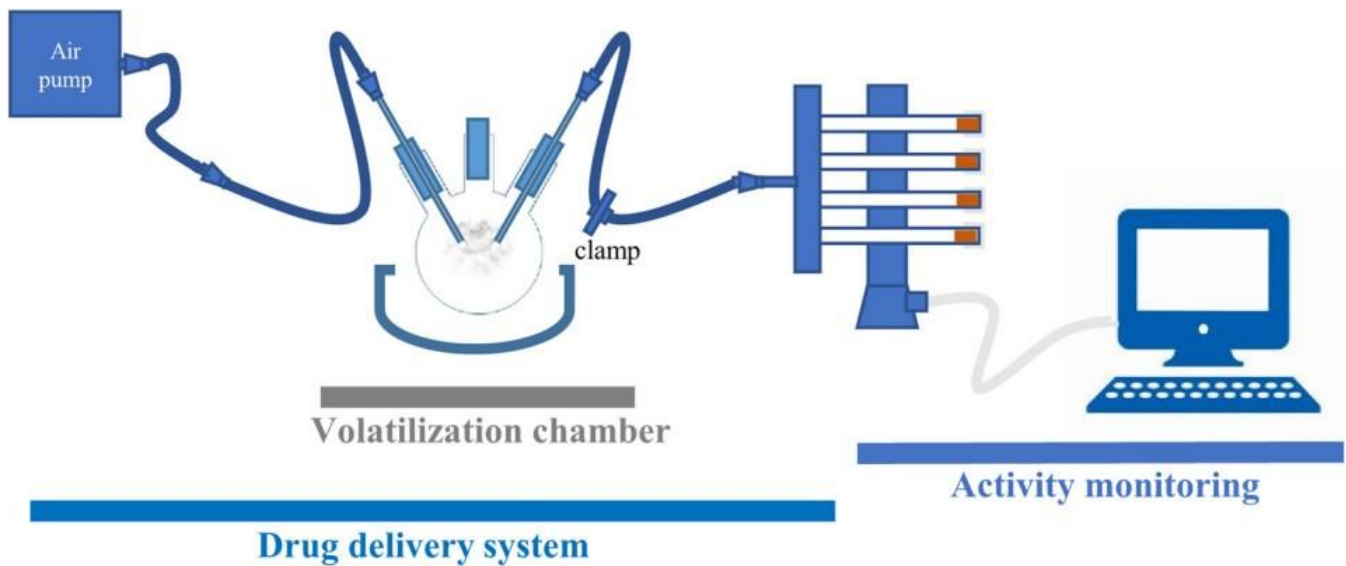


Figure 1. FlyBong platform for monitoring locomotor activity changes of *D. melanogaster* after administration of volatilized drugs (6).

3.6. Sleep and activity monitoring

Flies sleep and activity were monitored with DAM system (TriKinetics, USA). First, flies were collected under CO₂ anesthesia and individually transferred to glass tubes. One end of the tube was filled with food and sealed with parafilm, while the other end was sealed with a little cork. Glass tubes were placed in monitors that hold 32 tubes. Monitors were connected to the computer using PSIU9 Power Supply Interface Unit (TriKinetics, USA), and the data about the number of the infrared beam breaks per minute for each fly was captured and extracted using the DAMFileScan software. The raw data, saved as txt files, obtained from the DAM system were then analyzed using custom software (7).

Sleep is defined as a period of no movement within a five-minute interval and is quantified as the total duration of sleep over a 24-hour period. Locomotor activity is measured as the cumulative number of infrared beam breaks within a one-minute interval, during 24 hours. Measurements were

conducted for seven days in conditions of 12 h light/ 12 h dark and in conditions of constant dark.

3.7. Data analysis

The analysis of raw data was done in MS Excel program. GraphPad Prism 9.1.2 program was used for all figures and statistical analysis. Experimental data obtained from FlyBong experiments where baseline, first and second dose were compared, was analyzed using the ordinary one-way ANOVA with Tukey's multiple comparison test. The rest of the data was analyzed using two-way ANOVA test with Tukey's multiple comparisons test.

4. Results

4.1. Influence of different length oral exposure to METH on motor-activating parameters of psychostimulants

To determine how oral administration of METH affects motor-activating parameters of psychostimulants, we used FlyBong to expose flies to vMETH and vCOC. Prior the exposure to volatilized psychostimulants, flies were orally fed for one, seven and fourteen days with food mixed with METH. Control groups were fed with regular food and administered with hot air.

The motor-activating parameters were measured as locomotor activity based on the number of breaks of the infrared beam in the middle of the tube using one-minute resolution. The activity was measured 10 minutes before exposure (baseline) to vMETH and 10 minutes after exposure, and 5 minutes before and after exposure to vCOC. The exposure to volatilized psychostimulants was done two times. Data was shown at the population level where we compared the average population difference between flies fed with regular and METH mixed food and compared their baseline locomotion, and the change after the first and second dose.

4.1.2. Influence of METH feeding on vMETH exposure

METH feeding did not influence basal locomotor activity after one, seven and fourteen days of exposure (Fig. 2A). In both flies orally pretreated for one day with regular and METH mixed food, the first (1st) dose of vMETH increased locomotor activity (Fig. 2B). However, the activity of flies pretreated for seven and fourteen days was significantly lower after 1st vMETH exposure in comparison to those pretreated for one day (Fig. 2B). Similarly, flies pretreated for one day after second (2nd) exposure show increased activity (Fig. 2C), however flies pretreated for seven and fourteen days have significantly lower activity levels than those pretreated for one day (Fig. 2C). It is important to note that we did not observed influence of

pretreatment (control or METH feeding) or experimental conditions (hot air or vMETH) on changes in locomotor activity. However, we have shown that age decreases vMETH motor activating effects but there was no difference between METH pretreated and control flies.

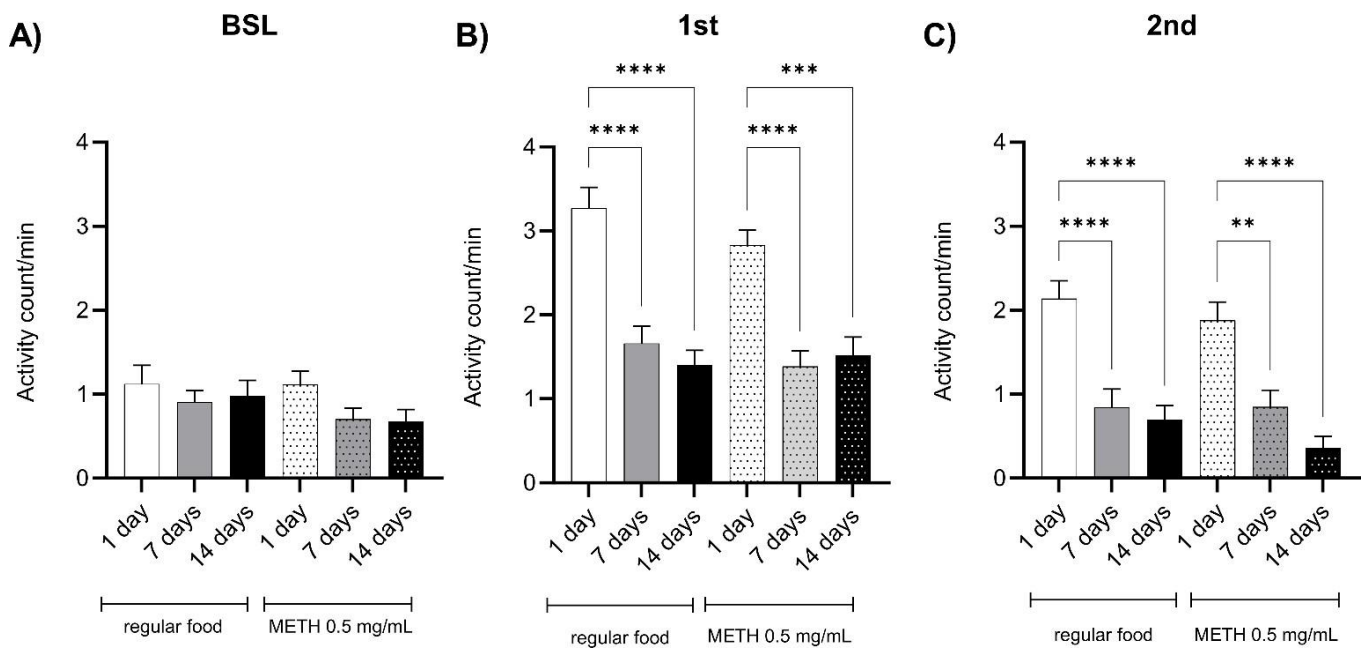


Figure 2. Effect of METH feeding on vMETH exposures. 75 μ L of vMETH administered to groups (n=32) of flies raised on regular and METH food in two doses (in 10h interval) via FlyBong, control groups were administered with hot air. Activity(count/min) was measured using DAMS. Locomotor activity of population is compared 10 minutes before and after vMETH administration to flies. Data is plotted as mean \pm SEM. Statistical test used was ordinary one-way ANOVA with Tukey's multiple comparison tests. **A**, Baseline (BSL) activity for flies exposed to METH and regular food for one, seven and fourteen days. **B**, Activity after first exposure to vMETH for flies exposed to METH and regular food for one, seven and fourteen days. ****: p<0,0001, ***: p=0,001. **C**, Activity after second exposure to vMETH for flies exposed to METH and regular food for one, seven and fourteen days. ****: p<0,0001, **: p=0,0035.

4.2.2. Influence of METH feeding on vCOC exposure

We wanted to investigate if the effect of METH feeding causes changes in the nervous system which influence the response to vCOC. Positive result would indicate overlap in the neural mechanisms that regulate changes in the brain after the administration of COC and METH. To determine that, we orally treated flies with METH for different length of time, as above, followed by the exposure to vCOC.

METH feeding influenced basal locomotor activity after seven days of exposure, where flies showed significantly lower activity (Fig. 3A). After 1st exposure to vCOC, flies pretreated for one day with regular food showed increased activity in comparison to flies pretreated for seven and fourteen days (Fig. 3B). Flies pretreated with regular food for seven days showed significantly decreased activity (Fig. 3B). For flies pretreated with METH after 1st exposure is observed gradual decrease in activity through the period of fourteen days. After 2nd dose, both flies pretreated with METH and regular food, show increased activity after pretreatment of one day and lower activity after pretreatment of seven and fourteen days (Fig. 3C).

In summary, it was shown that flies with age have weaker response to administration of psychostimulants (Fig. 3B, C). Furthermore, METH feeding did not influence vCOC administration based on the results of population analysis. With this approach, we have also shown influence of age on the decrease of vCOC motor activating effects, but without effect of the METH pretreatment.

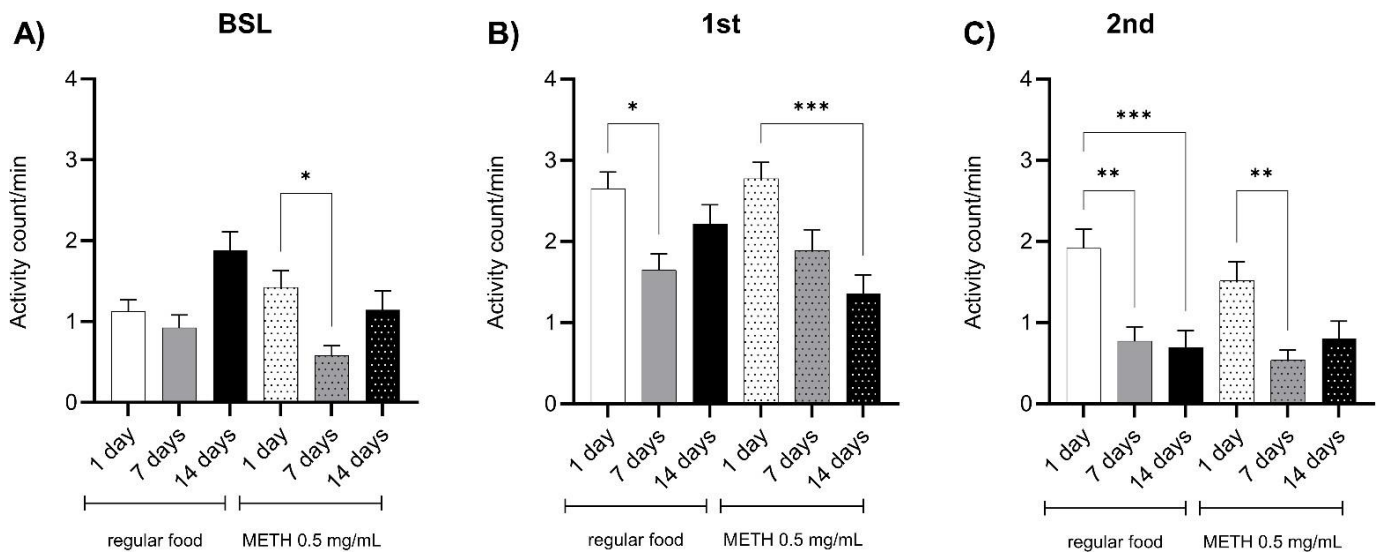


Figure 3. Effect of METH feeding on vCOC exposure. 75 μ L of vCOC administered to groups (n=32) of flies raised on regular and METH food in two doses (in 6h interval) via FlyBong, control groups were administered with warm air. Activity(count/min) was measured using DAMS. Locomotor activity of population is compared 10 minutes before and after vCOC administration to flies. Data is plotted as mean \pm SEM. Statistical test used was ordinary one-way ANOVA with Tukey's multiple comparison tests. **A**, Baseline (BSL) activity for flies exposed to METH and regular food for one, seven and fourteen days. *: p=0,0916 **B**, Activity after first exposure to vCOC for flies exposed to METH and regular food for one, seven and fourteen days. ***: p=0,0001, *: p=0,0187. **C**, Activity after second exposure to vCOC for flies exposed to METH and regular food for one, seven and fourteen days. ***: p=0,0004, **: p=0,0011.

4.3. Influence of different length oral exposure to METH on the activity and sleep

To investigate changes in the amount of activity and sleep after different length of oral exposure to METH, we fed flies with food mixed with METH for either one, seven or fourteen days. Their locomotor activity was afterward monitored in Drosophila Activity monitoring system (DAMS) and during that time they were fed with regular food. Control flies were fed only with regular food for the entire duration of the experiment. The activity was expressed as number of times that fly crossed infrared beam within a one-minute interval during 24 hours. Sleep amount was expressed as a period of no movement within a five-minute interval and was quantified as the average duration of sleep per hour during 24-hour period. Measurements were conducted in conditions of 12 h light/ 12 h dark (LD) and in conditions of constant dark (DD).

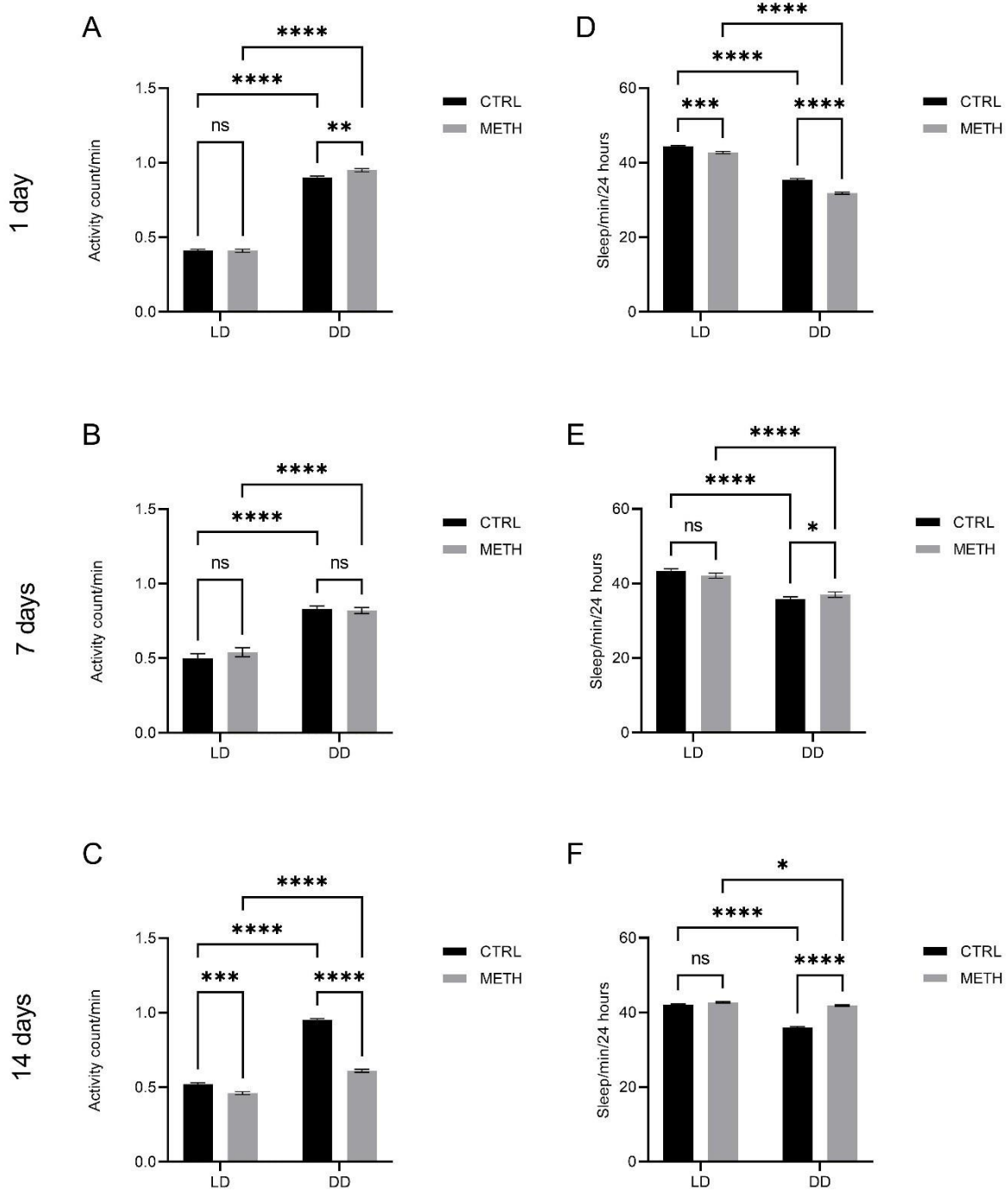


Figure 4. METH increases sleep and decreases activity in DD conditions through fourteen days of oral exposure to 0,5 mg/mL METH. Activity (count/min) and sleep (min/24hours) of flies (n=32 for each group) using DAMS in conditions of 12 h light/ 12 h dark (LD) and in conditions of constant dark (DD). Data is plotted as mean \pm SEM and analyzed with Two-way ANOVA with with Tukey's multiple comparisons test. **A**, Activity of flies pre-exposed to METH food

for one day. ****: $p < 0,0001$, **: $p = 0,0023$. **B**, Activity of flies pre-exposed to METH food for seven days. ns: no significance, ****: $p < 0,0001$. **C**, Activity of flies pre-exposed to METH food for fourteen days. ****: $p < 0,0001$, ***: $p = 0,0001$. **D**, Sleep of flies pre-exposed to METH food for one day. ns: no significance, ****: $p < 0,0001$, ***: $p = 0,0003$. **E**, Sleep of flies pre-exposed to METH food for seven days. ns: no significance, ****: $p < 0,0001$, ***: $p = 0,0004$. **F**, Sleep of flies pre-exposed to METH food for fourteen days. ns: no significance, ****: $p < 0,0001$, *: $p = 0,0393$.

There is no difference in activity in LD conditions between flies pretreated with METH and regular food for one day (Fig. 4A). However, in DD conditions their activity is significantly increased, and flies pretreated with METH show higher activity than those pretreated with regular food (Fig 4A). Flies pretreated with METH for one day exhibit decreased sleep, in both LD and DD conditions in comparison to flies on the regular (Fig. 4D). Sleep is overall decreased in DD conditions for both groups of flies (Fig. 4D).

No differences in activity are observed, in both LD and DD conditions, between flies pretreated with regular and METH food for seven days (Fig. 4B). It is only observed the effect of DD conditions, where flies have increased activity compared to LD (Fig. 4B). Furthermore, both groups of flies exhibit similar sleep amounts in LD conditions (Fig. 4E). In DD conditions, flies pretreated with METH for seven days show increased sleep amount in comparison to those pretreated with regular food (Fig. 4E).

Flies pretreated with METH for fourteen days, exhibit decreased activity in both LD and DD, compared to those pretreated with regular food (Fig. 4C) and this difference is the largest in DD conditions (Fig. 4C). Flies pretreated for fourteen days with METH show increased sleep in DD, however the increase in sleep is not as large as is the decrease in activity (Fig. 4F).

In summary, activity is increased (Fig. 4A, B, C) and sleep is decreased (Fig. 4D, E, F) in DD conditions for both group of flies. The effect

of METH pretreatment is evident on sleep in DD after 7 days of treatment, and on the amount of activity and sleep after 14 days of treatment (Fig. 4A, B, C).

4.4. Influence of oral exposure to METH on biochemical parameters of oxidative status

Since in previous experiments we did not observe the effect of METH feeding on behavioral changes, we were interested to see if it will influence biochemical parameters. It is known that the administration of METH leads to increased oxidative stress and production of ROS (17,20). Furthermore, formation of fAGEs is one of the biochemical indicators of extended oxidative stress (23). To determine how oxidative status in heads of flies is affected by acute and chronic exposure to METH, the amount of H₂O₂ was measured after one, seven and fourteen days of feeding on food containing 0,5 mg/ml METH. Flies fed with regular food were used as control (CTRL).

There is no statistically significant difference in the H₂O₂ levels in head homogenates after one, seven and fourteen days of METH feeding compared to CTRL (Fig. 5). However, significant decrease in H₂O₂ levels was observed after fourteen days in both groups compared to one day exposure. Measurements show gradual decrease in the H₂O₂ amount through fourteen days of exposure in METH fed flies and controls (Fig. 5).

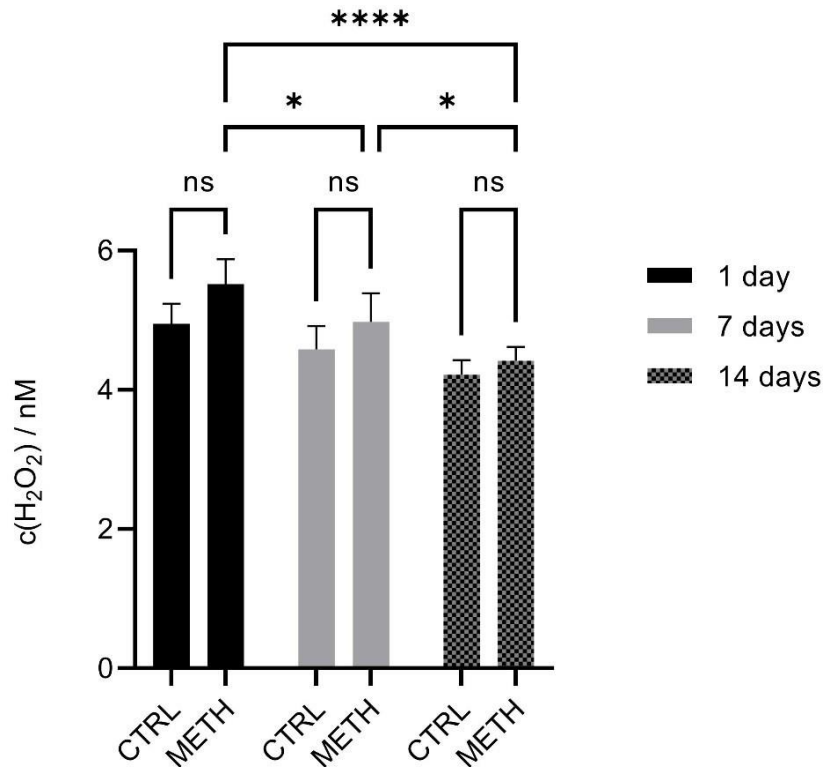


Figure 5. H₂O₂ formation decreases with time regardless of the presence of METH. Concentration of H₂O₂/nM in head homogenates after one, seven and fourteen days of exposure to food with 0,5 mg/mL METH (n=20 for each group). The peroxide concentration was measured as fluorescence intensity at an excitation wavelength of 480 nm and emission wavelength of 625 nm. **ns**: no significance, ********: p<0,0001, *****: p=0,0269, mixed effects two-way anova analysis with Tukey's multiple comparison tests.

To test if acute and chronic exposure to METH influences fAGEs formation, we measured fAGEs in head homogenates after one, seven and fourteen days of oral feeding with METH 0,5 mg/ml.

There is no statically significant difference between fAGEs levels in METH samples and related CTRL samples (Fig. 6). However, the fAGEs levels were gradually increased through the period of fourteen days (Fig. 6). The levels were increased in both CTRL and METH samples, regardless of the exposure to METH (Fig 6).

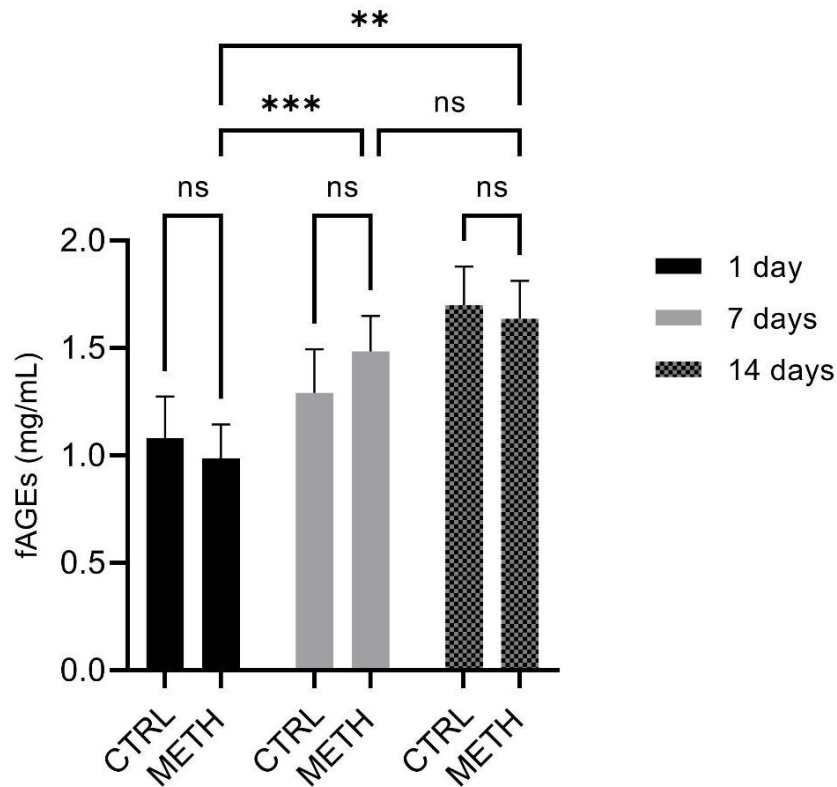


Figure 6. fAGEs formation increases with time regardless of the presence of METH. Concentration of fAGEs mg/mL in head homogenates after one, seven and fourteen days of exposure to food with 0,5 mg/mL METH (n=20 for each group). The fAGEs concentration was measured as fluorescence intensity at an excitation wavelength of 360 nm and emission wavelength of 440 nm. **ns**: no significance, *******: p=0,0008, ******: p=0,0011, mixed effects two-way ANOVA analysis with Tukey's multiple comparison tests.

5. Discussion

In this thesis we were interested to see how different duration of METH feeding affects behavioral and biochemical factors. In the first experiment we examined how different duration of oral treatment with METH influences vMETH motor activating parameters. We exposed flies to METH through food for one, seven and fourteen days and then we administered vMETH to them using FlyBong. We tracked the locomotor activity of flies at baseline, and after 1st and 2nd exposure. Our results suggest that METH did not influence basal locomotor activity after one, seven and fourteen days. Furthermore, we show that METH feeding did not influence changes in locomotor activity and that decrease in activity through fourteen days is consequence of aging.

Based on previous studies, where flies develop locomotor sensitization to vMETH (7) we expected to see increase in locomotor activity after longer period of METH administration. The lack of expected METH effect on locomotor activity could be due to insufficient concentration of METH in food. Furthermore, control groups of flies administered with hot air have shown sensitization to it. This indicates that our platform FlyBong should be validated and optimized. However, we observed the major effect of aging without influence of METH feeding itself or its duration. As the flies get older, they become less sensitive and unable to develop LS.

We also wanted to determine if oral METH feeding mechanisms are involved in regulating the response to vCOC. This would indicate similar neural mechanisms that regulate response to vMETH and vCOC administration. To examine this, we exposed flies fed with METH for either one, seven or fourteen days to vCOC via FlyBong protocol.

We did not confirm our expectation that METH feeding will influence response to vCOC administration. After 1st and 2nd dose, flies show higher activity after one day METH pretreatment and lower activity after pretreatment of seven and fourteen days. These results suggest that neural changes caused by METH are governed by different mechanisms to those

that control neural changes influenced by COC. However, our results confirm that vCOC has motor activating influence, which was shown in previous studies (7). Furthermore, we observed that flies with age have weaker response to administration of psychostimulants and that age decreases vCOC motor activating effects without the influence of METH feeding itself or its duration.

METH enhances DA release that is shown to decrease sleep amount (19). Previous studies have confirmed that flies exposed to METH have increased DA levels which makes them sleep less (10). Our aim was to see if different duration of oral feeding with METH has a long-term effects on the amount of sleep and activity in flies. Measurements were conducted after METH pretreatment in conditions of 12 h light/ 12 h dark (LD) and in conditions of constant dark (DD).

Flies treated with METH for one day, exhibited increased activity in DD conditions in comparison to those treated with regular food. We suspect that this is due to combination of DD conditions and METH activating effect. Unexpectedly, this difference is not observed after seven days where flies show no statistical significance between flies pretreated with METH and regular food. However, after fourteen days flies treated with METH exhibit decreased activity compared to their related controls. It is possible that long-term consumption of METH causes neurodegenerative changes that cause decrease in locomotor activity.

In DD, flies pretreated with METH for one day show decreased sleep when compared to control. However, those pretreated for seven and fourteen days exhibit increased sleep compared to control. This suggest that METH increases sleep in DD conditions through the period of fourteen days. This was not expected because previous studies have suggested that one of the main effects of METH feeding to *D. melanogaster* causes overall increased arousal mostly shown in a decrease in average sleep time (24). Additionally, different regulation of sleep and activity levels indicate that molecular

mechanism that controls sleep is separated from mechanism that regulates activity.

In our previous experiments the effect of METH oral treatment on behavioral changes in flies was not observed in all experiments. However, we did observe the ageing effect. Then, we were interested to see if oral pretreatment of different length affects biochemical parameters of oxidative stress such as ROS and fAGEs. We measured the levels of H₂O₂ in flies heads after one, seven and fourteen days of exposure to METH through food. Our data indicates that ROS production in flies' heads decreases over fourteen days regardless of the presence of METH.

Similar experiments showed different results which suggested that administration of psychostimulant METH increases the amount of created ROS, thus increasing levels of oxidative stress (19). Acute administration of METH to rats and mice induced ROS production in brain (25,26). The increase in ROS production had concentration and time dependent kinetics (25). Based on this, we were surprised to see that in our experiment production of ROS in flies fed with regular food did not differ from those that were fed with METH food. However, previous research was conducted on rodents whose metabolism and molecular pathways by which METH is metabolized can differ from those in *D. melanogaster*. Therefore, the results may vary.

The possible reason for decrease in ROS production in flies fed with METH is that organism adapts to METH exposure over time and compensates its original effect of increased ROS production. When METH activates ROS production, organism responds and activates defense which include enzymes SOD and CAT (27). SOD converts O₂^{·-} to H₂O₂, which can then be neutralized by CAT (27). Another explanation is that METH influences metabolism and it reduces the need for food consumption in METH treated flies by 60-80% (28). Studies have shown that METH decreases triglycerides and glycogen which are main energy storage leading to negative caloric balance (28). It is possible that long term METH

administration in our experiment resulted in disrupted metabolism and consequently redox balance, which led to limited consumption of METH food. Since our results focus only on one indicator of oxidative stress, H₂O₂, in future experiments focus can be on other ROS species, which might produce a different outcome.

Furthermore, our results suggest that decrease in ROS levels are not accompanied by decrease in fAGEs levels. On the contrary, fAGEs levels have increased through the fourteen days regardless of the presence of METH. Increased fAGEs formation over fourteen days in both CTRL and METH was expected, because fAGEs are proven to be indicators of ageing in *D. melanogaster* (29,23). Although we did not expect that flies fed with METH will show no differences in fAGEs amount compared to CTRL flies, this finding agrees with the observation that we noticed no difference in hydrogen peroxide between treated and untreated flies.

Previous studies showed opposite results, where DA signaling altered by psychostimulant administration led to modification of extracellular proteins and increased formation of fAGEs (11). However, administration of METH in our experiment did not influence the formation of fAGEs. It is possible that METH concentration of 0,5 mg/mL was not sufficient or that METH was degraded in food over the period of fourteen days. Difference in results could be because previous experiments were focused on conducting measurements on whole body (11), while our measurements of fAGEs were in head of *D. melanogaster*.

In summary, our results have shown that age affects biochemical parameters of oxidative stress and behavioral changes. Unfortunately, the effect of METH oral administration of different duration was not significant as we expected. To obtain more clearly results, for future experiments we suggest that they include changing the food during the pretreatment and using higher METH concentrations. Additionally, flies could be starved prior the exposure to oral METH, to be sure that they consume the food.

6. Conclusion

We explored the effect of the different duration of METH feeding on subsequent behavioral and biochemical parameters. The effect of METH feeding on motor-activating effects of vCOC and vMETH was not observed. However, we demonstrated that flies with age exhibit weaker response to administration of psychostimulants by decreasing its motor activating effects. Flies with age become less sensitive and fewer number exhibits changes in locomotor activity after the administration of vMETH and vCOC. Furthermore, results suggest that neural changes from METH feeding are governed by different mechanisms to those that control neural changes influenced by COC administration. The effect of METH feeding was observed on the amount of sleep and activity. METH feeding decreases activity and increases sleep through the period of fourteen days, indicating molecular mechanism that controls sleep is separated from mechanism that regulates activity. Analysis of biochemical indicators showed decrease of ROS and increase of fAGEs concentration through fourteen days. Decrease in ROS production can be the effect of antioxidative defense activation and increase in fAGEs levels are due to aging. However, it was unexpected that METH did not lead to differences in the majority of measured parameters, which requires the repetition of experiment. We propose using higher concentrations of METH in food, starving flies before the experiment and changing of food media that contains METH.

7. Literature

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8. Appendices

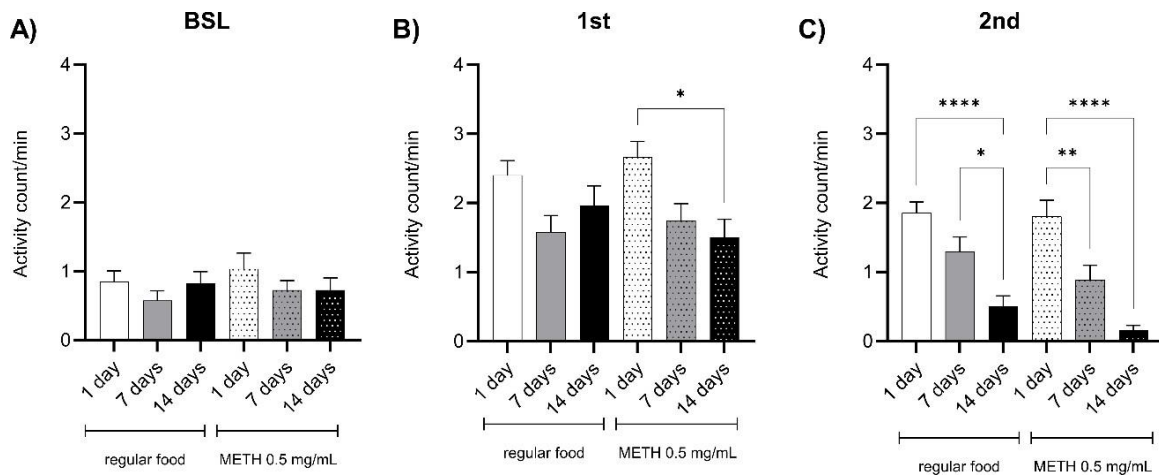


Figure 7. Effect of METH feeding on hot air exposure in control groups for vMETH. Hot air administered to control groups (n=32) of flies raised on regular and METH food in two doses (in 6h interval) via FlyBong. Activity(count/min) was measured using DAMS. Locomotor activity of population is compared 10 minutes before and after hot air administration to flies. Data is plotted as mean \pm SEM. Statistical test used was ordinary one-way ANOVA with Tukey's multiple comparison tests. **A**, Baseline (BSL) activity for flies exposed to METH and regular food for one, seven and fourteen days. **B**, Activity after first exposure to hot air for flies exposed to METH and regular food for one, seven and fourteen days. *: $p=0,0133$. **C**, Activity after second exposure to hot air for flies exposed to METH and regular food for one, seven and fourteen days. ****: $p<0,0001$, **: $p=0,0057$, *: $p=0,0264$.

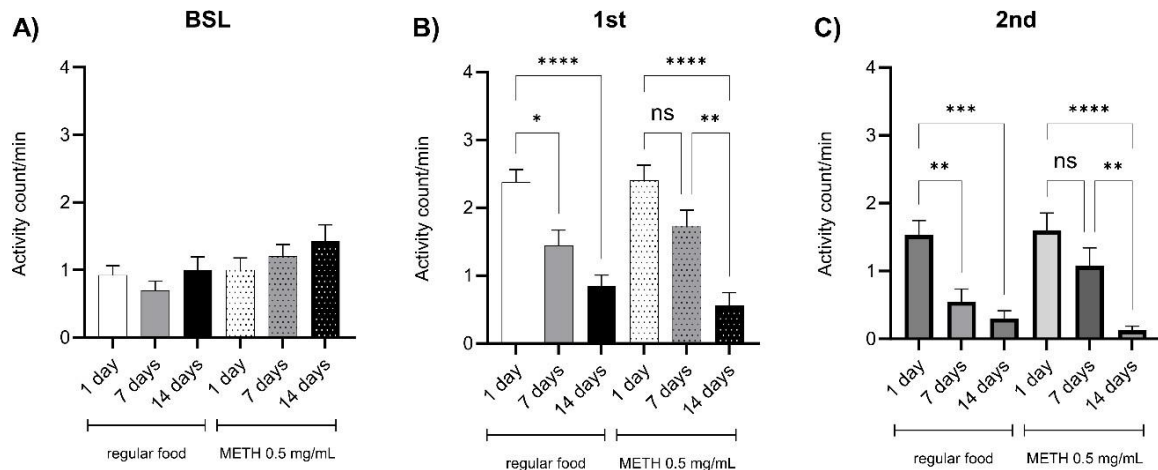


Figure 8. Effect of METH feeding on hot air exposure in control groups for vCOC. Hot air administered to control groups (n=32) of flies raised on regular and METH food in two doses (in 6h interval) via FlyBong. Activity(count/min) was measured using DAMS. Locomotor activity of population is compared 10 minutes before and after hot air administration to flies. Data is plotted as mean \pm SEM. Statistical test used was ordinary one-way ANOVA with Tukey's multiple comparison tests. **A**, Baseline (BSL) activity for flies exposed to METH and regular food for one, seven and fourteen days. *: p=0,0916 **B**, Activity after first exposure to hot air for flies exposed to METH and regular food for one, seven and fourteen days. **ns**=no significance, ****: p<0,0001, **: p=0,0010, *: p=0,0127. **C**, Activity after second exposure to hot air for flies exposed to METH and regular food for one, seven and fourteen days. **ns**=no significance, ****: p<0,0001, ***: p=0,0002, **: p=0,0099.



Ana Klasan

Home : Bana Josipa Jelačića, 22, 53220, OTOČAC, Croatia

Email: anaklasan25@gmail.com Phone: (+385) 977665332

LinkedIn: <https://www.linkedin.com/in/ana-klasan-a9a484223/>

Gender: Female Date of birth: 25/01/2000 Nationality: Croatian

EDUCATION AND TRAINING

[2021 – Current]

Drug research and development, Master's degree

Department of Biotechnology, University of Rijeka

[2018 – 2021]

Biotechnology and drug research, Bachelor's degree

Department of Biotechnology, University of Rijeka

Final grade: Cum laude

Thesis: The influence of circadian modulation on oxidative status in *D.Melanogaster*

[2013 – 2018]

High school diploma

Gimnazija Otočac

WORK EXPERIENCE

[02/2023]

Student Laboratory Assistant on the course "Biochemistry"

University of Rijeka

Assisted in the preparation and demonstration of laboratory exercises on the course Biochemistry.

[10/2021 – 01/2022]

Promotion Demonstrator of Dietary supplements

Sensapharm d.o.o.

City: Rijeka

[07/2021]

Student Internship

Croatian institute of public health

City: Rijeka

Gained insight in the quality control regulation and microbiological analysis of food, water, sea and air.

[02/2021 – 03/2021]

Bachelor Thesis Project

University of Rijeka

Experimental work in the laboratory for behavioral genetics on bachelor's thesis The influence of circadian modulation on oxidative status in *D.Melanogaster*.

ADDITIONAL EDUCATION

[11/2022]

Industrial Biotechnology, University of Manchester

Certificate provided by Coursera

[2007 – 2014] **Elementary Music School Diploma**

Music school Vjenceslav Novak, Senj

VOLUNTEERING

[2019 – 2021] **Biotechnology Student Association at University of Rijeka-USBRI**

In the project "Student mentor" mentoring three 1st year students, helping them with an orientation at the Department of Biotechnology.

LANGUAGE SKILLS

Mother tongue(s): Croatian

Other language(s):

English

LISTENING C1 READING C1 WRITING B2

SPOKEN PRODUCTION B2 SPOKEN INTERACTION B2

German

LISTENING A1 READING A1 WRITING A1

SPOKEN PRODUCTION A1 SPOKEN INTERACTION A1

Levels: A1 and A2: Basic user; B1 and B2: Independent user; C1 and C2: Proficient user

DIGITAL SKILLS

Competent with Microsoft Office programmes. | PyMOL, Avogadro, ChemAxon, Marvin, VMD, GAMESS, MacMOLPit

CONFERENCES AND SEMINARS

[2021] **Conference Future and perspective of Biotechnology (passive participant)**
Rijeka

DRIVING LICENCE

Cars: B

HONOURS AND AWARDS

[2023] **Science Arena- 2nd place Awarding institution: STEM GAMES**