BEHAVIORAL AND GENETIC CHARACTERISTICS OF PSYCHOSTIMULANT-INDUCED NEURONAL PLASTICITY IN DROSOPHILA MELANOGASTER

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DOCTORAL THESIS

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Abstract

Addiction is characterized by compulsive drug seeking and taking despite the negative consequences. In the laboratory, addiction is studied as set of simple forms of behaviors (endophenotypes), some of which are locomotor sensitization (LS), a phenotype of the motor-activating effects and preferential consumption, a phenotype of rewarding effects. The aim of the thesis is to develop tests for inducing and quantifying cocaine (COC) and methamphetamine (METH) motor-activating and rewarding effect, and investigate genetic mechanisms underlying such neural plasticity.

We developed and optimized two new methods, FlyBong for measuring motor-activating effects, and a self-administration method. We show that repeated exposures to volatilized COC and METH induce LS in flies, and that flies prefer COC and METH containing food to regular food. Pharmacodynamics of COC and METH LS and preferential consumption depends on the dopamine transporter. Reduction of dopamine, serotonin and octopamine, did not completely abolish the locomotor sensitization to COC and METH, suggesting the involvement of other monoamines in this processes. Manipulation of dopaminergic and serotoninergic vesicular monoamine transporters (VMAT) using RNA interference (RNAi), indicated that locomotor sensitization and preference to COC and METH depend on functional VMAT.

LS to COC depends on several circadian genes, *period* (*per)*, *cycle* (*cyc)* and *Clock* (*Clk)*, leads to high levels of reactive oxygen species (ROS) production and is susceptible to the exogenous redox perturbation. LS to METH depends only on one circadian gene, *Clk*, leads to lower levels of ROS and is less susceptible to exogenous redox perturbation. The motor-activation and rewarding effects of COC depend on the circadian gene *per*, while METH depends on *Clk*, indicating differential involvement of circadian genes in these two processes. Pre-treatment with H_2O_2 led to different effects on sensitivity and LS to COC and METH suggesting that H_2O_2 might function as a neuromodulator, and that its role is drug-specific.

Here we developed new behavioral tests and described the effects of COC and METH on two forms of neuronal plasticity. Our new high throughput test for measuring LS is ideally suited for unbiased genetic screen or selection. We described likely circadian and redox influence on behavior that correlates with monoaminergic

regulation. We hypothesize that circadian genes and redox regulation work together to change the brain functioning after consumption of addictive drugs.

Key words: *Drosophila melanogaster*, psychostimulants, neuroplasticity, circadian/redox feedback loops

Prošireni sažetak

Jednostavna definicija ovisnosti podrazumijeva kompulzivno traženje i primjenu sredstva ovisnosti unatoč negativnim posljedicama koje ima za organizam. Ovisnost se kod laboratorijskih životinja proučava kao niz jednostavnih ponašanja (endofenotipova) upotrebom optimiziranih bihevioralnih testova koji moraju biti objektivni, reproducibilni i kvantitativni. Ovisnost o psihostimulansima (PS) kokainu (COC) i metamfetaminu (METH) se može razložiti na jednostavnija ponašanja povezana s motoričkim (senzitizacija) i nagrađujućim (preferencijalna konzumacija) učinkom. Cilj ovog doktorskog rada je ispitati utjecaj COC i METH na motorički i nagrađujući učinak povezan s genetskim promjenama koje dovode do neuralne plastičnosti primjenom novo razvijenih testova za induciranje i kvantifikaciju ponašanja.

Tijekom mog doktorskog studija razvila sam i optimizirala novu metodu za mjerenje motoričke aktivnosti potaknute primjenom PS koju smo nazvali FlyBong. Upotrebom novog optimiziranog protokola „FlyBong“, utvrđeno je da akutno i opetovano izlaganje *Drosophile* volatiliziranom COC i METH inducira povećanje motoričkog odgovora. Istražena je farmakodinamička sličnost između mehanizma djelovanja COC i METH te utjecaj monoamina dopamina, serotonina i oktopamina na bihevioralni odgovor kod akutne i opetovane primjene PS. Testirala sam hipotezu da su cirkadijalni geni *period*, *timeless*, *clock*, *cycle* i *pigment-dispersing factor* uključeni u endofenotip senzitizacije na COC i METH. Senzitizacija potaknuta s COC nije prisutna kod per^{01}, cyc^{01} i Clk^{Jrk} mutanata, dok senzitizacija na METH nije prisutna kod Clk^{Jrk} mutanata. Izmjerila sam utjecaj COC i METH na sistemske biomarkere redoks statusa: katalazu, superoksid dismutazu, vodikov peroksid i reaktivne kisikove vrste čime sam pokazala da COC sistemski inducira veći, a METH manji pomak oksidativnog statusa Testirala sam utjecaj predtretmana s anti- i prooksidansima na motorički odgovor kod akutne i opetovane primjene COC i METH, čime sam pokazala da antioksidansi utišavaju senzitizaciju na METH, dok kod COC senzitizacija nije prisutna. Istražila sam potencijalnu neuromodulatornu i oksido-redukcijsku ulogu vodikovoga peroksida povezanu s predtretmanom koji nije uzrokovao sistemski pomak oksidativnog statusa, ali je utjecao na endofenotip senzitizacije. Testirana je hipoteza korelacije broja cirkadijanih gena i osjetljivosti na redoks status u ovisnosti o PS. Nađeno je da PS koji ovisi o više cirkadijalnih gena te uzrokuje veću sistemsku redoks preturbaciju pokazuje veću osjetljivost na promjenu redoks statusa u endofenotipu

senzitizacije ako je pred tretman anti- ili prooksidativan, dok PS koji ovisi o manjem broju cirkadijalnih gena, uzrokuje manju sistemsku redoks preturbaciju te pokazuje manju osjetljivost na promjenu redoks statusa više orijentiranu na predtretman s molekulama manjeg antioksidativnog kapaciteta.

Kako bi ispitala da li PS utječu ma motivaciju za konzumacijom optimizirala sam test samo-administracije (Capillary Feeding, CAFE) gdje mušice biraju između otopine šećera i otopine šećera pomiješane s PS. Ovim istraživanjem je nađeno da mušice kojima je ponuđen izbor između otopine šećera i otopine šećera pomiješane s PS pokazuju preferenciju prema otopini s PS, koja je posljedica nagrađujućeg efekta PS. Nađeno je da su procesi učenja i pamćenja povezani s periferijalnom konzumacijom te da će mušice preferencijalno konzumirati otopinu s PS unatoč dodatku supstance gorkog okusa. Ukoliko se mušicama koje su izložene PS nekoliko dana za redom, jedan dan ne ponudi PS te ih se nakon toga ponovno izloži izboru između otopine šećera i otopine šećera i PS, mušice će pokazivati jednaku ili veću preferenciju za PS kao što je bila prije perioda apstinencije. Istražen je utjecaj cirkadijalnih gena te receptora i prijenosnika povezanih s farmakodinamičkim djelovanjem COC i METH na nagrađujući učinak. Pokazala sam da se preferencijalna konzumacija COC i METH razlikuje po tipu cirkadijalnih gena koji na nju utječu, a neki od njih kontroliraju motoričku senzitizaciju. Ovi rezultati upućuju na mogući zajednički genetski mehanizam koji je u podlozi preferencijalne konzumacije, kao oblika nagrađujućeg utjecaja PS i motoričke senzitizacije.

Ishodi mojeg doktorskog rada rezultirali su razvojem dvije nove objektivne i visokoprotočne metode za kvantifikaciju ponašanja *Drosophile* koja će omogućiti naredna istraživanja genetske osnove neuralne plastičnosti izazvane PS korištenjem metode genetskog probira ili genetske selekcije. Daljnja istraživanja cirkadijalnih/redoks povratnih spregi potaknutih primjenom COC i METH trebala bi pomoći u razumijevanju mehanizma kojima PS mijenjaju neuronske mreže, te usmjeravanju u nove potencijalne farmakološke smjerove razvoja lijekova za prevenciju i liječenje ovisnosti.

Ključne riječi: *Drosophila melanogaster*, psihiostimulansi, neuralna plastičnost, cirkadijalne/redoks povratne sprege

Contents

1. INTRODUCTION

1.1. Substance addiction

Addiction is a complex brain disorder characterized by neuroplasticity, or changes in the genes and proteins that regulate brain functioning. Neuroplasticity is a change in the neural pathways and synapses of the brain (Kauer 2007), and in respect to addiction it is related to changes in the reward circuits of the brain (Volkow and Morales 2015).

Drugs induce the neurochemical and behavioral phenomenon of sensitization, which refers to a progressive increase in the effect of a drug with repeated treatments (Robbinson and Berridge 2000, Robbinson and Berridge 2008). In animal models, an acute response to psychostimulants is described as sensitivity, while repeated exposures to the same amount of a psychostimulant can induce a stepwise increase in locomotion or locomotor sensitization (Robbinson and Berridge 2000). In mammalian studies, the sensitization endophenotype is studied as a mechanism by which repeated exposure to a drug-related cue results in a progressive amplification of the response, craving for the drug (Steketee and Kalivas 2011).

The effects of drugs of abuse lead to compulsive drug seeking and taking behavior, as a result of craving which the drugs induce (Robbinson and Berridge 1993). When animals are offered choice between a drug and a non-drug liquid, they show preference to the drug-containing liquid. Animals self-administer such drugs more robustly if the drug is associated with a cue. Since the rewarding effect of the drugs engage dopaminergic neuromodulation, the processes of learning and memory are also involved in addiction (Nestler 2013).

After a period of abstinence from the drug, a drug-related cues can cause activation of mechanisms of positive reinforcement and the animal relapses, with their preference for the drug being the same as, or higher than, it was before the period of abstinence (Berridge et al. 2009). If the drug is associated with negative reinforcement (e.g. electric shock), addicted animals will consummate drug in spite of negative consequence induced by the drug.

1.1.1. Animal models and endophenotypes of psychostimulant addiction

Drug addiction is a complex behavior that is usually studied in animal models through use of endophenotypes, relatively simple and quantifiable phenotypes related to the addiction (Kaun et al. 2012). Endophenotypes studied in model organisms range from response to an acute drug dose to more complex behaviors such as self-administration and relapse. The higher is the complexity of the behavior which is induced and measured, the greater is the relevance of the model for human study. On the other hand, simpler forms of behavior can be more easily induced and measured, which makes them more convenient for high-through genetic screening and understanding the mechanisms that govern them.

Rats and mice have provided crucial insights into the mechanisms underlying drug-related behaviors (Sanchis-Segura and Spanagel 2006), but they are not ideal for unbiased, forward genetic approaches aimed at identifying novel and unpredicted genes or mechanisms. More ethical use of animals in scientific testing is refers to 3Rs principle (**r**eplacement, **r**eduction and **r**efinement). The model organism *Drosophila melanogaster*, a fruit fly, is great example of 3Rs principle, replacing the use of higher laboratory animals in scientific experiments . Firstly, *Drosophila* uses many of the same neurotransmitters as mammals and has similar mechanisms of neurotransmitter storage, release and recycling. *Drosophila* offers a variety of powerful molecular-genetic methods for the study of novel mechanisms using forward genetics (Owald et al. 2015). Additionally, flies are easy and inexpensive to grow in laboratory conditions, with a developmental cycle of around 14 days. *Drosophila* were used for many years to identify the molecular and neural mechanisms that regulate acute drug responses (McClung and Hirsch 1998), while recently developed assays that measure drug self-administration and reward now allow the analysis of more complex endophenotypes in flies (Devineni and Heberlein 2009).

1.1.1.1. Sensitivity and locomotor sensitization

Drosophila, in common with other animal models, shows sensitivity (SENS), to an acute psychostimulant (PS) dose by increasing locomotor activity. This behavioral endophenotype has been studied in flies, in response to cocaine (COC) (McClung and Hirsch 1998) and methamphetamine (METH) (Andretic et al. 2005, Van Swinderen and

Andretic 2011). Depending on the dose, administration of volatilized COC (vCOC) may induce different types of behavior in flies, ranging from increased locomotor activity at lower doses, stereotypical behaviors (buzzing and spinning in circles) at moderate doses, to akinesia or death at higher doses. Oral administration of METH promotes activity and reduces sleep, indicating motor activating and arousing effects. In mammals, METH induces behaviors similar to those observed with COC (Hyman 1996), but effects of volatilized METH (vMETH) have not been described in flies. Locomotor sensitization (LS) represents increase in the amount of locomotor activity to repeated doses of the same amount of PS. It is more complex behavior compared to SENS, and engages mechanisms of neuronal plasticity. While flies develop LS to repeated exposures to vCOC (McClung and Hirsch 1998), it has never been shown if flies increase amount of locomotion and develop LS to vMETH.

Over the past decades, a couple of behavioral assays for induction and quantification of SENS and LS in flies have been developed. The assay published by McClung and Hirsh in 1998 was based on volatile COC administration, using freebase COC (form of COC that volatilizes at low temperature). COC was volatilized from a heated filament in a vial, after which the flies were transferred to a video arena where the behaviors were filmed. Behavioral response was then scored using a descriptive scale after analyzing the video recording, and data were represented as a population response to either single or repeated exposures. This assay had a few disadvantages. Firstly, the flies experienced stress during transfer from the cultivation vial to the chamber for COC administration, and again during transfer to the video arena. The amount of COC delivered to each individual fly was not determined, and could vary between experiments, although there was an attempt to improve this method using a graphical arch airbrush (Lease and Hirsh 2005). Furthermore, scoring of the behavior was time consuming, subjective and population based. In spite of this, the assay had the advantage that all flies were exposed to COC at the same time, and that both SENS and LS could be tested using the same assay. A similar assay was developed by Bainton et al. in 2000. This assay used volatilized administration of COC as previously described, but quantification of the behavior after administration was automated, using the automated video analysis software Dynamic Image Analysis System (Solltech Inc.). The disadvantage of this assay is in ability to only score SENS in flies and not LS.

There was an attempt to automate vCOC administration to flies, which allowed simultaneous exposure of vCOC to all flies in the assay (Gakamsky et al 2013). The authors tried to minimize the environmental perturbation during exposure to the volatile drug by transferring flies to the video arena 2 hours before the assay. This habituated the flies to the novelty of the arena and to the presence of the other flies. The drug delivery system consisted of a chamber with multiple wire filaments that volatilized COC, which was then delivered to the flies in the arena using an air pump. Since neither food nor water was supplied to the fly during the entire 3 hours long experiment, there were possible effects of starvation and dehydration on the fly behavior. In addition, while the response to vCOC was quantified by automated video analysis software, it was still population based.

The crackometer assay was developed to measure the ability of flies exposed to vCOC to climb quickly to the top of a column, given their innate propensity for negative geotaxis (Heberlein et al. 2009). Compared to previous assays, crackometer provided an easily quantifiable score enabling a high-throughput approach. Ultimately, it was used in genetic screens that lead to discovery of new genes involved in sensitivity, but not in LS (Heberlein et al. 2009).

Finally, Dimitrijevic et al. in 2004 developed an assay in which COC was administered to the flies by injection, which provided an objective quantification of behavior using the DAM (Drosophila activity monitoring) system, which allows for quantification of locomotor activity of large numbers of flies at the same time. The locomotion is quantified as a number of times a fly crosses an infrared beam in the middle of the tube in a given time interval. The disadvantage of injection method was that the CO_2 anesthesia during injection protocol could have an effect on behavior after PS administration.

All of the mentioned assays had shortcomings in some of the following features: reproducibility, objectivity, throughput, and amount of COC to which flies were exposed or level of animal handling. The majority of them lacked adequate controls and their correlation to COC-exposed animals. They include complex data analysis that are population based, and therefore an inconvenient base for forward genetic screening.

Additionally, no data on vMETH induced LS in flies has published so far, as has been for vCOC (Rothenfluh and Heberlein 2002).

The importance of using data analysis based on individual animals has been demonstrated in rats, as not all rats will show a response to an acute dose of COC, or sensitization on repeated administration (Gulley et al. 2003). Thus, in the wild type (*wt)* populations there are always some individuals that do not respond to COC. The same was also found for METH (Kamens et al. 2004.). Furthermore, population based approaches provide less information, compared to individual-based approaches, as some information is lost or misinterpreted. Therefore, this work has focused on the development of a new method that would be reproducible, objective, high-throughput, with minimal animal handling and with possibility to analyses data at both the population and individual level.

1.1.1.2. Rewarding effect of psychostimulants

Most of the work done to date on the reward effect in flies lies in the field of learning and memory. This was possible because flies associate odors with food and food-related odors, and these odors can be used as cues for food reward (appetitive) or aversion (repulsive) stimuli (Tempel et al. 1983, Schwaerzel et al. 2003).

One of the more complex endophenotypes in response to COC, compared to SENS and LS, is self-administration. While testing of SENS and LS in flies, involves administration of COC by the researcher, in the self-administration approach the animal chooses when to consume the drug, likely because of the reward effect of the drug. This approach was first introduced in experiments based on ethanol preference (Devineni and Heberlein 2009). There was potential bias in the self-administration of ethanol, because of evolutionary attractiveness of the ethanol associated with ethanol-containing food with caloric value. However, self-administration enabled a new behavioral endophenotype to be studied in *Drosophila* addiction research.

In the pioneering work on ethanol self-administration, ethanol consumption was measured using a two-choice assay, similarly to the two bottle choice assay used in rodent studies. Flies have shown preference for ethanol in dose-dependent way, with the preference persisting in the absence of olfactory or gustatory input (Devineni and Heberlein 2009). In the same study, it was found that flies have two additional

addiction-like behaviors. Flies overcame an aversive stimulus, the bitter-tasting compound quinine, in order to consume ethanol, and they rapidly return to high levels of ethanol consumption after ethanol deprivation, modelling a relapse-like effect (Devineni and Heberlein 2009).

In summary, SENS and LS were investigated in terms of the response of flies to COC administration (Wolf 1999), however to date there are no published data on COC or METH preferential consumption in flies.

1.2. Psychostimulant-induced neuronal plasticity

The exact mechanism by which addictive drugs change the brain is still unknown (Sulzer 2011), but there is evidence that the likelihood of developing addiction is dependent on sources of reinforcement, neuroadaptive and neurochemical changes, which ultimately lead to the brain reward system (Koob et al. 1998).

Different psychostimulants have different molecular targets in the central nervous system (CNS), but they all lead to increases in extracellular concentrations of dopamine (DA) in specific regions of the brain (Laakso et al. 2002). In mammals, two transcription factors, CREB (cAMP response element binding protein) and ΔFosB are induced in the reward region of brain (the nucleus accumbens) following DA interaction with the dopamine 1 like receptor on the postsynaptic neuron, and contribute to drug-induced changes in gene expression. ΔFosB is a molecular switch, which gradually converts acute drug responses into relatively stable adaptations that contribute to the long-term neural and behavioral plasticity that underlies addiction (Nestler et al. 2001). CREB mediates a form of tolerance and dependence, in contrast to ΔFosB, which instead mediates a state of relatively prolonged sensitization to drug exposure (Nestler 2004).

In *Drosophila,* neuronal plasticity is under the control of an immediate early transcription factor (AP-1), a heterodimer of the proteins Fos and Jun (Sanyal et al. 2002). While the *Drosophila* and human Jun proteins have the same phosphorylation site, *Drosophila* Fos contains only one domain to be common to all mammalian Fos family, sharing only moderate similarity at the primary sequence level (Kockel et al. 2001). AP-1 was found to be involved in learning behavior in rodents, developmental

plasticity in insect models, and COC addiction in mammals (McClung and Nestler 2003).

Based on the minimal similarity between *Drosophila* Fos and the mammalian Fos gene family, synaptic plasticity induced by PS in this work will be focused on monoaminergic neurotransmission, circadian genes and ROS-induced by DA and PS oxidative metabolism, as these processes are more conserved between *Drosophila* and mammals.

1.2.1. Monoaminergic neurotransmission

Drosophila use many of the same neurotransmitters as mammals including gamma-aminobutyric acid (GABA), glutamate and acetylcholine, as well as some of the same monoamines including DA, serotonin and histamine (Martin and Krantz 2014). Additionally, flies possess octopamine, a fly analogue of noradrenaline, and trace monoamine tyramine.

COC and METH have different pharmacokinetics and different pharmacodynamics mechanisms of action, primarily in relation to DA neurons (Hyman 1996). As a result, the following chapters will be focused on COC and METH pharmacodynamics in mammals, specifically in dopaminergic neurons, with some analogies to *Drosophila*. The influence of mutation or pharmacological inactivation of targets for COC and METH on behavioral endophenotypes of self-administration and locomotor sensitization will also be discussed.

1.2.1.1. Cocaine and methamphetamine pharmacokinetics

The time required for the maximal effect of COC and METH differs as a result of their mode of the administration (Pandey and Nichols 2011). In humans, intravenous administration (IV) of COC causes an onset of action at 10-60 minutes, with the peak around 3-5 minutes after administration and total duration of 20-60 minutes. In comparison, inhalation of COC causes onset of action at 3-5 seconds, peak at 1-3 minutes, and duration of 5-15 minutes (Lange and Hillis 2001). In contrast, oral administration of METH in humans has a half-life of 6-12 hours, and the time before maximal effect is 1-3 hours (Baselt 2004). These data suggest not only different COC

and METH pharmacokinetics, but also varied onset of action depending on the method of administration.

1.2.1.2. Cocaine and methamphetamine pharmacodynamics

In mammals, DA is synthesized in the cytoplasm from tyrosine by tyrosine hydroxylase (TH) and DOPA decarboxylase (DDC), after which it is packed in vesicles by the vesicular monoamine transporter (VMAT) and stored prior to release in the synaptic cleft (Muñoz et al. 2012, German et al. 2015). In the basal state of the presynaptic neurons, when it becomes polarized, it starts to release DA by vesicular exocytosis (Figure 1A). Released DA in the cleft then binds to presynaptic and postsynaptic receptors. After the signal is transmitted to the postsynaptic neuron, DA is removed from the cleft via dopamine transporter (DAT) on the presynaptic neuron. It is then either repacked into vesicles by VMAT, or degraded by monoamine oxidase (MAO) and catechol-*O*-amine transferase (COMT) into inactive components (Figure 3), such as homovanillic acid (HVA) through the intermediate products 3,4-dihydroxyphenylacetic acid (DOPAC) or 3-methoxytyramine (3-MT) (Muñoz et al. 2012.).

Both processes, dopamine release and reuptake, are followed by DAT and VMAT integration on the presynaptic neuron membrane (German et al. 2015, Kahlig and Galli 2003). When DA is cleared from the synaptic cleft, early endosomes (EE) deliver DAT to the presynaptic neuron membrane trough recycling endosomes (RE). Claritin-coated vesicles (CCV), meanwhile, remove DAT from the membrane once all DA is removed from cleft and sent to either the early or late endosome and finally to the lysosome. VMAT, incorporated on presynaptic neuron membrane after releasing DA, is also removed by CCV.

a) COC mechanism of action

COC blocks reuptake of DA from the synaptic cleft to the presynaptic neuron by binding to DAT (Espana and Johnes 2013). Through blocking of DAT, the concentration of DA in the synaptic cleft becomes elevated, in parallel to an increase of DAT integration in order to compensate for elevated levels of DA (German et al. 2015). Simultaneously, the number of vesicles carrying additional DA in the cytoplasm increases, with a reduced number being integrated into the membrane (Figure 1C).

Increased levels of DA also affect dopamine receptors (DR) at the postsynaptic neuron, causing reduced integration into the membrane. Together, the increased DA in the cleft, and the blockade of DAT, stimulate the degradation of DA by MAO and COMT (Figure 3). This reduces postsynaptic neuron polarization, which causes an increase in ROS (Meiser et al. 2013), as it will be discussed in more detail later. COC binds differentially to the dopamine, serotonin, and norepinephrine transport proteins and directly prevents the re-uptake of dopamine, serotonin, and norepinephrine into pre-synaptic neurons, subsequently elevating the synaptic concentrations of each of these neurotransmitters.

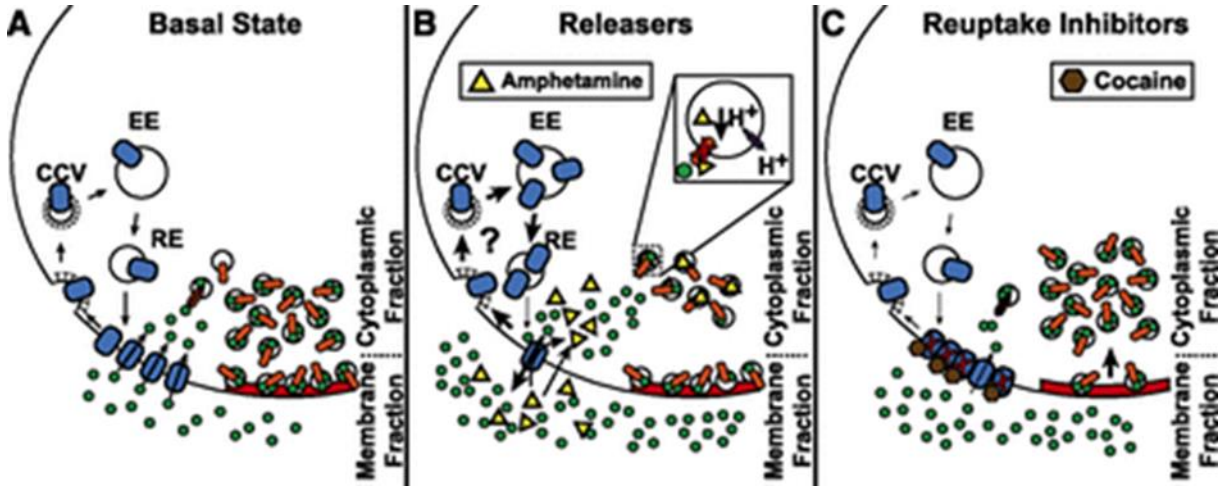


Figure 1. Basal state A) and pharmacodynamics of B) METH and C) COC in DA neuron. Blue – dopamine transporter (DAT), red – vesicular monoamine transporter (VMAT), green – dopamine (DA), CCV-clathrin-coated vesicle, EE- early endosome and RE – recycling endosome. (German et al. 2015).

b) METH mechanism of action

In contrast to COC, METH enters the presynaptic neuron by diffusion through the membrane. Once in the cytosol, METH binds to VAMT and reverses its mechanism, causing depletion of DA stored in the vesicles (Figure 1B). This process causes elevated DA in the cytosol, and reduced DA in vesicles. Translocation of DA from the presynaptic neuron to the cleft is then mediated by DAT, whose function is reversed, while VMAT-mediated vesicular transport is still occurring. DAT integration into the membrane may also occur, but this has not been demonstrated *in vivo*. However, a reduction of VMAT synaptic vesicles within the cytoplasmic fraction has been shown.

Additionally, METH causes a decrease in DA metabolism catalyzed by MAO, as METH can inhibit MAO, forcing DA removal from the cleft through autoxidation (Figure 3).

1.2.1.3. *Drosophila* DA anabolism, signal transduction and catabolism

a) Anabolism

In *Drosophila*, as in mammals, conversion of tyrosine to L-DOPA is mediated by tyrosine hydroxylase (TH), after which L-DOPA is converted to DA by DOPA decarboxylase (DDC). Blocking of dopamine synthesis using the TH inhibitor, 3-iodo-tyrosine (3IY) leads to a reduced effect of COC (Bainton et al. 2000). Decreased DA synthesis can be restored by feeding flies with L-DOPA or DA (Riemensperger et al. 2011). Ingested DA can restore the concentration and function of DA in the nervous system of flies (Budnik et al. 1989), which is in direct contrast to mammals where supplemental DA cannot cross the blood-brain barrier (BBB).

b) Storage and release

DA is synthesized in the cytoplasm and stored into synaptic vesicles (Fleckenstein et al. 2009, German et al. 2015). Transport of DA across the vesicle membrane is mediated by VMAT. The *Drosophila* isoform of VMAT (dVMAT-A) is expressed in both dopaminergic and serotonergic neurons. Reserpine, a drug that blocks mammalian VMATs, is also effective in *Drosophila* and has been used to inhibit DA signaling *in vivo (*Bainton et al. 2000). Flies pre-treated with reserpine, did not display sensitization to repeated COC administration (Bainton et al. 2000).

Flies mutant for dVMAT-A exhibit numerous behavioral abnormalities (Simon et al. 2009), although some of these defects may be due to serotonin and octopamine, since VMAT packages these monoamines in addition to DA. VMAT2 mutant heterozygote mice are hypersensitive, but do not show sensitization to COC, while acute exposure to METH increases their locomotion as is the *wt* animals (Wang et al.1997). Repeated METH exposures in VMAT2 mutant heterozygote mice lead to delayed LS (Fukushima et al. 2007) and reduction in reward effect of METH, when compared to *wt* mice, while the COC reward effect remains intact (Takahashi et al. 1997).

Overexpression of dVMAT-A in flies induces spontaneous stereotypic grooming behavior and locomotion, effects that can be reversed by blocking dVMAT-A activity or by administration of a dopamine receptor antagonist, such as COC. In addition, dVMAT-A overexpression decreases the fly's sensitivity to COC, suggesting that the synaptic machinery responsible for this behavior may be down-regulated by dVMAT-A overexpression (Chang et al. 2006). It was found that COC, like METH, rapidly alters VMAT, suggesting that alterations in cytoplasmic DA concentrations may contribute to stimulant-induced changes in vesicular DA uptake (Brown et al. 2001).

c) Signal transduction

Signal transduction involves binding of DA in the synaptic cleft to the postsynaptic dopamine receptor. In *Drosophila* four G-protein-coupled DA receptors have been identified: two D1-like receptors (*DopR* and *DopR2*) and one D2-like receptor (*D2R*). Similar to humans, *Drosophila* D1-like receptors act through activation of the cAMP pathway, while D2-like receptors inhibit this pathway (Yamamoto and Seto 2014).

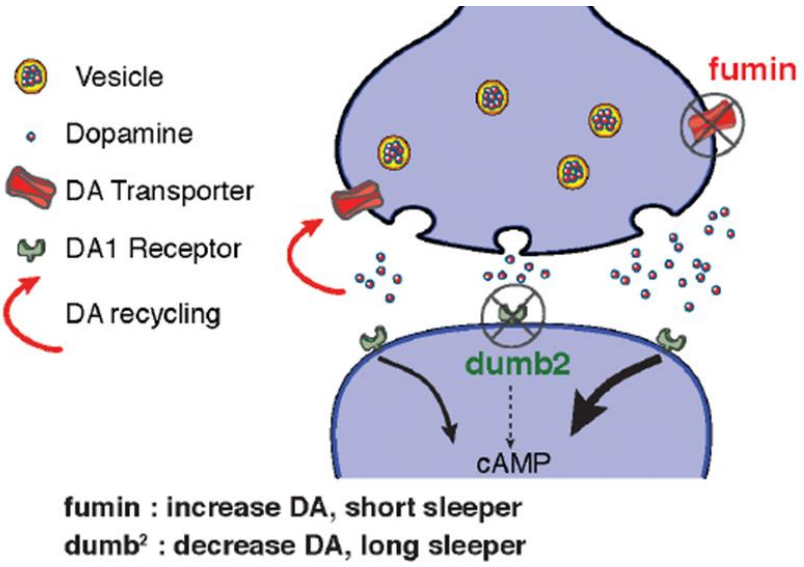


Figure 2. Scheme of DA signal transduction and recycling in *wt* flies, *dumb²* and *fmn* mutants (Faville et al. 2015)**.** *dumb²* is a hypomorphic allele of the dDA1 receptor (also called DopR), *fmn* is shorten from fumin (insomniac in Japanese) strain that contains a mutation in the *Drosophila* dopamine transporter gene (dDAT).

Mutations in D1 and D2 receptors block locomotor hyperactivity (Tella 1994) and self-administration (Bergman et al. 1990) of COC in mammals. The *Drosophila* D1 like receptor mutant *dumb* has decreased DA in the synaptic cleft causing the mutants to

sleep more and have lower learning and memory scores (Figure 2). *dumb* mutants also fail to increase locomotor activity after COC exposure (Lebestky et al. 2009).

Blocking of D1 and D2 receptors with an antagonist in mammals has been shown to reduce METH self-administration (Brennan et al. 2009), while mutation in the D1 receptor lowers sensitivity to METH (Xua et al. 2000).

d) Reuptake and metabolism

In order to maintain proper function after the signal transduction, DA needs to be cleared from the synaptic cleft. Removal of DA from the cleft can occur through its reuptake into the presynaptic neuron via DAT (Zahniser and Sorkin 2004), or by metabolism into inactive compounds (Figure 3).

A mutation in *Drosophila* DAT named *fumin (fmn)*, causes elevated levels of DA (Figure 2), increase in basal activity and decrease in sleep (Faville et al. 2015). In mammals, DAT mutants do not show sensitivity to acute doses of COC, or LS after repeated COC exposures (Hall et al. 2009). Mutation in DAT did not eliminate the reward effects of COC in the conditioned place preference (CPP) or self-administration paradigms in mammals (Rocha et al. 1998, Sora et al. 1998). Additional studies have shown that mutations in the serotonin transporter (SERT) and DAT eliminated the reward effects of COC in the CPP (Sora et al. 2001). Mutation in DAT did not affect the response to acute METH exposure, while repeated exposures to METH delay and attenuate LS in mammals (Fukushima et al. 2007).

DA synthesis, secretion, and signaling is conserved between *Drosophila* and mammals, but degradation of DA differs significantly because direct orthologs of MAO and COMT genes have not been identified in flies (Paxon et al. 2005). However, the DA oxidative products DOPAC and HVA have been detected in *Drosophila*, suggesting that an analogue pathway may exist in flies (Paxon et al. 2005). Monoamines in *Drosophila* could be metabolized by dopamine N-acetyltransferase, also known as arylalkylamine N-acetyltransferase (aaNAT) (Paxon et al. 2005). Flies with reduced aaNAT activity show defects in sleep homeostasis, a phenotype that is affected by aberrant DA signalling (Brodbeck et al. 1998).

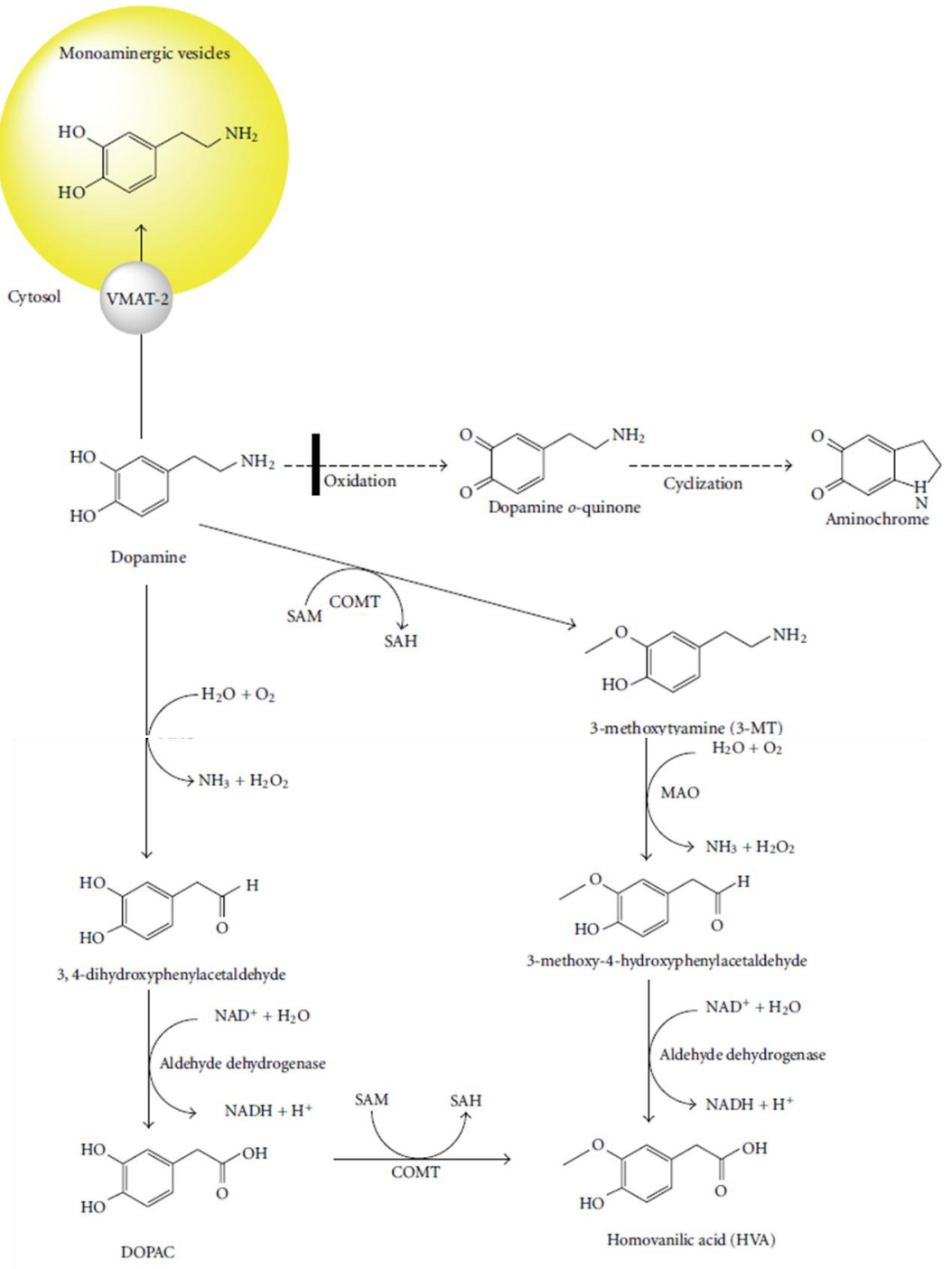


Figure 3. Dopamine degradation pathway (Muñoz et al. 2012)**.**

1.2.1.4. Involvement of other monoamines

In *Drosophila,* DA is not the only monoamine to be involved in the mechanisms of neuronal plasticity induced by drugs. Tetanus toxin light chain (TNT) expressed in dopamine and serotonin neurons of living flies blocked behavioral sensitization to

repeated COC exposures (Li et al. 2000). Flies that lack both tyramine and octopamine, as a result of mutation in tyramine decarboxylase-encoding genes (TDC), have dramatically reduced basal locomotor activity levels and hypersensitivity to acute COC exposure (Hardie et al. 2007). Flies with a null mutation in the tyramine-β-hydroxylase gene, which is responsible for conversion of tyramine to octopamine, produce low levels of octopamine and high levels of tyramine, display normal locomotor activity and COC responses (Hardie et al. 2007). Tyramine has also been implicated in COC sensitization (McClung and Hirsh, 1999).

Taken together, these results indicate that the dopaminergic, serotonergic systems and the trace-amine tyramine mediate acute and repeated cocaine-induced behaviors in flies.

1.2.2. Circadian genes

The circadian rhythmicity with a period of around 24 hours is result of an endogenous biochemical oscillator (circadian clock) that cycles with a stabile period and phase, and is present in all plants and animals. Circadian clock inputs, such as light or food, modulate circadian clock outputs such as locomotor activity, metabolism and neuronal plasticity (Allada and Chung 2010).

Pleiotropy of the circadian genes, and their involvement in processes of neuronal plasticity induced by PS, were first identified in flies (Andretic et al. 1999), with the same endophenotype later being confirmed in mice (Abarca et al. 2002). These findings drew considerable attention towards the importance of finding a possible connection between development of drug addiction and the role of circadian genes in that process (Falcón and McClung 2010, Logan et al. 2014). The primary target of PS is the monoaminergic system, whose functioning has been shown to be associated with circadian genes (McClung 2007). The relationship between the circadian and reward systems is based on the effect of drugs on the clock genes, and the effect of clock genes on genes involved in neuronal plasticity (Parekh and McClung 2016, Parekh et al. 2015), with a focus primarily on DA. Perturbations in DA synthesis do not appear to have a significant impact on circadian locomotor activity rhythms *per se*, but do have an influence on general locomotor activity levels (Hanna et al. 2015).

1.2.2.1. The circadian clock of Drosophila

Out of the 100,000 neurons in the brain of *Drosophila*, only 150 are clock neurons and capable of autonomous circadian functioning. The simplified circadian clock in *Drosophila* consists of five major genes and proteins: *period* (*per*), *timeless* (*tim*), *Drosophila clock* (d*Clk*), *cycle* (*cyc*) and *double-time* (*dbt*). Regulation of the circadian clock is under the transcriptional and translational PER/TIM and CLK/CYC negative feedback loops (Peschel and Helfrich-Förster 2011, Tataroglu and Emery 2014).

Unlike flies, the circadian clock of mammals has three copies of the *per* gene, *Per1, Per2 and Per3*, while the mammalian analogue to *Drosophila tim* is *cryptochrome (cry)*, and the mammalian analogue to *Drosophila cyc* is *Bmal*1 (Yu and Hardin 2006).

a) PER/TIM negative feedback loop

During daytime, the protein heterodimer CLK-CYC acts as a transcription factor of the *period* (*per*) and *timeless* (*tim*) genes. In the cytosol PER and TIM proteins activate cellular mechanisms of their dimerization and degradation. The PER-TIM heterodimer is translocated from the cytosol to the nucleus early in the evening, causing CLK-CYC phosphorylation and inhibition of their transcription (Figure 4). All these processes are under control of other molecules such as light-induced cryptochrome (CRY) and *double-time* (*dbt*) to ensure stabile circadian period and phase.

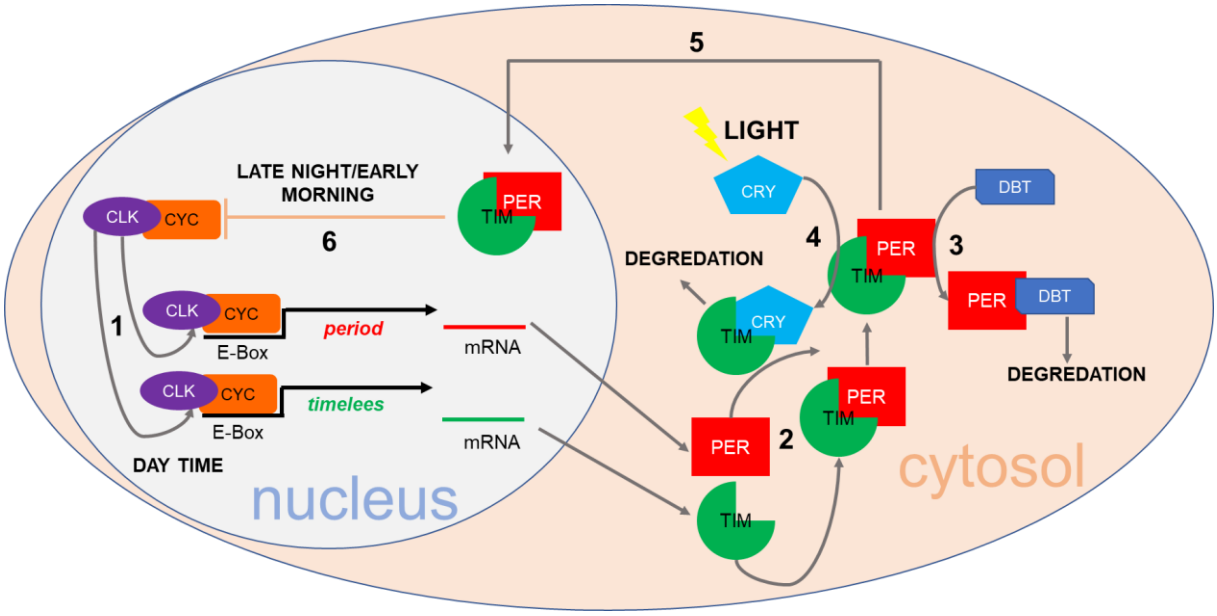


Figure 4. Scheme of PER/TIM negative feedback loop. 1) Binding of the CLK-CYC dimer to the E-box of the *per* and *tim* genes. 2) PER and TIM dimerization in the cytosol. 3) Degradation of TIM by light-induced CRY. 4) Degradation of PER by DBT phosphorylation. 5) Translocation of PER-TIM into the nucleus and 6) PER-TIM inhibition of their own transcription.

b) CLK negative feedback loop

Late in the day, or early at night, the CLK-CYC dimer is located in nucleus and acts as a transcription factor for Par domain protein 1ε (PDP1ε) and Vrille (*Vri*) (Cyran et al. 2003). *Vri* is a negative transcription factor for *clk* and reduces CLK synthesis. *PDP1ε* is a *clk* positive transcription factor, which induces CLK synthesis. Once synthesized, CLK binds to CYC and a new cycle of the feedback loop starts (Figure 5).

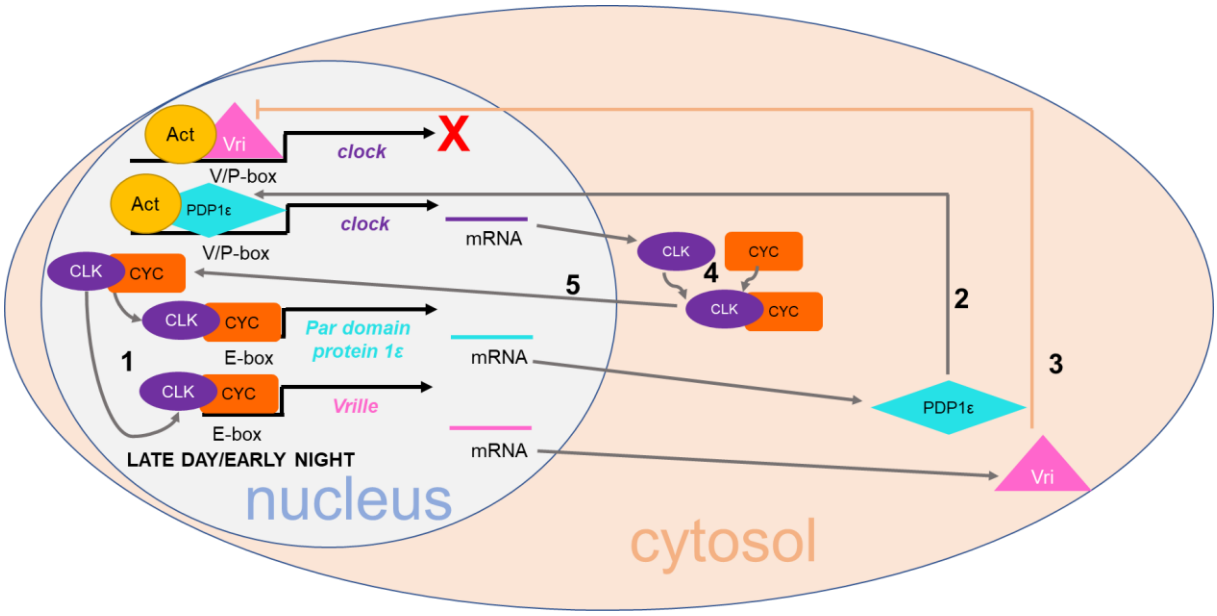


Figure 5. Scheme of the CLK negative feedback loop. 1) CLK-CYC binds to the E-box of Par domain protein 1ε (PDP1ε) and Vrille (Vri). 2) Binding of PDP1ε to the V/P-box of *Clk* is facilitated by activator (Act) and induces CLK synthesis. 3) Binding of Vri to the V/P-box of *Clk* is facilitated by activator (Act) and inactivates CLK synthesis. 4) CLK and CYC bind together and 5) translocate from the cytoplasm to the nucleus to act as transcription factors in new cycle of the feedback loop.

It has been shown that the *Drosophila* mutants *per*^{01}, *cyc*^{01}, *Clk*^{Jrk} and *dbt* do not develop LS to repeated COC administration, while their response to acute exposure was same as in *wt* flies (Andretic et al. 1999). Because LS of *tim* mutants was the same as in *wt* flies, it suggested that circadian genes regulate COC-induced LS through a different mechanism than the one for circadian regulation. The same phenotype was also observed in mice (Abarca et al. 2002). Mice with a mutation in *Per1* do not develop LS to repeated COC injections, while *Per2* mutants, in contrast, show hypersensitivity to the same treatment. Reward effect of COC, as measured by the CPP assay was abolished in *Per1* mutant but present in *Per2* mutant mice. *Clock*Δ19 mutant mice have normal COC sensitization and an increased preference for cocaine (Abarca et al. 2002).

c) Pigment-Dispersing Factor Signaling

Despite the evidence supporting a synchronizing function of pigment-dispersing factor (PDF) within the clock neuron network (Edery et al. 2000), other observations suggest a more complex role for PDF in circadian timekeeping (Shafer et al. 2014).

One possible mechanism is through PDF feedback to suppress CLK activity and induce its own transcription (Mezan et al. 2016, Mertens et al. 2005).

The *pdf*^{01} mutants are arrhythmic, indicating that the ability to produce endogenous circadian rhythms is dependent on PDF (Renn et al. 1999). The *pdf*^{01} mutants, on the other hand, show normal sensitivity to COC (Heberlein et al. 2009, Tsai et al. 2004).

After method optimization, we have used *pdf*^{01} and *tim*^{01} mutants as positive controls, while *per*^{01}, *cyc*^{01} and *Clk*^{Jrk} were used as negative controls for vCOC induced LS. Same endophenotype was tested on vMETH using *per*^{01}, *cyc*^{01}, *Clk*^{Jrk}, *pdf*^{01} and *tim*^{01} mutants.

1.2.2.2. Circadian genes and monoamine synthesis

In mammals, DA neurotransmission is under the influence of circadian genes in presynaptic neurons, largely because they can act as transcription factors for proteins involved in DA synthesis and degradation (Figure 6). Several mechanisms have been proposed through which circadian genes may influence rhythmicity, namely DA synthesis trough TH, DA release trough D2 auto-receptors or DA degradation by MAO.

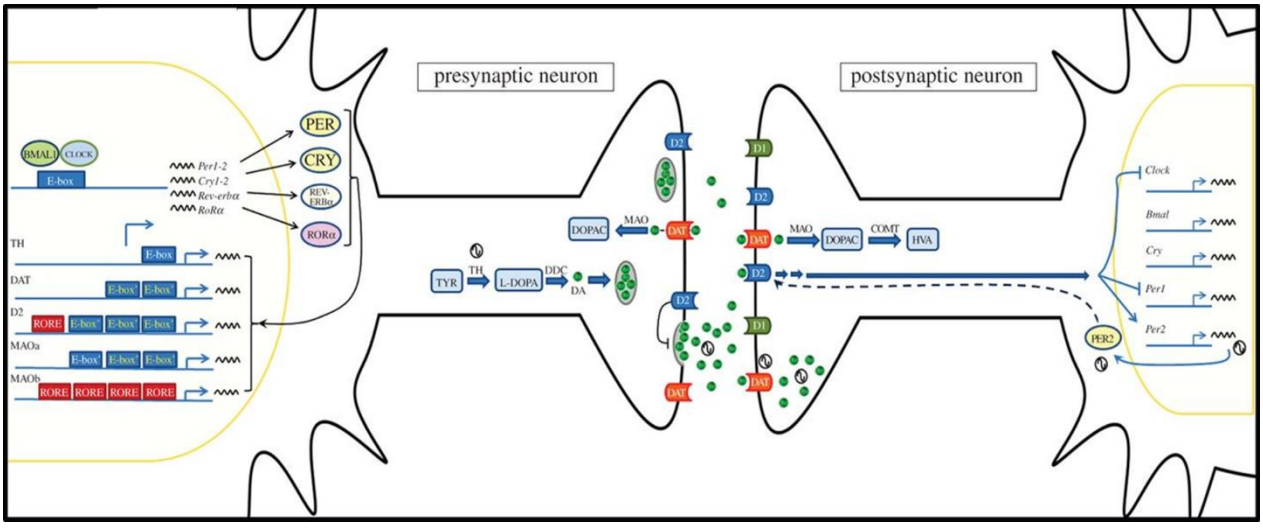


Figure 6. Possible mechanism of circadian genes influence on DA synthesis, release and degradation. COMT - Catechol-O-methyl transferase; D1 - DA receptor type 1; D2 - DA receptor type 2; DA - dopamine; DAT - DA transporter; DDC - DOPA decarboxylase; DOPAC - 3,4-dihydroxyphenylacetic acid; E-box - non-canonical E-box; HVA - homovanillic acid; MAO - monoamine oxidase; ROR - retinoid-related orphan receptor; RORE - ROR response element; TH - tyrosine hydroxylase; TYR - tyrosine. Mammalian model (Golombek et al. 2014).

Reciprocal regulation has also been observed where expression of clock genes *per* and *cry* is affected by DA, through the activation of D2 receptors on the postsynaptic neuron (Golombek et al. 2014).

1.2.2.3. Circadian genes and redox changes

Numerous lines of evidence strongly suggest interaction and relationship between oxidative stress and circadian rhythms (Fanjul-Moles and López-Riquelm 2016). Reactive oxygen species can be produced in cells endogenously as a by-product of mitochondria metabolism or induced environmentally by exposure to ultra violet (UV) light or chemical pollutants. Cellular concentrations and activity of antioxidant enzymes and other protective small molecules have been found to have circadian rhythmicity (Díaz-Muñoz et al. 1985). Circadian genes contain an evolutionary conserved PAS (**P**er: period; **A**RNT: aryl hydrocarbon receptor nuclear transporter; **S**im: single-minded protein) domain. PAS is a modular domain consisting of PAS sensor (input) domains that can detect physical and chemical stimuli, such as oxygen, redox potential or light. PAS sensor regulates the activity of an effector domain, which can include catalysis or DNA binding (Möglich et al. 2009). It has been reported that *Drosophila* per^{01} mutants show elevated oxidative stress (Krishnan et al. 2008), while elevation of ROS was reported for mice lacking clock protein BMAL1 (Kondratov et al. 2006). *Drosophila* circadian mutants per^{01}, cyc^{01}, Clk^{Jrk} and *dbt* do not develop LS to repeated COC administration (Andretic et al. 1999), while METH-induced expression of *per* gene in mice (Nikaido et al. 2001) is followed by an increase in ROS (Miyazaki and Asanuma 2008). It is therefore reasonable to suggest that circadian genes expression is under the regulation of ROS and that in this scenario, ROS represent a positive transcription factor.

1.2.3. Psychostimulant induced oxidative stress

Imbalance in cell redox status, caused by increased level of oxidants, leads to oxidation of proteins, lipids or DNA, and ultimately causes cell dysfunction. COC and METH induce redox imbalance (Figure 7) by producing ROS through auto-oxidation or enzyme oxidative metabolism of monoamines, primary DA (Miyazaki and Asanuma 2008), and their oxidative metabolites ultimately causing mitochondrial dysfunction (Cunha-Oliveira et al. 2013).

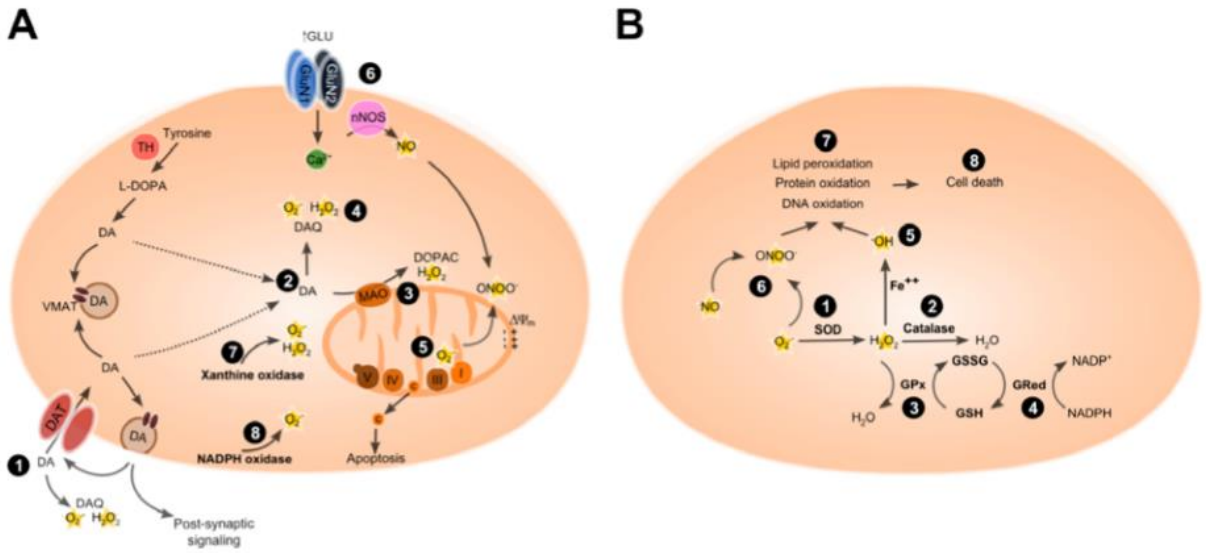


Figure 7. ROS formation induced by COC and METH. A) Central nervous system cell. 1) COC induced release of DA 2) METH increases intracellular DA. DA can be 3) oxidized by MAO to form DOPAC and H_2O_2 or 4) auto-oxidized to form H_2O_2, superoxide ($O_2^{\cdot-}$) and reactive dopamine quinones (DAQ). Through the influence of the mitochondria respiratory chain O_2^- can be produced 5) and cause overstimulation of N-methyl-D-aspartate (NMDA) glutamate receptors 6). Additional sources of ROS include xanthine oxidase 7) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 8). **B)** Cellular antioxidant system.1) Superoxide dismutase (SOD) converts superoxide to H_2O_2, which is further converted to H_2O through 2) catalase (CAT) or 3) glutathione peroxidase (GPx), by using glutathione regenerated by 4) glutathione reductase (GRed). In the presence of transition metal ions, such as Fe^{2+}, H_2O_2 can be converted to a hydroxyl radical ($OH^{\cdot-}$) 5). $ONOO^-$ is generated by the reaction of NO with $O_2^{\cdot-}$. ROS products react with cell proteins and DNA 7) ultimately causing cell death 8). (Cunha-Oliveira et al. 2013).

1.2.3.1. Psychostimulant induced neurotoxicity

a) Cocaine

Mammalian research has shown reduced catalase activity after COC administration (Macedo et al. 2005), followed by higher SOD activity, causing increase in ROS production (Dietrich et al. 2005). COC exposure has been reported to increase hydrogen peroxide (H_2O_2) levels in the prefrontal cortex and in the striatum of rats (Dietrich et al. 2005).

b) Methamphetamine

Increased levels of DA in synaptic cleft, caused by exposure to METH, are metabolized mainly by auto-oxidation, causing the generation of ROS and dopamine

quinones (Kita et al. 2003; 2009, Moszczynska 2017). METH-induced ROS production is DA dependent, as demonstrated by the fact that non-dopaminergic neurons do not show enhanced ROS production (Larsen et al. 2002). In addition to ROS produced by METH (Yamamoto et al. 2008, Fleckenstein et al. 1997), nitric oxidative species have also been reported (Kovacic 2005). METH induces an increase in levels of CAT and protein carbonyls in the brain (Koriem et al. 2012), whereas it decreases SOD activity (Frenzilli et al. 2007).

c) H_2O_2 as a neuromodulator

Through the increasing use of metabolomic methods, more evidence is accumulating that metabolites and metabolic by-products can act as signaling molecules. One example is H_2O_2 (Rice 2011), which is small and rapidly diffusible in cells, commonly taken as ROS source since H_2O_2 degradation can lead to water or hydroxyl radical (OH•) formation. H_2O_2 can modulate neuronal activity by affecting the transmitter release (Lee et al. 2015). Similar evidence was also found in *Drosophila,* where it was proposed that DA regulates the response to oxidative stress (Hanna et al. 2015).

In vitro studies have shown that endogenously generated H_2O_2, trough metabolism and the mitochondria respiratory chain can modulate vesicular neurotransmitter release of DA (Chen et al. 2001). Exogenously applied H_2O_2 can reversibly inhibit the evoked release of DA (Chen et al. 2001, Patel and Rice 2012). These data demonstrate an important new signaling role for ROS in synaptic transmission.

1.2.3.2. Monoamines as ROS scavengers

The chemical properties of monoamines that can be seen *in vitro* are in contrast to those found *in vivo*. Based on their chemical structure, all biological monoamines and their precursors show strong antioxidant activity *in vitro* (Gow-Chin and Chiu-Luan 1997). Additionally it was found that, due to their catechol structure, which is widely distributed in many naturally occurring antioxidants, these monoamines could scavenge ROS (Shimizu et al. 2010).

Several studies, discussed above, led to an emphasis on the effect of PS on ROS production induced by increased DA removal from synaptic cleft via oxidative

metabolism, which can be reduced by adding antioxidants such as TEMPOL (Jang et al. 2015). Together, these results indicate a dual role of monoamines in ROS production and scavenging, with implications for neuronal plasticity induced by drugs of abuse.

1.2.3.3. Exogenous antioxidant and prooxidant effects

Due to the very low permeability of many antioxidants, and the influence of blood-brain-barrier on its susceptibility, exogenous antioxidants ultimately fail to protect the brain from oxidative stress (Shimizu et al. 2010). Imbalance in the levels of free radicals, common to many neurodegenerative diseases (Valko et al. 2007), as well as drug addiction (Cunha-Oliveira et al. 2013), can affect normal ROS removal, which can ultimately lead to long lasting neurological changes (McCord 2000).

a) Polyphenols and antioxidants

Many naturally occurring antioxidants, and especially polyphenols, have been reported to have beneficial effects on health (Costa et al. 2016, Lobo et al. 2010, Poljsak et al. 2013, Rodríguez-Morató et al. 2016) and neurodegenerative diseases (Bhullar et al. 2013, Solanki et al. 2015, Vauzour 2012). Recent studies also show an effect of ROS scavengers on phenotypes related to the drug addiction. One example of this is TEMPOL, a molecule known to have ROS scavenging properties. By using TEMPOL, it was reported that locomotor sensitization in rats, induced by COC and METH, can be abolished, leading to the reduced production of ROS (Jang et al. 2015, Jang et al. 2017, Numa et al. 2008). Some naturally occurring antioxidant molecules have also been tested for the ability to lower ROS upon drug administration. Vitamin C, commonly known as a naturally occurring antioxidant, was shown to reduce METH-induced oxidative stress in the mammals (Huang et al. 2017). Another example is luteolin, polyphenol molecule, which was reported to inhibit behavioral sensitization to METH (Yan et al. 2014).

In this thesis, we have tested two molecules with potential antioxidant properties quercetin and tyrosol, and their effect on SENS and LS after METH and COC exposures. There is evidence to indicate that similar biological properties would be expected for quercetin and luteolin based on their structural and chemical similarities

(Cotelle 2001, Galati et al. 2001), and for naturally occurring polyphenol tyrosol, which has the same catechol structure as monoamines and has ROS scavenging properties (Domínguez-Perles et al. 2017, de la Torre et al. 2006).

b) Pro-oxidants paraquat and hydrogen peroxide

In *Drosophila* research, paraquat (PQ) is commonly used as an agent for inducing oxidative stress and modelling neurodegenerative diseases (Bonilla et al. 2006, Vrailas-Mortimer et al. 2012). Flies exposed to PQ show movement disorders (Chaudhuri et al. 2007), and have decreased food intake (Ja et al. 2007). This suggests that PQ is sufficient to induce oxidative stress, even at small dosages and short exposures. In this thesis, it is used as a way to induce oxidative stress and to measure the consequence on SENS and LS after METH and COC exposures.

Another approach to induce oxidative stress in flies is to feed them with hydrogen peroxide (Bonilla et al. 2006). It has been reported that flies continuously fed with hydrogen peroxide will initially increase their locomotor activity and subsequently their daily locomotor activity rhythms will became suppressed (Grover et al. 2009). Hydrogen peroxide is sufficient to induce oxidative stress, even in small dosages and for short exposures. In this work we used H_2O_2 as a way of inducing oxidative stress and measured the consequence on SENS and LS after METH and COC exposures.

2. AIMS

The main aim of this thesis is to identify molecular mechanisms of neuronal plasticity induced by psychostimulants. The thesis covers two endophenotypes that are relevant for development of addiction: locomotor sensitization and rewarding effects of psychostimulants. The focus is in describing characteristics of the COC- and METH-induced neuronal plasticity, and relation between the mechanism of locomotor sensitization and rewarding effect. In particular, I investigate the function of circadian genes, monoaminergic regulation and redox balance in the development of psychostimulant-induced plasticity. The activities were planned in five main aims.

The first aim is the development and optimization of a new method for inducing and quantifying sensitivity (response to an acute dose of a drug) and locomotor sensitization (response to repeated treatment) to volatilized COC and METH in *Drosophila melanogaster*. By applying the newly developed test, I will test if the vesicular monoamine transporter, dopamine receptor type 1 and dopamine transporter regulate sensitivity and locomotor sensitization to vCOC and vMETH, since these proteins are known targets in mammals. The involvement of monoamines in the development of sensitivity and sensitization to vCOC and vMETH will be tested by pharmacological reduction of dopamine levels using 3-iodo tyrosine (3IY), and reduction of dopamine, serotonin and octopamine levels using reserpine (res).

The second aim is to test if dependence of locomotor sensitization on the function of a selected group of circadian genes is linked with redox balance. To address this question, the requirement for circadian genes for development of sensitization to vCOC and vMETH will be tested. The influence of acute and repeated exposures of vCOC and vMETH on redox status will be determined by measuring CAT and SOD activity, and ROS and hydrogen peroxide production in *wt* flies. The influence of exogenous pre-treatment with pro- and antioxidant will be tested by investigating the development of sensitization to vCOC and vMETH in *wt* flies. The effectiveness of the anti- and prooxidants will be determined using the free radical scavenging DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, and compared to the effects that these substances have on locomotor sensitization to vCOC and vMETH. Finally, the redox status markers in non-treated flies and flies pre-treated with anti- and prooxidants will be compared with their degree of sensitization to vCOC and vMETH.

The third aim is to test the hypothesis that exogenous H_2O_2 can act as a neuromodulator. The level of perturbation induced by exogenous H_2O_2 will be determined by measuring CAT, SOD, ROS and hydrogen peroxide production. Behavioural responses to repeated vCOC and vMETH exposures will be measured in flies pre-treated with H_2O_2. In order to confirm the neuromodulator properties of H_2O_2, flies will be pre-treated with an antioxidant and H_2O_2, before being tested for sensitization to vCOC and vMETH.

The fourth aim is to test if flies voluntarily self-administer COC and METH by applying the modified capillary feeder (CAFÉ) assay. Positive outcome would indicate that psychostimulants in flies, as in mammals, act on the motivation for drug taking. If the preferential consumption of psychostimulants is established then the strength of the preference will be tested by increasing the aversiveness to psychostimulants, and by testing if flies show elements of relapse after drug deprivation.

The fifth step is to determine the role that circadian genes, the vesicular monoamine transporter, dopamine receptor type 1 and dopamine transporter have on preferential consumption of COC and METH, in order to define common genetic elements between two forms of neuronal plasticity, preferential consumption and locomotor sensitization.

3. METHODS

3.1. Fly stocks

All behavior and biochemical assays were performed on 3 to 5 day-old flies of the wild type *CantonS* background. Flies were raised in a light:dark (LD) cycle (12 hours:12 hours) at 25°C, 70% humidity on a standard cornmeal/agar medium. Circadian mutant strains used were: per^{01}, tim^{01}, cyc^{01}, Clk^{Jrk} and pdf^{01}. The autosomal recessive mutation in the dopamine transporter (DAT) used was *fumin (fmn)* (Kume et al. 2005). The mutant for the dopamine receptor type 1 (DR1) was *dumb* (Faville et al. 2015*).* Flies carrying transgenic constructs *UAS*-VMAT RNAi and *DDC-GAL4* were kind gifts from S. Birman.

3.2. Reagents and general procedures

The psychostimulants cocaine-hydrochloride (≥97.5%) and methamphetamine-hydrochloride (≥98%) were purchased from Sigma Aldrich. 96% ethanol used for preparing of psychostimulants stock solutions was purchased from VWR. Reserpine (res) (≥99%), 3-iodo-L-tyrosine (3IY) (≥95%), 3,4-dihydroxy-L-phenylalanine (L-DOPA) (≥98%), dopamine-hydrochloride (DA) (≥98%), tyramine-hydrochloride (TYRA) (≥98%), hydrogen peroxide (H_2O_2) (30%), paraquat (PQ) (≥98%), quercetin dihydrate (QUE) (≥95%), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL) (98%), quinine (QIN) (90%) and tyrosol (TYR) (98%) were purchased from Sigma Aldrich.

Fly food was prepared according to following recipe: 1 L of molasses food was prepared by adding 15 g of sugar, 35 g of dry yeast, 12 g of Agar type I and 60 mL of 50% molasses water solution to 970 mL of tap water. Mixture was cooked on the induction plate for 15-20 minutes. To prevent mold growth after cooking, 7.5 mL of propionic acid (Sigma Aldrich, 99%) and 7.5 mL of p-hydroxybenzoic acid methyl ester (NIPAGINE, Roth, 99%) in 15% ethanol solution were added.

3.2.1. Substance pre-treatments

To prepare food with supplements, supplemental stock solution/s were added to the 50 mL plastic tube and mixed with 10 mL of liquid molasses food. The mixture

was stirred and vortexed at 1,400 rpm for one minute to avoid the possibility of the final concentration of supplement in food not being evenly homogeneous. After cooling, the same preparation was used both for pre-treatment of flies and for preparing of DAM system tubes.

Table 1. Food supplements used in pre-treatments of flies.

Molecular properties	Supplement	Concentration in fly food	Time of exposure to substance before assay
Blocking VMAT	Reserpine	10 μM	48 h (Bainton et al. 2000, Andretic et al. 2005)
Blocking TH	3IY	5 mg/mL	
Dopamine precursor	L-DOPA	1 mg/mL	
Blocking VMAT + dopamine precursor	L-DOPA + reserpine	1 mg/mL+10 μM	
Prooxidants	H_2O_2	0.4 %	17 h (Jang et al. 2015, 2017, Chaudhuri et al. 2007, Grover et al. 2009)
	PQ	4 mM	
Antioxidants	TEMPOL	3 mM	
	QUE	4.8 mM	
	TYR	12.45 μM	
Pro- and antioxidants	H_2O_2 + QUE	0.4 % + 4.8 mM	
	H_2O_2 + TYR	0.4 % +12.45 μM	

3.2.2. Collecting flies for behavioral assays

Each assay was done with flies of a single sex, and sorting of flies was done using carbon dioxide (CO_2) platform under the microscope using a small paint brush for no more than 5 minutes. Selected flies were transferred to new culture vial for their recovery from anesthesia, at least 2 hours to a day before the start of the behavioral assays.

3.2.3. Preparation of tubes for DAM system

DAM system tubes (diameter 5 mm, length 65 mm, TriKineticks) are made from polycarbonate with two small holes at the side of the tube to enable air flow. Regular food or food with supplements was taken out of a plastic vial and cut into 5 mm thick slices. Food was then left on paper tissue for not more than 5 minutes in order to adsorb excess fluid from the food. This is important, as flies can stick to wet food during transfer from the culture vial to the DAM tube. Polycarbonate DAM tubes were pressed vertically onto the food surface in order to push food into the tube. The food end was preserved from drying out by wrapping a small piece of parafilm wrap around it. Holes

on the tubes were oriented to food end to enable normal airflow and waste draining from the system during the assay.

An aspirator was used to transfer flies from the culture vial to the DAM tubes. In order to collect flies, the vial containing the flies was turned upside down and the pipet tip of the aspirator was put inside in such a way as to prevent the flies from escaping from the vial, while still allowing them to be collected by applying negative pressure. After collecting the flies, positive pressure was applied in order to transfer the fly from the aspirator into the DAM system tube. Flies were transferred to DAM tubes at least 12 hours before the assay in order to habituate to the new environment.

3.3. Behavior assays development and optimization

This section provides complete information on construction, optimization and execution of behavioral test for measuring motor/activating effects (FlyBong) and reward effect (CAFÉ assay).

3.3.1. FlyBong platform

The platform for psychostimulant administration and locomotor activity monitoring consists of: a volatilization chamber, a *Drosophila* activity monitor connected to the DAM system (TriKinetics) and a psychostimulant delivery system (Figure 8). The volatilization chamber (a 250 ml three neck flask, VWR) has a heat cap (SAF, LabHEAT, KM-G, for 250 mL flask), which is used to volatilize cocaine (COC) or methamphetamine (METH) by heating it for 8 min at 185-200 °C. The side necks of the volatilization chamber are connected via glass and rubber tubes to the *Drosophila* activity monitor (DAM) on one side, and an air pump (Crawfish 1800 air pump), as a psychostimulant delivery system on the other side. After the 8 minute heating period, the air pump was applied for 1 minute with air flow rate of 2.5 L/min, in order to deliver volatilized PS.

The central neck of the volatilization chamber is used for applying the psychostimulant-ethanol solution (PS are dissolved in 96% ethanol at a concentration of 10 mg/mL). To eliminate potential effect of ethanol fumes on fly behavior, the psychostimulant-ethanol solution was added into the volatilization chamber 4 to 6 hours before drug administration, to ensure that the ethanol evaporates. This assay

uses vertical DAMS monitors (TriKinetics, Waltham, MASS) with a disperser (Gas Distribution Manifold, TriKinetics) and polycarbonate tubes. The rubber tube which is connected to the volatilization chamber and monitor disperser is closed by clamp during the heating period, in order to eliminate the leaking of PS before it can be administered by air pump.

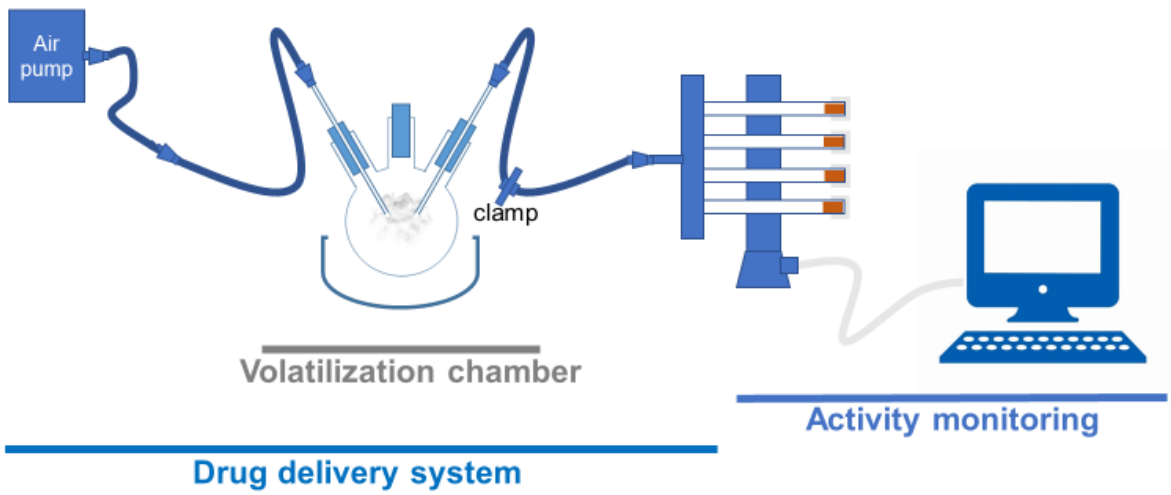


Figure 8. Scheme of the FlyBong platform for measuring changes in locomotor activity of *Drosophila* after delivery of vCOC and vMETH. PS dissolved in ethanol is pipetted into three-neck flask, which is then heated to the PS volatilization temperature. Single flies are housed in the individual tubes of the vertical *Drosophila* monitor (TriKinetics) and exposed to vCOC or vMETH for one minute by turning on the air pump and removing the clamp. Their locomotor activity is monitored as the number of crossings of the midline of the tube per minute for 30 minutes before and 30 minutes after PS exposure.

3.3.1.1. Optimization of volatilization temperature and psychostimulant distribution

Cocaine and methamphetamine in hydrochloride form begin to volatilize at around 185 °C. The time at which the heating cap of the FlyBong platform reaches this temperature, was tested using an empty system, with a thermometer in the central neck of the three-neck flask, placed 1-5 mm from the bottom of flask.

To test the homogeneity of distribution and stability of PS after volatilization, empty tubes without flies or food were immersed in 2 mL Eppendorf tubes filled with 500 µL of distilled water. For this test, the optimized protocol was applied (8 minutes of volatilization, 2.5 L/min air flow, 1 minute of drug delivery and 75 µg of PS). After the volatilization, all tubes were detached from the dispenser and washed with an additional 500 µL of distilled water. Tubes were additionally vortexed for 1 minute at 2,500 rpm. The monitor and dispenser are arranged with 4 rows and 8 columns in each row, so that for all 32 samples the UV-VIS spectra were recorded. 500 µL of each sample was recorded in triplicate using a 48-well plate and microplate reader TecanInfinitePro200 at λ_{max}=275 nm for COC and λ_{max}=250 nm for METH. Similarly, the amount of PS on each fly in the monitor was determined. For this, we have used flies exposed to optimized protocol. After PS exposure flies were frozen at -20 °C and transferred in empty 2 mL Eppendorf tubes filled with 300 µL of distilled water. Tubes containing flies were additionally vortexed for 1 minute at 2,500 rpm and 250 µL of each sample was recorded in triplicate using a 48-well plate and microplate reader TecanInfinitePro200 at λ_{max}=275 nm for COC and λ_{max}=250 nm for METH. To test the stability of PS after volatilization, we performed UV-VIS on random PS samples after volatilization using Cary 60 UV-VIS in range from 200-800 nm.

3.3.1.2. Optimization of behavioral response

a) Air flow strength and duration

To test the influence of airflow on the behavior, different airflow strengths (2.5-12 L/min) were tested using the FlyBong platform. This test was important since flies are sensitive to bursts of air (Lebestky et al. 2009). Additionally, after heating of the volatilization chamber for 8 minutes (without psychostimulant or ethanol), various lengths of exposure of the flies to 2.5 L/min warm air flow were tested, ranging from 10 seconds to 7 minutes. Flies are sensitive to dehydration (Sayeed and Benzer 1996), so it is important to optimize their response to the duration of exposure to potentially dehydrating warm air. The optimum airflow was found to be 2.5 L/min, and the optimum duration of exposure to warm air after heating was 1 minute. The influence of evaporated ethanol (75 µL), present as solvent in PS stock solutions, was then tested in order to exclude the possibility that it may interfere with the behavior of the flies. The flies displayed an increased response following acute exposure to ethanol vapor (Parr

et al. 2001), and a control experiment was therefore performed to check that ethanol was not present in the system after an evaporation period of 6 hours and that any behavioral response was induced solely by the volatile PS.

b) Response to acute exposure

Using the optimized protocol (8 minutes of volatilization, 2.5 L/min air flow and 1 minute of drug delivery) different amounts of PS were tested, ranging from 0-200 µg. This was in order to determine the amount that will lead to a significant increase in the locomotor activity of the flies, when compared to the baseline and the activity of the control group, at both the individual and population level. All tests were performed at 09:00 in the morning. Flies show increased locomotion after acute exposure to vCOC (Bainton et al. 2000, Heberlein et al. 2009, McClung and Hirsh 1998), and same was observed for vMETH.

c) Response to repeated exposures

Different time interval between two exposures of flies to PS (3-30 hours) were tested for each PS, in order to determine which would lead to LS (8 minutes of volatilization, 2.5 L/min air flow,1 minute of drug delivery and 75 µg of PS). Data were analyzed at both the population and individual level.

Multiple administrations of vCOC and vMETH were performed using the same standard protocol as for single administrations (8 minute volatilization, 1 minute delivery, 2.5 L/min air flow), but with lower levels of COC (45 µg) and METH (25 µg). COC and METH were each administered three times: at 09:00, at 19:00 and at 09:00 on the following day.

d) Circadian modulation of response on acute and repeated exposures

To test the circadian modulation of sensitivity and locomotor sensitization, vCOC and vMETH assays were performed in constant dark conditions (DD), with flies previously entrained to 12 hours light:12 hours dark, using the standard protocol and 12 hours between exposures. Flies were in DD for one day before the experiment. The first group of flies in DD was exposed to the initial dose of vCOC or vMETH at 10:00, 2 hours after the light would have come on in a light:dark cycle, and to the second dose

at 22:00, two hours after light would have turned off in a light:dark cycle. The second group received the initial dose at 22:00 and the second at 10:00. All experimental manipulations were performed under dim red light.

3.3.1.3. Screening using the FlyBong platform

To validate FlyBong protocol we tested the involvement of: a) circadian gens, b) genetic and pharmacological manipulation of aminergic system, and c) prooxidant and antioxidant treatment, and measured sensitivity and LS of flies after exposure to vCOC and vMETH.

Sensitivity (SENS) screens were based on one administration of COC or METH at 09:00. LS screens required repeated administrations, which were 6 hours apart in case of COC, and 10 hours apart for METH, with first administration always at 09:00 (Table 2).

Table 2. Optimized protocols for inducing SENS and LS using FlyBong.

Psychostimulant	**COC**	**METH**
Volatilization temperature	≥185°C	
Heating period	8 min	
Air flow strength	2.5 L/min	
Duration of air flow	1 min	
Volume of 10 mg/mL psychostimulant-ethanol stock solution	75 µL	
First administration	09:00	
Second administration	15:00	19:00
Experimental conditions	25°C, 70 % humidity, LD 12:12	

3.3.1.4. DAM system data collection, analysis and interpretation

The DAM monitor captures locomotor activity of 32 flies simultaneously using Data Acquisition Software. An infrared beam placed in the center of each tube measures the number of crossings made by the individual fly in a one minute interval, and is recorded as number of counts per minute (Pfeiffenberger et al. 2010). Locomotor activity counts are collected (Table 3) on a computer using a PSIU9 Power Supply Interface Unit (TriKinetics). Using DAMFileScan software, the stored data are extracted, based on given parameters (Table 3). Raw data, in the form of .txt files, collected by the DAM system were analyzed at either the population or individual fly level using Microsoft Office Excel 2016. Data recorded during 8 minutes of heating and

1 minute of drug delivery were excluded from both population and individual data analysis.

Table 3. Summary of analyzed time periods for COC and METH.

	Time period of raw data collection		Duration (min)
	COC	METH	
Before or baseline	08:22-08:51	08:22-08:51	30
Heating period	08:52-08:59	08:52-08:59	8
Drug delivery	09:00	09:00	1
After 1st administration	09:01-09:30	09:01-09:30	30
Before or baseline	14:22-14:51	18:22-18:51	30
Heating period	14:52-14:59	18:52-18:59	8
Drug delivery	15:00	19:00	1
After 2nd administration	15:01-15:30	19:01-19:30	30

3.3.1.4.1. Population data analysis

For population-based data analysis, raw data was analyzed as a mean number of counts per minute from 32 flies, at a 1 minute resolution, both 30 minutes before and 30 minutes after the administration of PS. Locomotion was plotted on a kinetic graph as counts/min against time, from which conclusions can be made about the kinetics of perturbation induced by PS administration.

Table 4. Calculations used in population data analysis and comparisons needed for SENS and LS determination.

		vCOC	**vMETH**
Time interval		**5 minutes AVERAGE**	**10 minutes AVERAGE**
Number of flies		**32**	**32**
SENS	*Calculation*	Before – AVE 5 min After - AVE 5 min	Before – AVE 10 min After - AVE 10 min
	Comparison	a) Before to after in control (*wt* flies, mutants and treatments) b) After in control to after in vCOC c) After vCOC in *wt* flies to after vCOC in mutants and treatments	a) Before to after in control (*wt* flies, mutants and treatments) b) After in control to after in vMETH c) After vMETH in *wt* flies to after vMETH in mutants and treatments
LS	*Calculation*	Before- AVE 5 min After 1st - AVE 5 min After 2nd - AVE 5 min	Before- AVE 10 min After 1st - AVE 10 min After 2nd - AVE 10 min
	Comparison	a) After 1st to after 2nd in control (*wt* flies, mutants and treatments) b) After 1st to after 2nd vCOC in *wt* flies c) After 1st to after 2nd vCOC in mutants and treatments	a) After 1st to after 2nd in control (*wt* flies, mutants and treatments) b) After 1st to after 2nd vMETH in *wt* flies c) After 1st to after 2nd vMETH in mutants and treatments

Kinetic graphs give the information's about the strength and duration of PS administration on locomotion for the population.

As the strongest change in the locomotor activity after vCOC occurs within first 5 min after administration the comparisons were performed using data from the 5 minutes immediately before and after vCOC administration (Table 4). The data were then combined, and plotted as histograms of average counts per minute (from 32 flies) in the 5 minutes immediately before and after COC administration for SENS. For LS determination, data obtained 5 minutes before and after the first and second administrations were used.

Population analysis of METH was based on the data obtained 10 minutes before and 10 minutes after administration, since the effect of the optimized protocol on fly behavior detectable on the kinetic graph was significantly different from baseline during first for 10 min after administration. LS determination data was obtained 10 minutes before and after the first and second administrations. For population data analysis, we compared flies exposed to volatilized PS to the control group (who were exposed to hot air and evaporated ethanol only).

3.3.1.4.2. Individual data analysis

The individual fly response compares averaged counts/min of an individual fly (in the first 5 minutes in the case of COC and in the first 10 minutes in the case of METH), before and after PS administration (first and/or second). From this data set, several behavioral properties can be objectively calculated at the individual level (Table 5).

Sensitivity (SENS) was calculated as the number or percentage of the flies, which increase their activity when baseline locomotor activity (B) was compared to the activity after the first administration of PS (A1). Individual fly data was calculated as the average during the 5 minutes before and after administration of COC, or 10 minutes before and after administration of METH. Activity was then categorized as either the "same", "decreased" or "increased" following the administration. The flies that increase their activity represent the subset of the population that responds to the psychostimulant.

Table 5. Criteria for calculation of SENS and LS in the individual data analysis.

		COC	METH
Time interval		**5 minutes AVERAGE**	**10 minutes AVERAGE**
Number of flies		**1**	**1**
SENS	*Criteria*	B < A1	B < A1
	Calculation	SENS% = number of SENS / total number of flies in assay	SENS% = number of SENS / total number of flies in assay
	Comparison of wt flies with	a) control b) different mutants c) treatments	a) control b) different mutants and c) treatments
LS	*Criteria*	B < A1< A2	B < A1< A2
	Calculation	LS% = number of LS / total number of flies in assay	LS% = number of LS / total number of flies in assay
	Comparison of wt flies with	a) control b) different mutants c) treatments	a) control b) different mutants c) treatments

The same comparison can be performed for differences in behavior after first and second psychostimulant administration, with the caveat that the flies that respond to the second dose my not necessary include those who responded with an increased activity to the first dose. Therefore, comparison between first and second exposure only indicates sensitivity to the second exposure.

To calculate the number or percentage of flies that develop LS upon repeated psychostimulant administration, flies that show stepwise increase in locomotor activity: before (B) < after 1st (A1) < after 2nd (A2) are included. These same criteria were used for calculation of locomotor sensitization in individual flies that received more than two doses of psychostimulant.

3.3.1.4.3. Statistical data analysis

a) Population data

Population data statistical tests were performed in the program Statistica 13.3. Student t-test was used in the case of dependent samples (comparison within group). For comparison between 2 groups Student t-test for independent samples was used. One-way Analysis of variance (ANOVA) was used to analyze differences in locomotion between groups exposed to different concentration of vCOC and vMETH with a post-hoc Dunnett test to determine the significant differences between a single control group and the remaining treatment groups. ANOVA for repeated measurements was used to

analyze differences in locomotion within one group before, after first and second exposure with a post-hoc Bonferroni multiple comparison test. The level of statistical significance was considered to be $p < 0.05$ if not stated differently.

b) Individual data

For testing the statistical difference in individual data Mann-Whitney U-test for nonparametric analysis of two independent samples was used. For statistical analysis of data obtained from 3 or more groups, Kruskal-Wallis H-test for nonparametric analysis of independent multiple samples was used followed by Dunn's post-hoc test. The level of statistical significance was considered to be $p<0.05$.

3.3.2. CAFÉ assay

Testing of voluntary PS consumption by flies, was performed using the previously developed Capillary Feeding (CAFÉ) assay for measuring ethanol preference (Devineni and Heberlein 2009, Zer et al. 2016). CAFÉ assay chambers were made from two main parts: a container for the flies and a feeding lid (Figure 9). The container was a 50 mL *Drosophila* tube, on top of which was a feeding lid made of plastic Petri dishes (Φ 25 mm) with 4 holes, into which 20-200 µL micropipette tips were placed. Each micropipette tip was adjusted to hold a 5 µL glass capillary (3.00 cm long and 1 mm diameter, Hirschmann) which can be easily place inside the tip, but will not drop through it.

Flies used in the CAFÉ assay were collected under CO_2 anesthesia and transferred into the chamber, for a total of 6 flies per chamber. On the bottom of each chamber, a wet cotton ball was placed to prevent dehydration (1/5 of cotton ball was wetted with 1 mL of tap water). 5 µL capillaries, used as a container for liquid food, were first dipped in mineral oil (Sigma Aldrich) to fill 2–3 mm of the capillary, preventing liquid food evaporation, and then in liquid food (100 mM sucrose solution) or the same liquid sucrose food supplemented with PS.

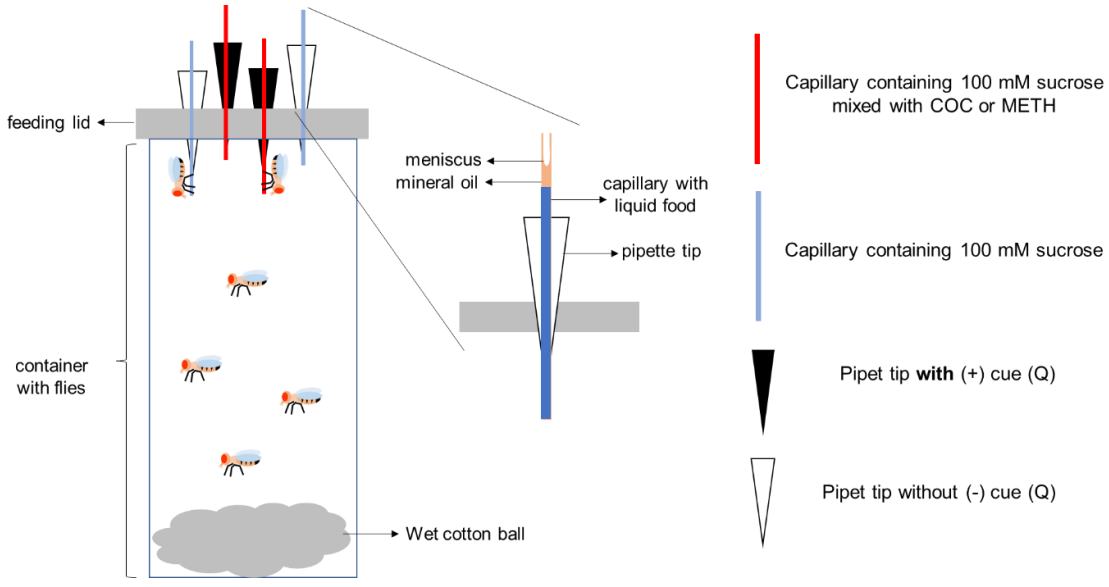


Figure 9. CAFÉ assay chambers.

Filled capillaries were wiped with tissue paper and the amount (in mm) of the food was measured using a ruler from the bottom of the capillary to the interface with mineral oil. The capillary was then placed inside of the pipette tip, with 2–3 mm of the capillary extending into the tube. Flies were then placed in an incubator at 25 °C, 70% humidity, light:dark 12:12, and after 24 hours the amount of remaining liquid food in each capillary was measured. This process was repeated each day for 3 to 5 days. On the first day, flies received sucrose in all capillaries, and from second day two capillaries contained PS.

3.3.2.1. Optimization of PS concentration

Since flies orally administered 100 mM sucrose solution supplemented with COC or METH, different concentrations were tested in order to determine concentration that will lead to the maximal preference. For COC, preferential consumption concentrations tested were: 0.05, 0.10, 0.15, 0.20, 0.50, 1.00, and 1.50 mg/mL, while METH concentrations tested were: 0.10, 0.20, 0.30 and 0.40 mg/mL. Testing was performed in naive flies (never exposed to PS before the assay), with the PS and liquid food locations fixed (every day in the same location), and without visual cues (Figure 10). Data were plotted as histograms of average preferential consumption over 4 days for each concentration of COC and METH, compared to that of the control group. These tests were necessary in order to eliminate the effect of the bitter taste of COC and METH, which is repulsive to flies (Amrein and Bray 2003).

Initial stock solutions of COC and METH were prepared in distilled water from HCl forms of both PS, at a concentration of 10 mg/mL. A water stock solution of sucrose was 1 M. All COC and METH concentrations tested were prepared by diluting these stock solutions using distilled water.

3.3.2.2. Influence of cue and capillary location

The first step was to test the influence of cues (Kaun et al. 2011) that correlate with drug addiction and underlying learning and memory (Sanchis-Segura and Spanagel 2006), associated with PS and drug preference. Cues represent environmental signals (such as odor, color or light) which are associated with either punishment or reward (Pitman et al. 2009). For the purpose of this research, the cue was a black colored pipette tip.

Additionally, it was tested whether, if the location of the cue was fixed, but associated with PS or liquid food differently on different the days, this had an effect on the preference.

3.3.2.2.1. Cue independent and dependent self-administration

In the non-cued assay, two capillary were filled with PS solution and two with sucrose solution, arranged diagonally with no marking on the pipette tips. In the cue location assay, the presence of PS was indicated with a black-colored pipette tip. Preference in both assays was measured over four consecutive days, and plotted as histogram of average daily preference to PS. Testing of the influence of cues (Figure 10) was performed using optimal COC (0.15 mg/mL) and METH (0.20 mg/mL) concentrations.

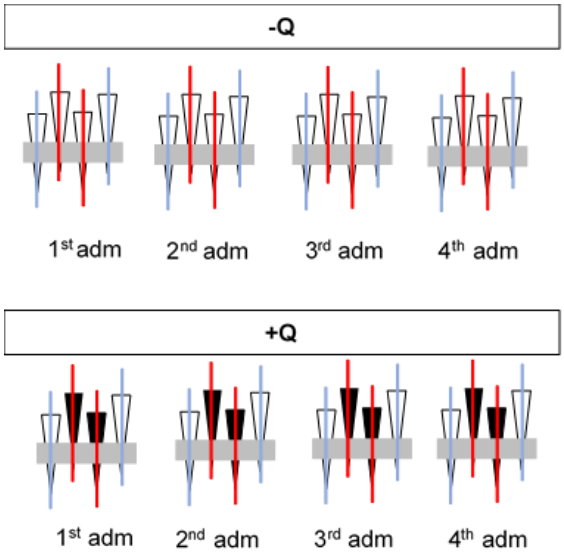


Figure 10. Scheme of the feeding lid, both without cues (-Q) and fixed capillary containing PS (red line) location, and with (+Q) fixed cues (black pipette tip) and fixed capillary containing PS (red line) location, over consecutive 4 days.

3.3.2.2.2. Capillary location

Since cues increased preference for COC and METH self-administration, we performed a series of control experiments with fixed or altered cue and capillary locations. Concentrations used were 0.15 mg/mL COC and 0.20 mg/mL METH.

a) Fixed cue and capillary location

The location of the cues and PS were fixed throughout the days of administration (Figure 11). Since the data for this approach showed an increased preference for PS compared to other approaches tested, fixed locations of both the cue and PS capillary were used for all further tests and screens, combined with optimized concentrations of PS.

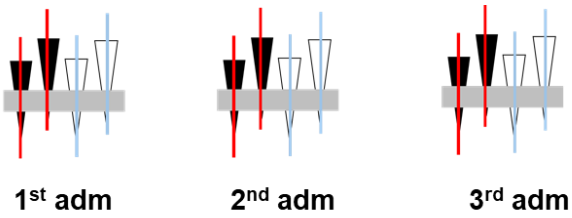


Figure 11. Scheme of the feeding lid with fixed locations of the cue (black pipette tip) and the capillary containing PS (red line), over 3 consecutive days. The pipette tip without a cue contained a capillary with sucrose solution (blue line), which was at the same location for all three days of the assay.

b) Fixed cue and altered capillary locations

In this approach the location of the cue was fixed, while the PS capillary location was changed, by switching the sides containing the PS and food capillary (Figure 12) or by diagonal rotation (Figure 13).

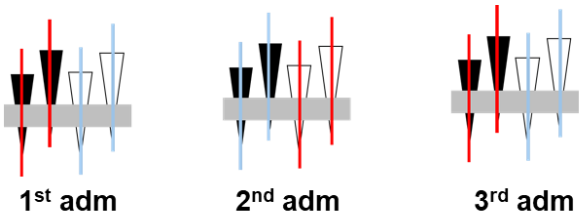


Figure 12. Scheme of feeding lid with fixed cues (black pipette tip), but changing of the sides of the capillary containing PS (red) over 3 consecutive days. Each day, the side where the PS and sucrose capillary was located is changed, with regard to a fixed cue location. On the first day of administration, the cue (black pipette tip) was associated with the PS capillary (red line), while on the second day regular food (blue line) was associated with cue, and the third day was the same as the first.

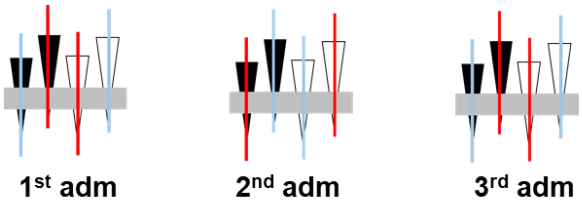


Figure 13. Scheme of feeding lid with fixed cues (black pipette tip) and diagonally changing location of the capillary containing PS (red), over 3 consecutive days. On the first day of administration, the cue (black pipette tip) was associated with one PS (red line) capillary and one regular food (blue line) capillary, as were the pipette tips without cues. On the second day, capillaries were rotated clockwise by 90°, meaning that the cue was associated again with one PS and one regular food capillary, but in a different order, as were the pipette tips without cues. The third day was the same as the first.

3.3.2.3. Testing features of addiction

Features of addiction, relapse and consumption in the presence of a negative consequence were tested using the optimized protocol: fixed cue location associated with PS over a period of 4 or 5 consecutive days, with concentrations of 0.15 mg/mL COC or 0.20 mg/mL METH.

2.3.2.3.1. Relapse induced by deprivation

To test for relapse following deprivation from a drug, the preference of flies was measured over 5 consecutive days, for both a drug-deprived group and a non-deprived control group. The non-deprived group received each day two capillaries with sucrose and two with sucrose and PS. The deprived group received two capillaries with sucrose and two with sucrose and PS for the first two days only. On days 3 and 4, all 4 capillaries were filled with sucrose, while on day 5 the setup was restored to that used before the deprivation.

2.3.2.3.2. Consumption against negative consequence

The 300 mM quine was added to liquid food containing PS, in order to enhance the bitter taste of liquid food, which is aversive to the flies (Devineni and Heberlein 2009). The optimized protocol (Table 6) was applied to three groups of flies: a) a group which had a choice between sucrose solution alone or a sucrose solution supplemented with quine, b) sucrose solution alone or a sucrose solution containing

PS, and c) sucrose solution alone or a sucrose solution containing PS and quine solution.

3.3.2.4. Screening using the CAFÉ assay

The optimized CAFÉ assay protocol (Table 6) was used to test the involvement of: a) circadian genes (*per^{01}, tim^{01}, cyc^{01} and Clk^{Jrk}*) and b) genetic manipulation of aminergic system (*fmn, dumb and Gal4DDC-UAS VAMT RNAi*) on the preferential consumption of COC and METH.

Table 6. Optimized protocol for testing self-administration of PS in flies.

	COC	METH
Concentration of PS in 100 mM sucrose solution	0.15 mg/mL	0.20 mg/mL
Sucrose liquid food concentration	100 mM	
Number of capillary and cue	4 in total – 2 with sucrose and 2 with PS + sucrose	
Measurement and exchanging of capillary	10:00	
Cue and capillary location	fixed cue and capillary location	
Cue associated with	two PS containing capillary	
Number of flies per a chamber	6	
Humidity inside of chamber	1/5 of cotton ball wetted with 1 mL of tap water	
Number of days in assay	4	
Experimental conditions	25 °C, 70% humidity, LD 12:12	

3.3.2.5. Data collecting

Preferential consumption is calculated as a preference index (PI), based on the total consumption over 24 hours of food containing drug (from both capillaries), minus drug-free food (from both capillaries) and normalized by total food consumption (food with and without drug from all 4 capillaries).

3.3.2.5.1. Statistical data analysis

Statistical tests were performed in the program Statistics 13.3. ANOVA for repeated measurements was used to analyze differences in PI within one group over several days. For comparison of the average PI between different groups, a t-test for independent samples (for 2 groups) or one-way ANOVA (for 3 or more groups) were used. Following all ANOVA analyzes a post-hoc Tukey's multiple comparison test was performed. The level of statistical significance was considered to be $p<0.05$.

3.4. Biochemical assays

Biochemical assays were performed to investigate the influence of COC and METH on the antioxidant enzymes activity and induction of oxidative stress, since it was found that both biomarkers are perturbed upon PS administration (Dietrich et al. 2005, Hanna et al. 2015). All assays were performed on whole body extracts. To test the antioxidant properties we used the DPPH antioxidant assay, and tested dopamine, dopamine precursors, L-DOPA and tyramine (Gow-Chin Y 1997, Dimić et al. 2017). The same assay was applied in order to test the antioxidant properties of other molecules investigated in this thesis (Table 7).

3.4.1. Antioxidant enzymes activity assay

The 0.05 M potassium phosphate buffer [pH 6,9] was prepared by mixing 49 mL 0.1 M K_2HPO_4 with 51 mL of 0.1 M KH_2PO_4 and diluting with 100 mL of distilled water. The 20 mM potassium phosphate buffer [pH 10], was prepared from 0.05 M potassium phosphate buffer [pH 10] by dilution. The 0.05 M potassium phosphate buffer [pH 10] was made by mixing 100 mL of 0.05 M Na_2HPO_4, 5 mL of 0.1 M NaOH, and 95 mL of distilled water. K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaOH, and EDTA (ethylenediaminetetraacetic acid) were purchased from Kemika. H_2O_2 (30%), TEMED (N,N,N′,N′-Tetramethylethane-1,2-diamine) (≥99%), and quercetin dihydrate (≥95%) were purchased form Sigma Aldrich, while Triton X-100 was purchased from Bio-Rad.

3.4.1.1. Catalase enzyme activity assay

This assay was adapted from (Luck 1965, Sun and Tower 1999). Enzyme extracts were prepared by homogenization of five adult flies in 800 µL of ice-cold homogenizing solution (0.05 M potassium phosphate [pH 6,9], 0.1% Triton X-100). Flies in 2 mL tubes were frozen at -20 °C for about 20 minutes, after which they were homogenized with a plastic pestle. 800 µL of ice-cold homogenizing solution was then added to the tubes and vortexed for 1 minute at 1,800 rpm. Samples were centrifuged at 14,000 rpm, 4 °C for 20 minutes. 300 µL of the resultant supernatant was then transferred in new tube and diluted in a ratio 1:2 with homogenizing solution (i.e. 600 µL of homogenization solution was added to 300 µL of enzyme extract). Reactions were initiated by adding 10, 15, 20 or 25 µL of diluted extract to 450 µL of substrate

solution, containing 0.05 M potassium phosphate buffer [pH 6.9] and 15 mM H_2O_2. Over 5 minutes, the decrease in the optical density at 240 nm (OD_{240}) was measured using a TecanInfinitePro200, and data were plotted as change in OD_{240} per minute. For each dilution, these data were linear regarding to time and amount of extract. The catalase activity is reported as the change in OD_{240} per minute per 0.1 µg of extract (mean and standard error of three experiments).

3.4.1.2. Percentage of SOD inhibition

This assay was adapted from one published previously (Kostyuk and Potapovich 1989, Sun and Tower 1999). Total SOD activity was determined using the same protocol for enzyme extraction as in the catalase assay. SOD activity was then measured as the degree by which the oxidation of quercetin by TEMED is inhibited in the presence of extract. 10, 15, 20 or 25 µL of extract were added to 450 µL of reaction mixture containing 20 mM potassium phosphate buffer [pH 10], 0.8 mM TEMED, 0.8 mM EDTA and 0.5 mM quercetin. The change in OD_{406} was measured on a TecanInfinitePro200 for 10 min, and compared to that of controls without extract. An average value of OD_{406} (from three experiments) was taken for each different concentration of extract added, along with the corresponding standard error. Enzyme activity is shown as a percentage of quercetin oxidation inhibition per minute and per 0.1 micrograms of enzymatic extract (% QUE oxidation inhibition/minute/0.1 µg).

3.4.2. Measuring indicators of oxidative stress

The Tris-HCl (Trizma hydrochloride) was purchased from Sigma Aldrich, and buffer solution was prepared by dissolving Tris-HCl in distilled water. The pH was adjusted with 35% HCl titration. Other chemicals used were: NaCl and DMSO purchased from Kemika, SDS (sodium dodecyl sulfate, GE Healthcare), PBS (Phosphate Buffered Saline, Roche), while HEPES (99.5%), CHAPS hydrate (98%), DDT (98%), and sodium deoxycholate (98%) were purchased from Sigma Aldrich.

3.4.2.1. Determination of hydrogen peroxide concentration

This assay was adapted from (Wang et al. 2011, Jakubowski and Bartosz 1997). The concentration of hydrogen peroxide (H_2O_2) present in whole fly extracts was measured using 2,7-dichlorofluorescein (H_2DCF), purchased from Sigma Aldrich.

Three flies were homogenized in 150 µL RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-400, 5% sodium deoxycholate, 0.1% SDS) and samples were centrifuged for 20 min at 14,000 rpm, 4 °C. Aliquot of 10 µL of homogenate was then placed in one well of a 96-well plate (black bottomed), to which 140 µl of PBS containing 50 µM H_2DCF was added (initially prepared as DMSO stock solution). The reaction was incubated for 60 minutes at room temperature. A TecanInfinitePro200 microplate reader was used to measure fluorescence at 515 nm excitation and 680 nm emission. The relative fluorescence intensity was normalized to protein concentration. The relative hydrogen peroxide concentration was indicated by the ratio of fluorescence intensity at 515 nm/680 nm (mean and standard error based on 3 experiments).

3.4.2.2. Determination of superoxide anion radical concentration

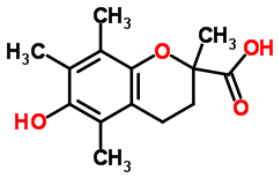
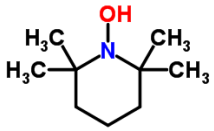
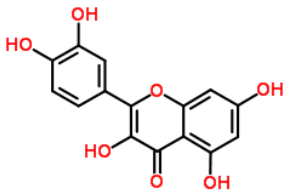
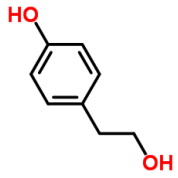
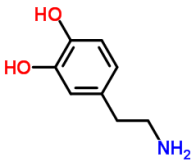
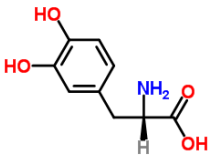
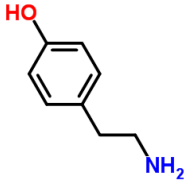
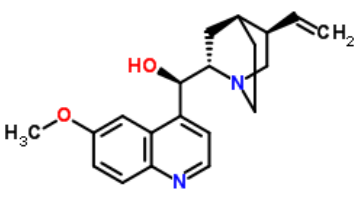
This assay was adapted from (Wang et al. 2011, Jakubowski and Bartosz 1997). The concentration of superoxide anion radicals ($O_2^{\bullet -}$) in all fly extracts was determined using dihydroetidium (DHE), purchased from Sigma Aldrich. Three flies were homogenized in 150 µL of lysis buffer (50 mM HEPES pH 7.4, 5 mM CHAPS and 5 mM DTT) and samples were centrifuged at 14.000 rpm for 20 minutes at 4 °C. Aliquot of 10 µL of homogenate was put in one well of 96-well plate (black bottomed), to which 140 µl of PBS containing 10 µM DHE was added (initially prepared as DMSO stock solution). The reaction was incubated in the dark at room temperature for a period of 10 minutes. A TecanInfinitePro200 microplate reader was used to measure fluorescence at 485 nm excitation and 585 nm emission. The relative fluorescence intensity was normalized to protein concentration. The relative concentration of the superoxide anion radical was indicated by the ratio of fluorescence intensity at 485 nm/585 nm (mean and standard error from three tests).

3.4.3. DPPH radical scavenging assay

The α,α-diphenyl-β-picrylhydrazyl (DPPH) is a molecule used for quantification of antioxidant capacity of different substances (Kedare and Singh 2011). The first step is to prepare 1 mM methanol stock solutions of TRX (Trolox, 99% Sigma Aldrich), DA (dopamine), L-DOPA, TYRA (tyramine), QUE (quercetin), TYR (tyrosol), TEMPOL and QIN (quinin), as well as a 0.1 mM methanol solution of DPPH (99%, Sigma Aldrich).

Aliquot of 160 µL of 0.1 mM DPPH methanol solution was added to a 96-well plate, to which 30 µL of the testing molecule solutions were added. Kinetics was measured at 0, 5 and 10 minutes, based in absorbance at 515 nm using a TecanInfinitePro200.

Table 7. Tested molecules and their structures.

Name	Structure	Expected properties *in vitro*
Trolox		antioxidant
TEMPOL		antioxidant
Quercetin		antioxidant
Tyrosol		antioxidant
Dopamine		antioxidant
L-DOPA		antioxidant
Tyramine		antioxidant
Quinin		antioxidant

The UV-VIS spectrums of hydrogen peroxide, dopamine, L-DOPA, octopamine, tyramine, tryptophan, quercetin, tyrosol, and TEMPOL were measured using Cary 60 (Agilent Technologies), with methanol (p.a., Kemika) as solvent, in a quartz cuvette. The wavelength range used for measurement was 800-200 nm.

3.4.4. Statistical tests used

All the data were plotted as mean and standard error of experiments performed in triplicate of the appropriate measurement (CAT and SOD activity, ROS and H_2O_2 production, DPPH free radical scavenging). Statistical tests were carried out in the program Statistics 13.3. One-way ANOVA was used for comparison of SOD and CAT enzyme activity, ROS and H_2O_2 production before and after the first and the second exposures, comparison between different groups, such as pre-treated and non-treated treated groups. Post-hoc Dunnett test was used to determine the significant differences between a single control group and the remaining treatment groups. The level of statistical significance was considered to be $p < 0.05$.

Pearson correlation coefficient was determined for correlation between oxidant status marker and antioxidant enzymes, with interpretation being performed on Petz's scale. The same approach was used to test correlation between free radical scavenging, oxidant status marker and antioxidant enzymes. The level of statistical significance was considered to be $p<0.05$.

4. RESULTS

Results are separated into three main sections. The first section covers optimization of assays for the induction and quantification of the motor-activating effect to COC and METH. In this part, circadian, monoaminergic and redox modulation of neuronal plasticity, induced by COC and METH are tested. The second part is focused on optimization of the CAFÉ assay for measuring self-administration of COC and METH in flies. This assay was used to test the circadian and monoaminergic modulation of the reward effect induced by COC and METH. The third part is focused on biomarkers of COC and METH influence on redox status.

4.1. Locomotor activating effect of PS

One of the main aims of this thesis is the construction and optimization of a new platform for the induction and quantification of the locomotor activation effect in flies following acute or repeated COC and METH administration.

The platform presented, “FlyBong” is cheap, user friendly and easy to build in any laboratory conditions. In addition, it is high-throughput, reproducible, and objective and allows data analysis on both the population and individual fly level.

4.1.1. FlyBong platform optimization

The optimization of the platform was focused on defining parameters of the duration of the volatilization and uniformity of PS distribution to individual flies in the system. Next concern was delivery of PS with minimal environmental perturbations, in order to minimize the artefacts of the mode of the drug delivery from biological effects of vCOC and vMETH.

That was followed by optimization of the amounts of COC and METH that would have specific motor-activating effects. Additionally, the time interval between two administrations had to be optimized in order to induce a stepwise increase in locomotor activity or locomotor sensitization.

4.1.1.1. Volatilization and distribution of PS

The first step was to optimize the length of time required for the heating cap to raise the temperature of the three-neck flask to 185 °C. This is the temperature at which volatilization of COC and METH in hydrochloride form has been reported to occur. We showed that, starting from room temperature, the heating cap should be active for at least 8 minutes for internal temperature of the flask to reach 185-200 °C (Figure 14A). Based on this evidence, we selected a heating period of 8 minutes.

The system for delivery of the drugs is composed of a gas disperser, a three-neck flask, rubber and glass tubes. This can cause a lot of condensation of COC and METH on these surfaces, before the desired amount reaches the flies in the monitor. Some of the consequences of this may be: non-uniform distribution of COC and METH across rows and columns of the dispenser or possible decomposition of COC and METH due to the high temperatures applied in the volatilization procedure. To test these potential weaknesses of the system, we quantified the amount of COC and METH that was delivered to empty polycarbonate tubes (without flies or food). For this test we used 75 µL of 10 mg/mL COC and METH ethanol solution, which was heated for 8 minutes, after which we applied air flow 2.5 L/min for 1 minute. All tubes were immersed in 2 mL Eppendorf tubes containing 500 µL of distilled water during heating and delivery. After the assay, all tubes were detached and washed with an additional 500 µL of distilled water. After exposure to PS flies were frozen at -20 °C, transferred into 2 mL Eppendorf tubes containing 300 µL of distilled water, and vortexed for 1 minute at 2,500 rpm. We showed that the distribution of COC and METH inside of tubes and on the flies was uniform across all the rows and columns (Figure 14B and 14C), and since there was no additional peaks in UV spectra after COC and METH quantification, we concluded that 8 minutes of volatilization does not cause significant decomposition of COC and METH (Suppl. Mat. Figure 1A and 1B).

Based on these data, we selected an optimized protocol consisting of: 8 minutes of volatilization, 1 minute of 2.5 L/min airflow and 75 µg of COC and METH. With this protocol, the same amount of COC and METH is uniformly delivered to the all flies in the system at the same time, allowing high reproducibility using the FlyBong platform. The system is high-throughput, since COC and METH can be administered to 32 flies in the assay at the same time.

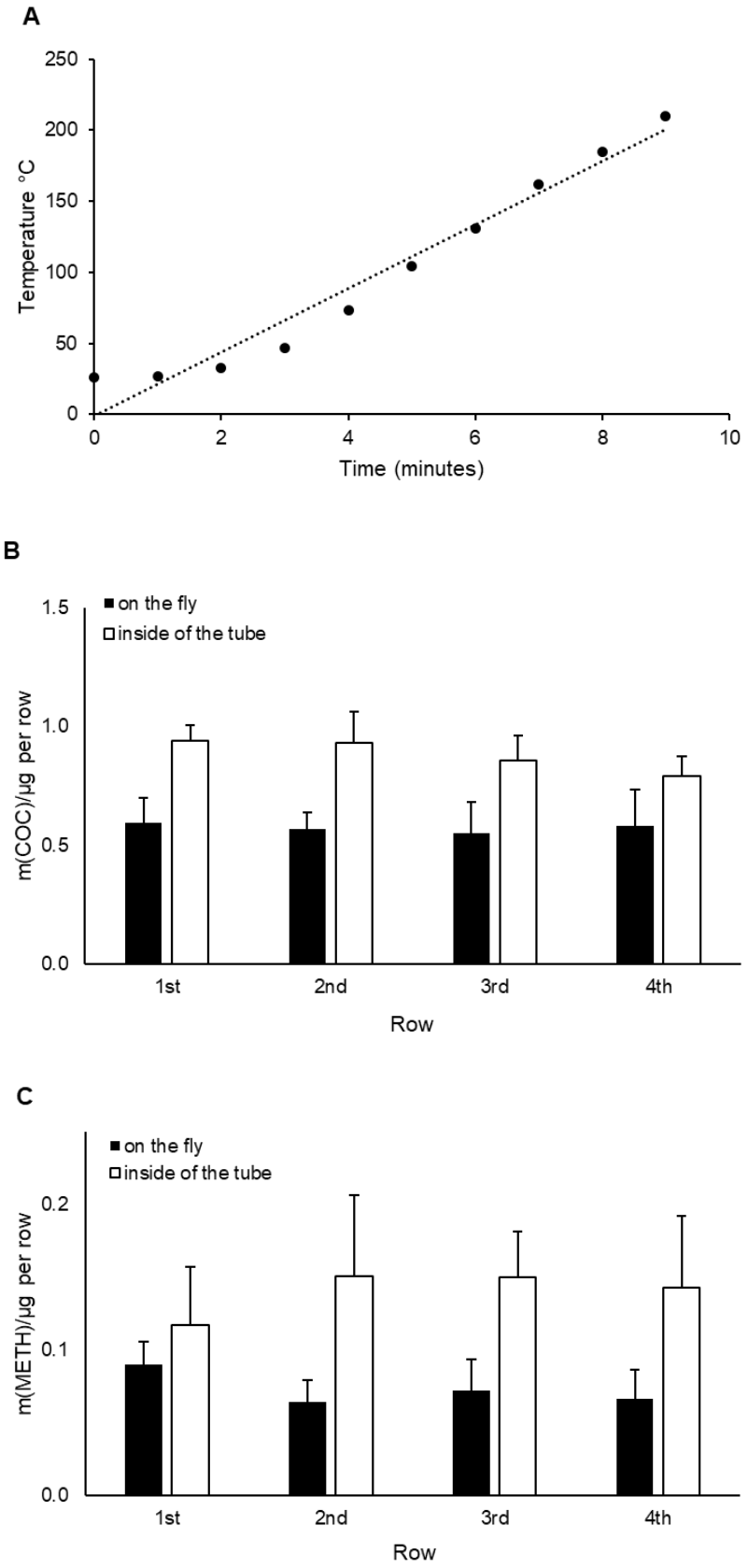


Figure 14. Optimization of the volatilization temperature, and distribution of COC and METH in DAM system tubes. **A)** Volatilization of COC and METH depends on the temperature of the flask and the duration of heating. After 8 minutes, the internal temperature reaches 185 °C, the vaporizing

temperature for HCl form of COC and METH. **B)** Volatilized COC is uniformly distributed in all tubes of the DAM monitor. Mean amount of COC ± standard error of the mean (SEM) delivered per row of the vertical DAM monitor consisting of 32 slots (4 rows and 8 columns). Data are plotted as histograms showing the average of three tests. Using one-way ANOVA no statistical difference was found between amounts of COC per row. **C)** Volatilized METH is uniformly distributed in all tubes of the DAM monitor. Mean amount of METH ±SEM delivered per row of the vertical DAM monitor consisting of 32 slots (4 rows and 8 columns). Data are plotted as histograms showing the average of three tests. Using one-way ANOVA no statistical difference was found between amounts of METH per row.

4.1.1.2. Minimizing the handling of flies during PS administration

Flies are sensitive to startle (Lebestky et al. 2009), a sudden or unexpected environmental stimuli, and as a consequence increase the locomotor activity. Because of this, different strengths of airflows (2.5-12 L/min) were tested, and we found that the maximal airflow rate that could be used is 2.5 L/min (Figure 15A). This airflow rate caused low perturbation of the locomotion behavior of flies at the population level, when compared to a control group that did not receive any treatment. Airflow of 6 L/min or higher led to significant increase in locomotor activity.

The second parameter to be optimized was the duration of 2.5 L/min warm airflow. We exposed flies to the different 2.5 L/min airflow durations from a flask heated for 8 minutes without COC (Figure 15B). Minimal perturbation of the flies locomotor activity was seen when the airflow was applied for 1 minute (Suppl. Mat. Figure 1C).

In contrast, durations shorter or longer than one minute led to a significant increase in locomotor activity. We suspect that shorter exposures (<1 minute) cause a startle-like response (Lebestky et al. 2009), while longer exposures (>1 minute) decreased humidity and increased temperature in the recording tubes, causing increased locomotion (Sayeed and Benzer 1996).

Additional control experiments were performed to eliminate any possible influence of the 96% ethanol used as solvent for the preparation of the PS stock. One minute of 2.5 L/min airflow rate, with or without heating, has not been previously seen to increase locomotion at the population level, and the same result was observed when 75 µL of 96% ethanol was added 6 hours before the airflow (Figure 16). In summary, the protocol was optimized with 8 minutes of heating, 2.5 L/min air flow and 1 minute of drug delivery.

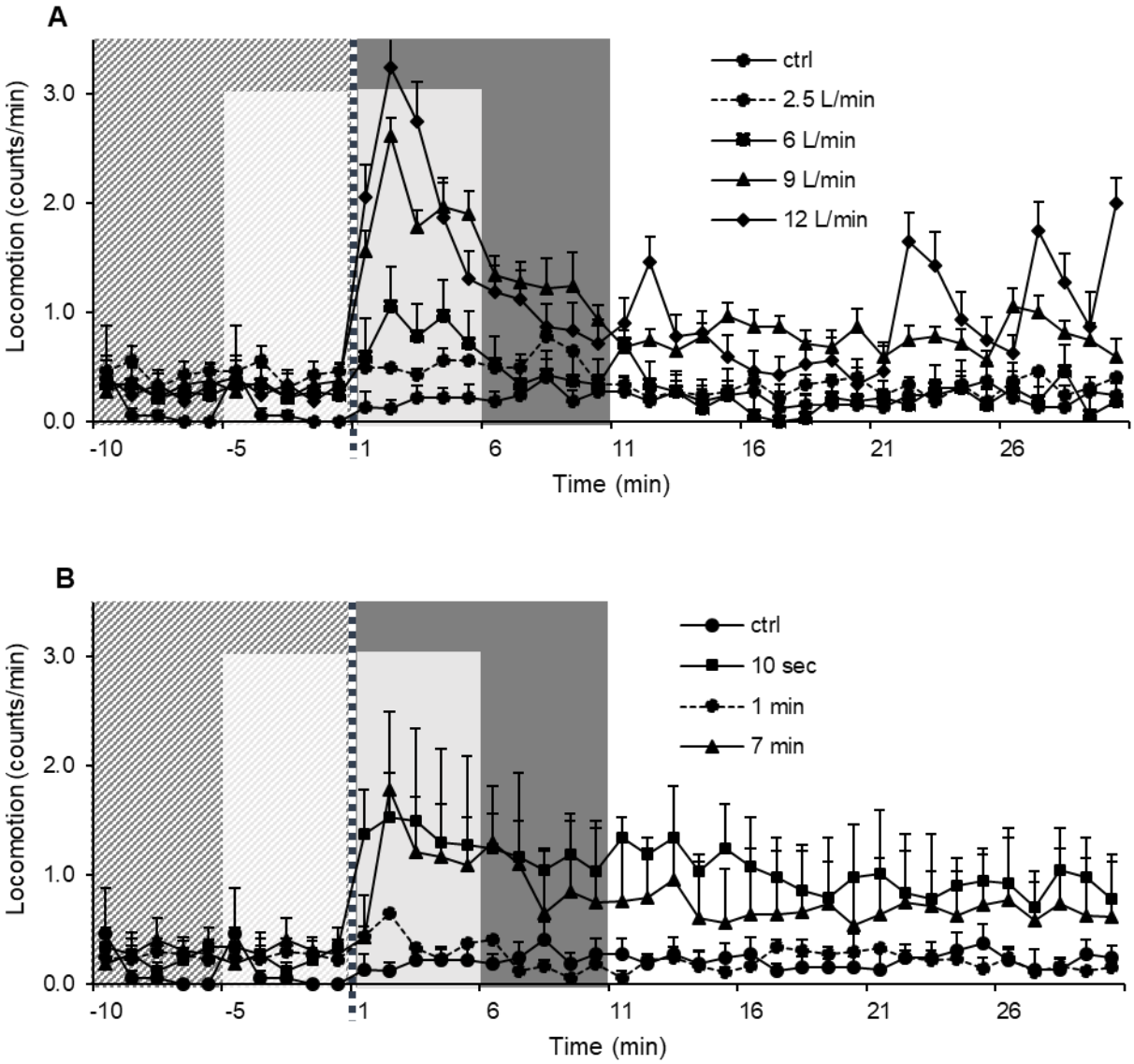


Figure 15. The amount of locomotor activity depends on the intensity and duration of airflow in male *wt* flies. Locomotor activity was measured in the DAM system at one minute resolution and plotted on kinetic graphs as mean activity ± SEM for 32 flies (all tested groups). Times before exposure are shaded gray, lighter for 5 minutes and darker for 10 minutes, the dotted line indicates the time of the exposure and times after are shaded gray, lighter for 5 minutes and darker for 10 minutes. **A)** Flies exposed to different rates of airflow (without COC or heating of the flask) for one minute. **B)** Mean activity ± SEM for flies exposed to different duration of 2,5 L/min airflow from a flask heated for 8 minutes, without COC.

After testing the same optimized protocol on females (8 minutes of heating and one minute of 2.5 L/min airflow), it was observed that female flies are more sensitive to the warm air and the intensity of the airflow than males (Suppl. Mat. Figure 2.A). To test this, different durations, from 1 to 7 minutes of 2.5 L/min airflow were used which

resulted in non-significant effect on duration of increase in locomotion (Suppl. Mat. Figure 2.B). Females therefore, cannot be tested for a motor-activating effect of PS using the protocol optimized on males, as environmental perturbation causes significant increase in their locomotion.

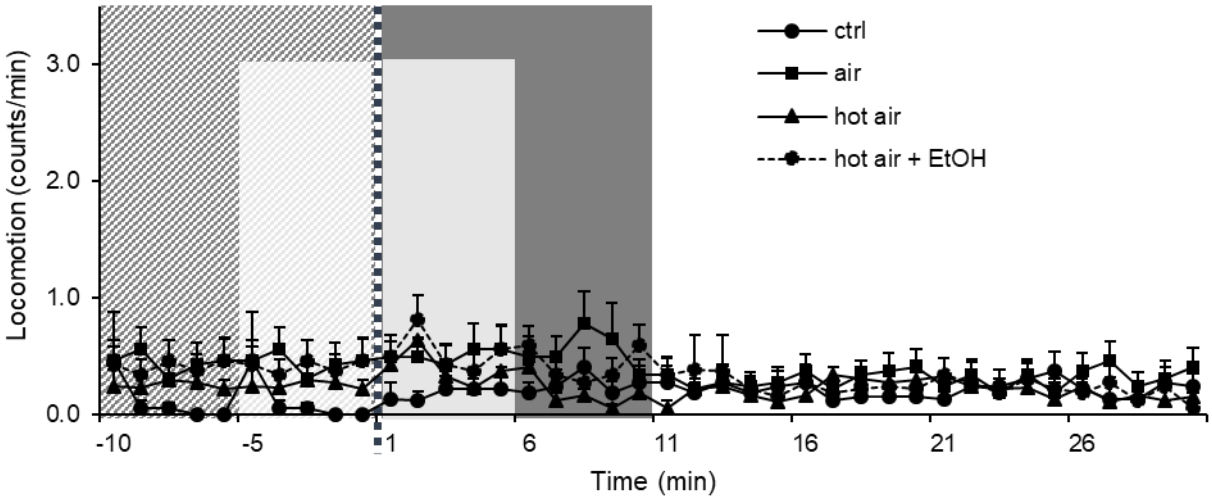


Figure 16. Optimized protocol causes minimal perturbation of *wt* males locomotor activity. Locomotor activity was measured in the DAM system at one minute resolution and plotted on kinetic graphs as mean activity ± SEM for 32 flies (all tested groups). Times before exposure are shaded gray, lighter for 5 minutes and darker for 10 minutes, the dotted line indicates the time of the exposure and times after are shaded gray, lighter for 5 minutes and darker for 10 minutes. Ctrl - represents the group without any treatment. The air - group has received 1 minute of 2.5 L/min airflow. The hot air- group received 1 minute of 2.5 L/min air flow after 8 minutes of heating. The hot air + EtOH - group received 1 minute of 2.5 L/min air flow after 8 minutes of heating of volatilization chamber, into which 75 µL of 96% ethanol had been added before the assay.

4.1.1.3. Sensitivity to vCOC nad vMETH is dose dependent

Applying different amounts of COC and METH: 0-150 µg for COC and 0-200 µg for METH using standard protocol, we tested sensitivity, or the increase in locomotion response after acute exposure to PS.

All of these drug amounts were tested at the same time of the day, 09:00 in the morning, to eliminate any circadian effect on sensitivity (Baird and Gavin 2000). This time point was chosen because flies are diurnal animals (Helfrich-Förster 2009), active during the daytime (08:00 to 20:00) and inactive or sleeping during the night time (20:00 to 08:00). Flies show a bimodal (Helfrich-Förster 2009) activity profile, with maximal activity at the beginning (08:00) and end (20:00) of the day. It is known that

flies respond with increases in locomotion after administration of low amounts of COC (McClung and Hirsh 1998). To test FlyBong as a platform for drug delivery of vCOC, different amounts of COC were added to the three-neck flask 6-12 hours before the assay, and locomotion of the flies was observed 30 minutes before and after drug administration. From kinetic graphs (Figure 17A), it can be seen that the flies increase

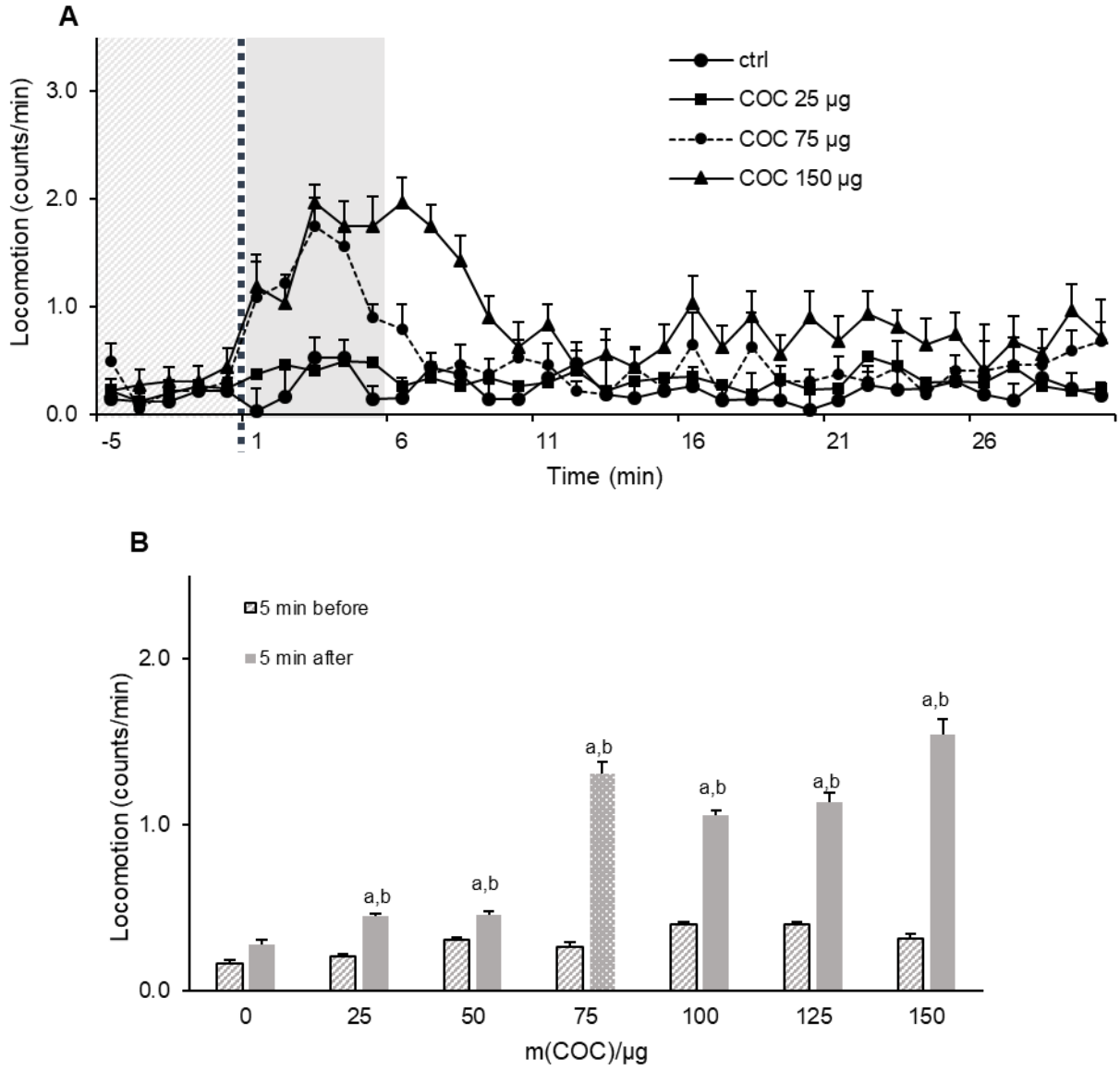


Figure 17. Exposure to volatilized COC transiently increases locomotor activity in *wt* males in a dose-dependent manner. A) Kinetic graph of locomotion, expressed as number of counts per minute for control group of flies (ctrl) (n=32) exposed to warm air, and groups exposed to 25 µg (COC 25 µg) (n=32), 75 µg (COC 75µg) (n=32) or 150 µg (COC 150µg) (n=32) of volatilized COC at 09:00. Data are plotted as mean activity ± SEM for 32 flies in one minute resolution (all tested groups). The shaded gray panel represents locomotor activity 5 minutes before drug exposure, the dotted line indicates the time of the exposure, and the gray panel is first 5 minutes after the exposure. **B)** Mean locomotor activity ± SEM for amounts of volatilized COC applied, ranging from 0 to 150 µg (n=32 flies per treatment) for 5

minutes before and 5 minutes after COC exposure at 09:00. Statistical significance at p≤0.05; a-comparison of before to after (within the group using Student's t-test for dependent samples), b-after in control group compared to after in groups exposed to volatilized COC (one-way ANOVA with Dunnett post-hoc test).

their locomotor activity in the first 5 minutes after application of 75 µg vCOC. After that time, locomotion decreases to the level of baseline before the exposure and to the level of control group, which received same treatment but without COC. Lower amounts of vCOC did not induce increases in locomotion, while higher amounts yielded similar results as 75 µg (Figure 17B). Amounts above 75 µg most likely did not show further increases in locomotion since higher COC doses can induce stereotypical behaviors, manifesting in flies as buzzing, twirling or spinning in circles (McClung and Hirsh 1998). These types of behavior cannot be quantified using the DAM system with one IR beam, as the system records only the number of crossings in the middle of the tube. Based on this data, 75 µg of COC was chosen as the optimum amount to induce a transient increase in locomotor activity.

Effects of vMETH have previously not been tested on fly's behavior. It is known that oral administration of METH through food reduces sleep during the night (Andretic et al. 2005). To test the motor-activating effect of vMETH on the behavior of flies, we exposed flies to different amounts of vMETH (0-200 µg) for 1 minute, 2.5 L/min airflow after 8 minutes of heating. From the kinetics graphs (Figure 18A), it can be seen that amounts of METH lower than 75 µg induce an increase in locomotion, which is dose dependent with similar but longer effects seen for amounts up to 150 µg (Figure 18B). Therefore, an optimum amount of 75 µg vMETH was chosen to induce sensitivity, since the kinetics of the behavioral response to this dose was the most stable and reproducible.

Based on this data, we have concluded that the FlyBong platform can be used in research of the motor-activating effect (sensitivity) of vCOC and vMETH. The optimized protocol for this consists of 1 minute of 2.5 L/min airflow, following 8 minutes of volatilization of 75 µg of COC or METH at 09:00 for *wt* male flies. From here on, we refer to this as standard protocol.

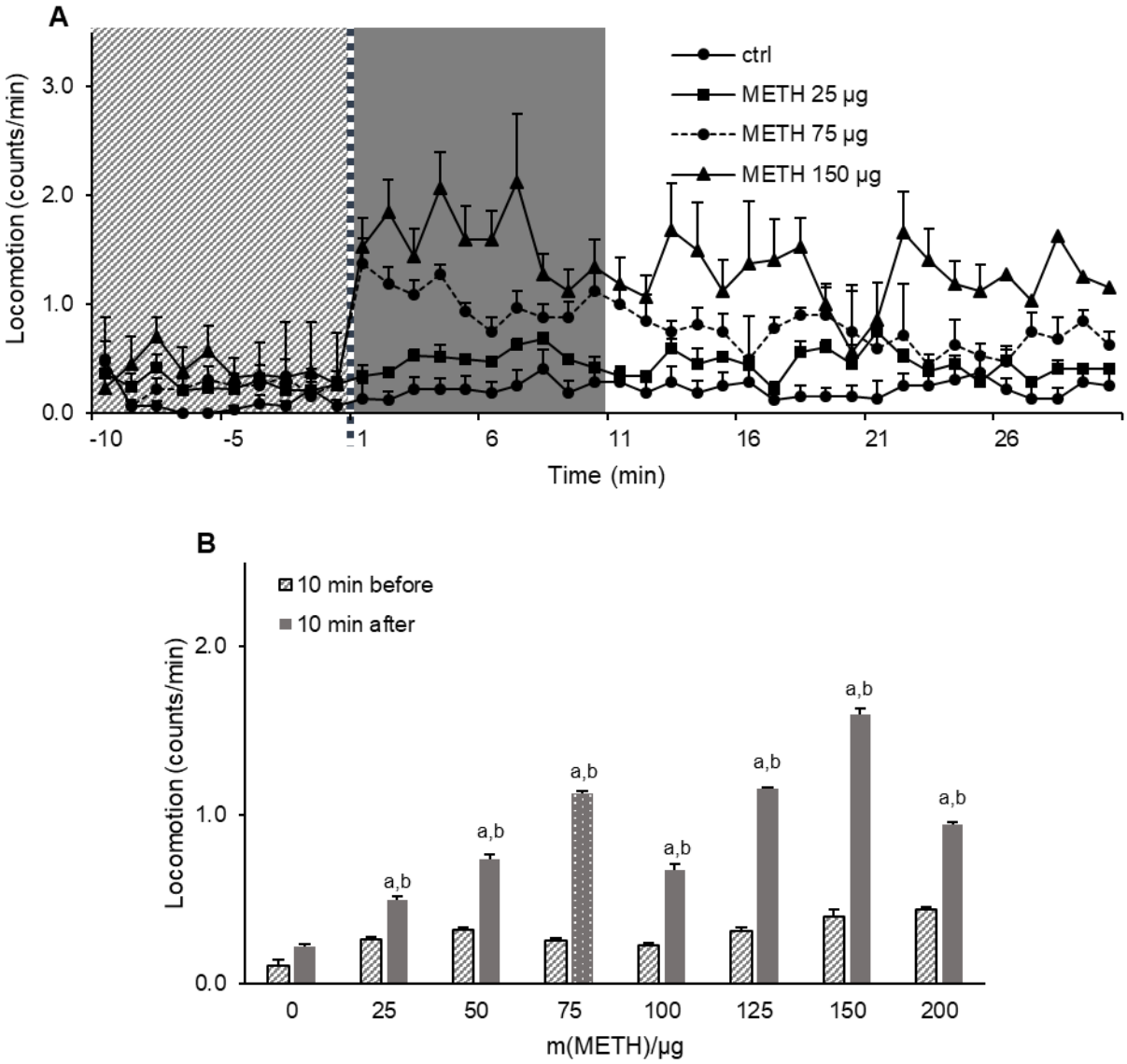


Figure 18. Exposure to volatilized METH transiently increases locomotor activity in a dose-dependent manner in *wt* males. A) Kinetic graph of locomotion, expressed as number of counts per minute for a control group of flies (ctrl) (n=32) exposed to warm air and groups exposed to various amounts of volatilized METH: 25 µg (METH 25 µg) (n=32), 75 µg (METH 75 µg) (n=32) or 150 µg (METH 150 µg) (n=32). Data are plotted as mean activity ± SEM for 32 flies in one minute resolution (all tested groups). The shaded gray panel represents locomotor activity 10 minutes before drug exposure, the dotted line indicates the time of exposure, and the gray panel is first 10 minutes after the exposure. **B)** Mean locomotor activity ± SEM for amounts of volatilized METH ranging from 25 to 200 µg (n=32 flies per treatment) for 10 minutes both before and after METH exposure. Statistical significance $p \leq 0.05$; a- comparison of before to after (within the group using Student's t-test for dependent samples), b- after in control group compared to after in groups exposed to volatilized METH (one-way ANOVA with Dunnett post-hoc test).

The *wt* female flies were also tested for the motor activation effect (sensitivity) of vCOC and vMETH, by applying the standard protocol (Table 2). From the kinetic graphs for vCOC (Suppl. Mat. Figure 3A), it can be seen that 75 µg of COC does not induce an increase in locomotion, and is instead comparable to the control group that received only warm airflow (1 minute 2.5 L/min air flow after 8 minutes of heating). An increase in locomotion compared to the control group is present when amounts of vCOC over 150 µg were applied (Suppl. Mat. Figure 3B). However, the level of sensitivity of the female flies is constant for all tested amounts of vMETH (Suppl. Mat. Figure 4A,B). These data confirm our previous conclusion that in female flies, the method of delivery causes strong motor-activating effect, which interferes with motor-activating effect of PS.

In order to further validate FlyBong, we compared population-based data (Figure 19A) of the standard vCOC and vMETH protocols to analysis of data from individual flies (Figure 19B). As for the population data analysis, the first 5 minutes before vCOC administration was averaged from all 32 flies in assay, and compared to the average locomotor activity of all 32 flies in assay in the first 5 minutes after vCOC administration. The same was done for vMETH but for the 10 minutes before and after administration. The time intervals selected for population data analysis (5 min for vCOC and 10 minutes for vMETH) are based on previously obtained kinetic graphs (Figure 17A COC and Figure 18A METH). From the population analysis, it can be seen that standard protocols for vCOC and vMETH cause a statistically significant increase in population locomotor activity compared to the baseline and the control group (Figure 19A).

The individual data analysis following vCOC administration compared the average locomotion of individual flies in the 5 minutes before administration with the average locomotion of individual flies 5 minutes after administration. The locomotion of the individual fly was then graded as “same” if the average of 5 minutes before and 5 minutes after were identical, “decrease” if the average of 5 minutes after the exposure was lower than 5 min before the exposure, and “increase” if the average of 5 minutes after the exposure was higher than before the exposure. The same approach was used for vMETH analysis, but with a 10-minute time interval. In control groups, around 30% of the flies increased their activity after exposure to warm airflow for 1 minutes of 2.5

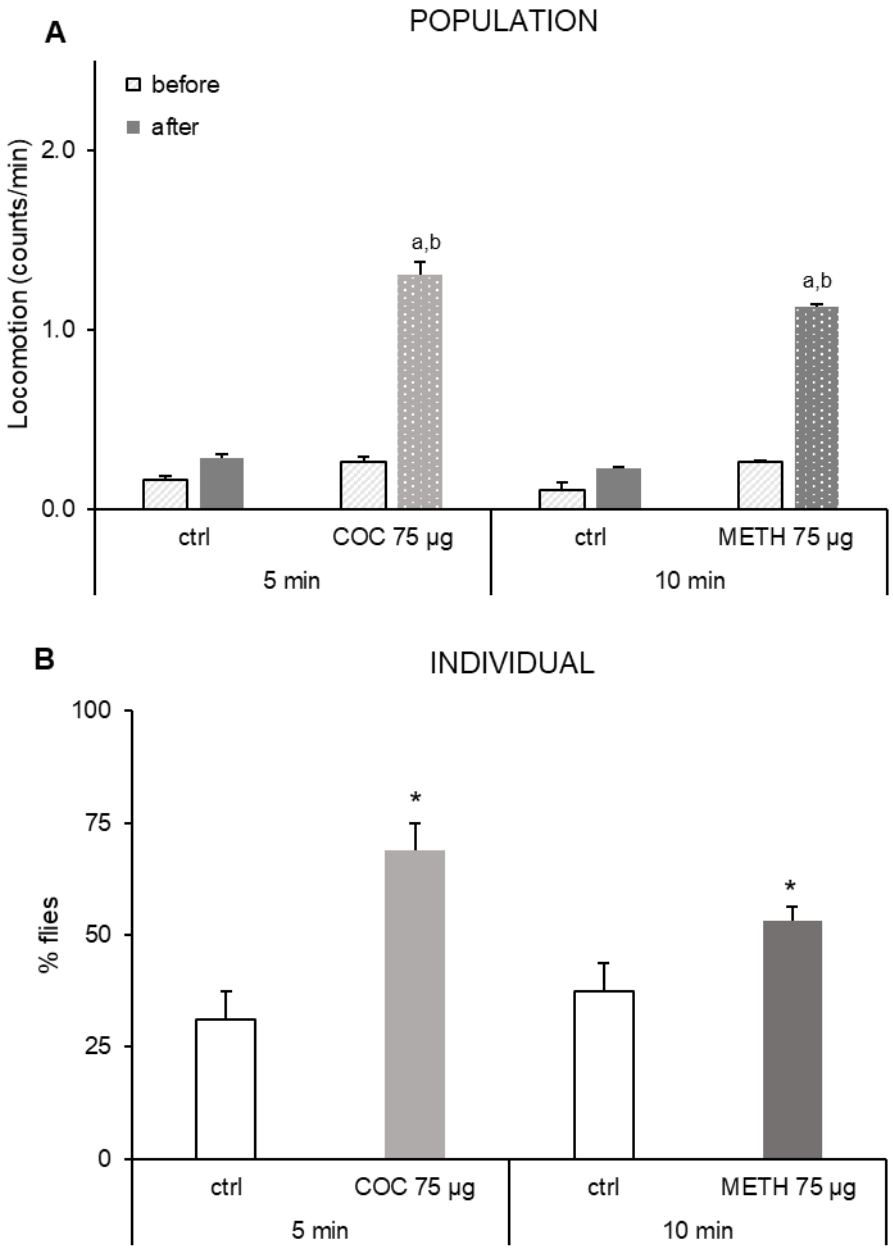


Figure 19. Standard protocols for administration of COC and METH increase population and individual response in *wt* males. A) Histogram of mean locomotor activity ± SEM for control groups, and groups exposed to 75 µg of volatilized COC or METH (n=32 flies per treatment). Activity compared before (5 minutes for COC, 10 minutes for METH) and after (5 minutes for COC, 10 minutes for METH) exposure. Statistical significance p≤0.05; a-comparison of before to after (within the group using Student's t-test for dependent samples), b- after in control group compared to after in groups exposed to volatilized COC and METH (Student's t-test for independent samples). **B)** Individual amount of locomotor activity before exposure (5 min for COC, 10 min for METH) compared to after exposure (5 min for COC, 10 min for METH) to 75 µg of volatilized COC (n=32) or METH (n=32). Data are plotted as histograms showing the mean of five tests ± SEM. Plotted values represent percentage of flies responding to PS, as those that increased locomotion after the first administration compared to the

baseline. Obtained values were compared to a control group (n=32) that received warm airflow only (8 minutes heating, 1 minute exposure and 2.5 L/min airflow). Mann-Whitney U-test for nonparametric analysis of two independent samples showed a statistically significant difference, p≤0.05 (*), in the locomotion of flies after COC and METH exposure, when compared to the control group.

L/min after 8 minutes of heating, while almost 70% of flies exposed to vCOC increased their activity, and after vMETH exposure over 50% of flies increased their activity (Figure 19B). In females, individual data analysis shown that the percentage of flies with the same, increased or decreased activity were identical between flies given 100 µg of vCOC and controls (Suppl. Mat. Figure 3C), and between those given 150 µg of vMETH and controls (Suppl. Mat. Figure 4C). These data indicate that analysis at the level of individual flies yields significant percentage of population that is sensitive to vCOC or vMETH. Reason why increase is not present in all the flies in the population remains to be investigated.

4.1.1.4. Locomotor sensitization and time between two administrations

Repeated PS administration of identical amounts of PS leads to a stepwise increase in locomotor activity, or locomotor sensitization. It was previously found that repeated administrations of vCOC lead to this behavioral endophenotype in flies (McClung and Hirsh 1998), but it has yet to be investigated whether flies develop this type of behavior after repeated administration of vMETH.

Using the standard protocol, we gave first vCOC administration of 75 µg at 09:00, followed by a second administration after different time intervals. The time intervals tested were: 3 hours after the first administration (at 12:00), 6 hours (15:00), 8 hours (17:00), 12 hours (21:00), 24 hours (09:00 of the following day), and 30 hours (15:00 of the following day). These tests were important in order to optimize the time between two exposures, in order to define the peak response to the second dose. The minimal time interval between two vCOC administrations that led to a LS phenotype was 6 hours (Figure 20A). Shorter time intervals, such as 3 hours (Figure 20B), have not increased population locomotor activity, suggesting that vCOC-induced neural modulation requires more than three hours for activation of cellular mechanism. The maximum time tested between the two exposures that still led to a LS phenotype was 24 hours (Figure 20B). The standard protocol with a first vCOC administration at 09:00

and a second at 15:00 was also tested on *wt* female flies. The amount of vCOC used in these tests was 100 µg (Suppl. Mat. Figure 5A), since females are sensitive to higher

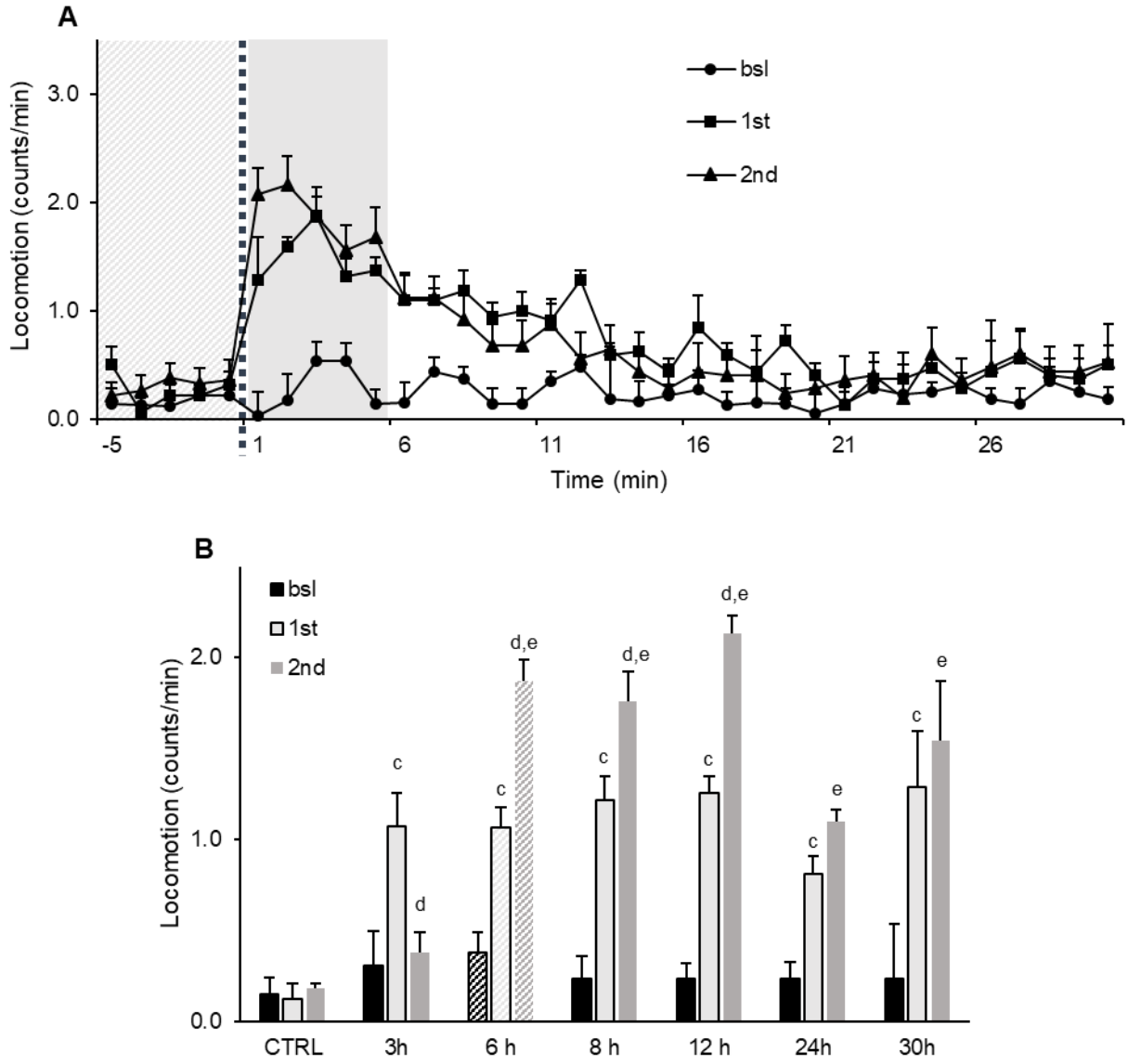
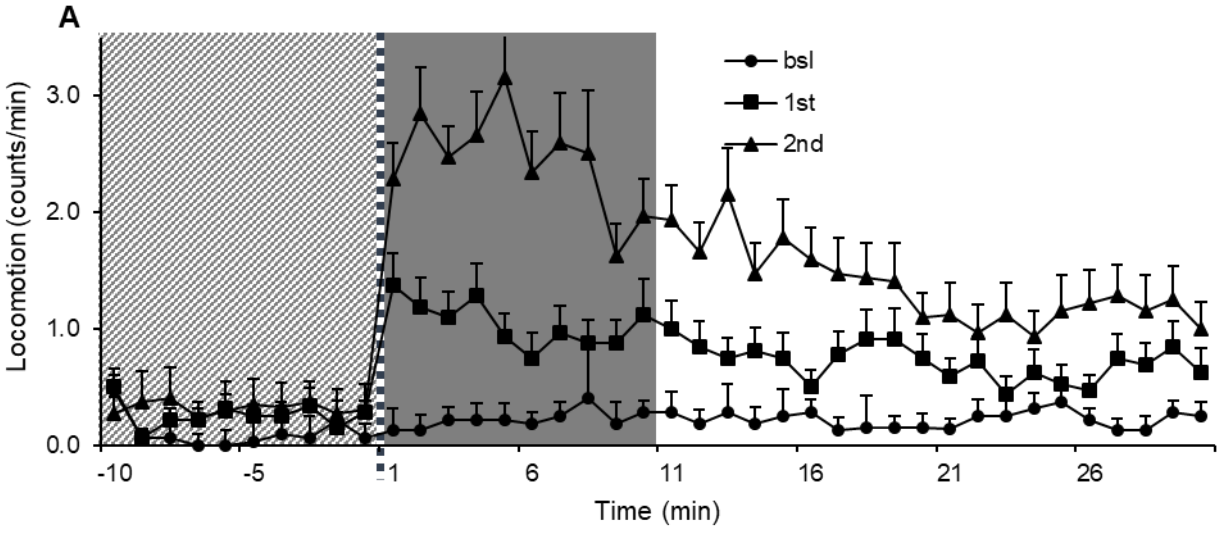


Figure 20. Locomotor sensitization to volatilized COC depends on the time interval between exposures. A) Kinetic graph of the average locomotion (counts per minute) of groups of flies (n=32) before (bsl) administration, and after administration of 75 µg volatilized COC, initially at 09:00 (1st) and then at 15:00 (2nd). Data are plotted as mean activity ± SEM for 32 flies in one minute resolution (all tested groups). The time point 5 minutes immediately before the exposure is represent with a shaded gray panel, the dotted line indicates the time of the exposure, and the 5 minutes after the administration is represented by a gray panel. **B)** Histogram of different time intervals between two administrations of volatilized COC (75 µg), plotted as an average of population (32 flies) locomotor activity in the 5 minutes before (bsl) and after (1st and 2nd) exposure to COC ± SEM. The control (CTRL) group was exposed to warm air only (8 minutes of heating, 1 minute exposure, and 2.5 L/min airflow rate). Statistical significance was p≤0.017 within groups; c- baseline to after first administration, d- after first to after

second and e - baseline to after second administration (ANOVA for repeated measurements with Bonferroni post-hoc test).

amounts of vCOC (Suppl. Mat. Figure 3B). From the kinetic graphs (Suppl. Mat. Figure 5A) it can be seen that females respond to the first vCOC dose with an increase in locomotion, but after the second, the response is same as for the control group. This is suggesting that for females both sensitivity and locomotor sensitization require different mode of drug administrations in order to exclude artifacts and that a longer period between two administrations should be applied.

We used similar procedure to test whether flies can develop LS to vMETH. Flies develop LS after repeated administrations of vMETH when the time between the two exposures is 10 hours, which is 4 hours longer than for vCOC (Figure 21A). Shorter or longer periods than 10 hours (Figure 21B) did not induce a stepwise increase in locomotor activity. This difference between COC and METH may be the consequence of their differing pharmacokinetic and pharmacodynamic properties. Testing was also conducted on female *wt* flies in order to see if they can develop LS to repeated administration of vMETH. For this test, 150 µg of vMETH was used for both the first administration at 09:00 and for the second administration at 19:00. Female flies increase their locomotor activity to the first administration of vMETH, but second administration did not lead to further increase in locomotor activity (Suppl. Mat. Figure 6A). Sensitivity and locomotor sensitization in females require different mode of drug administrations in order to exclude artifacts and a longer period between two administrations should be applied.



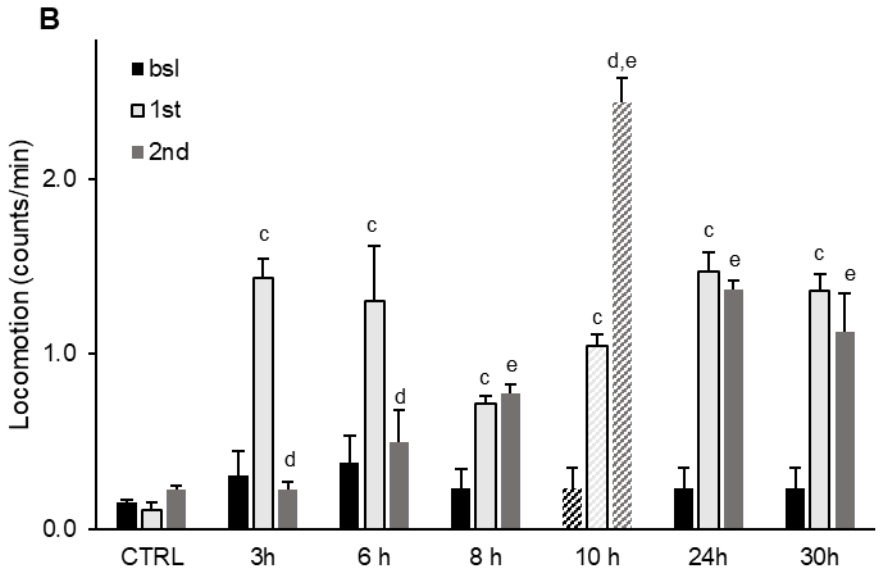


Figure 21. Locomotor sensitization to volatilized METH depends on the time interval between exposures. A) Kinetic graph of average locomotion (counts per minute) for groups of flies (n=32) before (bsl) administration, and after administration to volatilized 75 µg METH, initially at 09:00 (1st) and then again at 19:00 (2nd). Data are plotted as mean activity ± SEM for 32 flies in one minute resolution (all tested groups). The 10 minutes immediately before the exposure is shown with shaded gray panel, the dotted line indicates the time of the exposure, and the 10 minutes after administration is represented with a gray panel. **B)** Histogram of different time interval durations between two administrations of volatilized METH (75 µg), plotted as a mean of population (32 flies) locomotor activity in the 10 minutes before (bsl) and after (1st and 2nd) exposures to METH ±SEM. The control (CTRL) group was exposed to warm air only (8 minutes of heating, 1 minute exposure, and 2.5 L/min airflow rate). Statistical significance was p≤0.017 within groups; c- baseline to after first administration, d- after first to after second and e - baseline to after second administration (ANOVA for repeated measurements with Bonferroni post-hoc test).

In summary, male flies can develop LS after repeated administration of vCOC or vMETH (75 µg) with a time interval of 6 hours (for vCOC) or 10 hours (for vMETH), between two administrations. This suggests different pharmacodynamics and pharmacokinetics mechanisms of COC and METH (German et al. 2015). The sexual dimorphism suggests that different protocols would be required for studying male and female flies, with regards to airflow, dose and time interval between the two exposures.

For analysis of population data, the locomotor activity in the 5 minutes immediately before vCOC administration were averaged from all flies in the assay, and compared to the average activity in the 5 minutes immediately after the first and second administrations of vCOC. The same analysis was done for vMETH administration, but

comparison was performed between the average activity 10 minutes before and after the first and second administrations. The time intervals selected for population data analysis are based on previous data (Figure 20A and Figure 21A). At the population level, both vCOC and vMETH optimized protocols led to a statistically significant increase in locomotor activity, when compared to base line and first exposure (Figure 22A).

Data analysis for vCOC at the individual level required comparing the average locomotion of a single fly in the 5 minutes before and the 5 minutes after first and

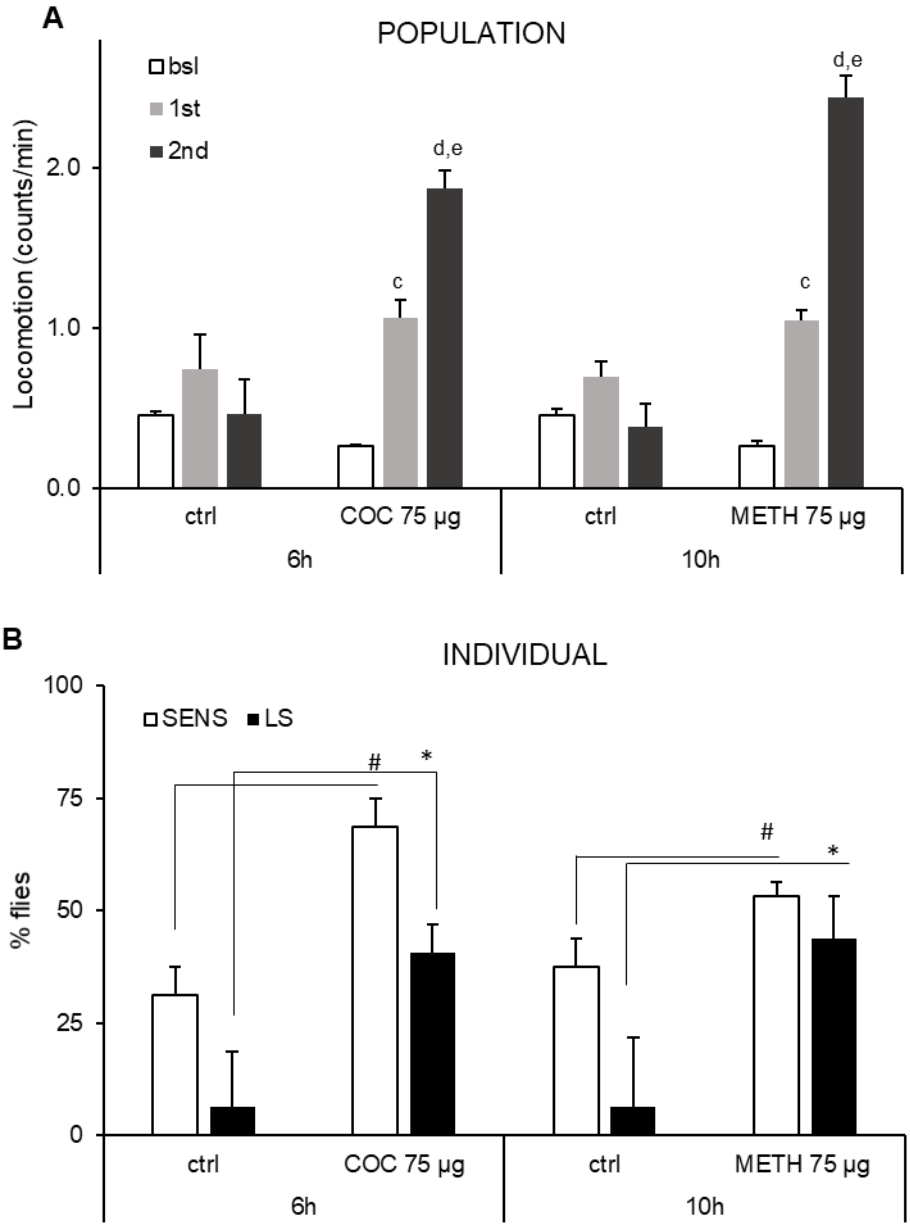


Figure 22. vCOC and vMETH induce LS at population and individual level in *wt* males. A) Histogram of mean locomotor activity ± SEM for the control group, and groups exposed to 75 µg of volatilized COC and METH (n=32 flies per treatment) before (bsl 5 minutes for COC, bsl 10 minutes for

METH) and after the first and second exposures (5 minutes for COC, 10 minutes for METH exposure). Statistical significance was p≤0.017 within groups; c-comparison of baseline to after first administration, d- after first to after second and e - baseline to after second administration (ANOVA for repeated measurements with Bonferroni post-hoc test). **B)** Locomotor activity of individual flies before exposure (5 min for COC, 10 min for METH) was compared to after exposure (5 min for COC, 10 min for METH) to 75 µg of volatilized COC (n=32) or METH (n=32). Data are plotted as histograms showing the mean value of five tests ± SEM. Plotted values represent the percentage of flies that responded with an increase in locomotion when the baseline is compared to response to the first administration (SENS) and when the same fly shows further increase to the second (LS). Obtained values were compared to a control group (n=32) that received warm airflow only (8 minutes heating, 1 minute exposure and 2.5 L/min airflow). Mann-Whitney U-test for nonparametric analysis of two independent samples showed a statistically significant difference, *p≤0.05 for flies which increased locomotion after COC and METH acute exposure (SENS)and # p≤0.05 repeated exposure (LS), when compared to the control group.

second administration. The locomotion of each individual fly was then classified as „same", if average of 5 minutes before and 5 minutes after each administration were the same, as "decrease" if the average of 5 minutes before was higher than 5 minutes after each administration, and as "increase" if the average of 5 minutes before is lower than in the 5 minutes after each administration. This same approach was then performed for the vMETH analysis, but with a 10 minute-time interval. In the control group, around 30% of the flies increased their activity after the first exposure, almost 70% of flies exposed to vCOC increased activity, and following vMETH exposure over 50% of flies increased their activity (Figure 22B). In the control group only 6% of flies showed LS, as indicated by a stepwise increase in locomotor activity, while for both vCOC and vMETH over 40% of flies showed LS (Figure 22B). In females, individual data analysis showed that similar percentages of flies displayed same, increased and decreased activity for each of the control, vCOC and vMETH groups (Suppl. Mat. Figure 5B and 6B).

To test if flies will continue to increase their locomotor activity following multiple administrations, we exposed flies to three intermittent doses of vCOC and vMETH. Analysis of the population shows that flies continue to increase their locomotor activity following the third exposure (Figure 23A) however, an important characteristic of individual response is not obvious from the population data. Specifically, the percentage of flies that are sensitive to each given administration is similar for all three doses and varies by around 50%, but of these, only a subpopulation (22%) showed

the consecutively increased locomotion to each dose that would indicate locomotor sensitization in this assay (Figure 23B).

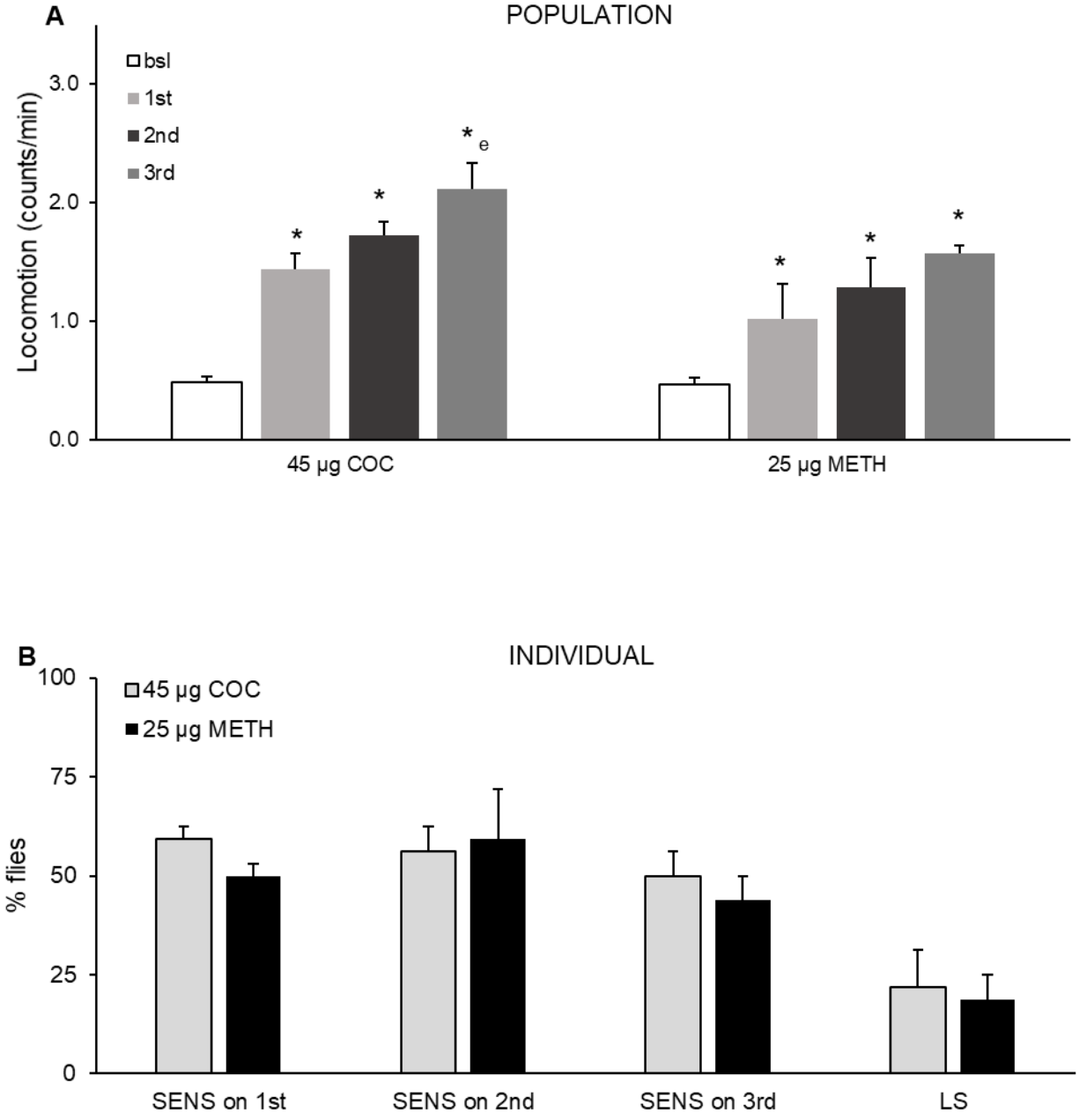
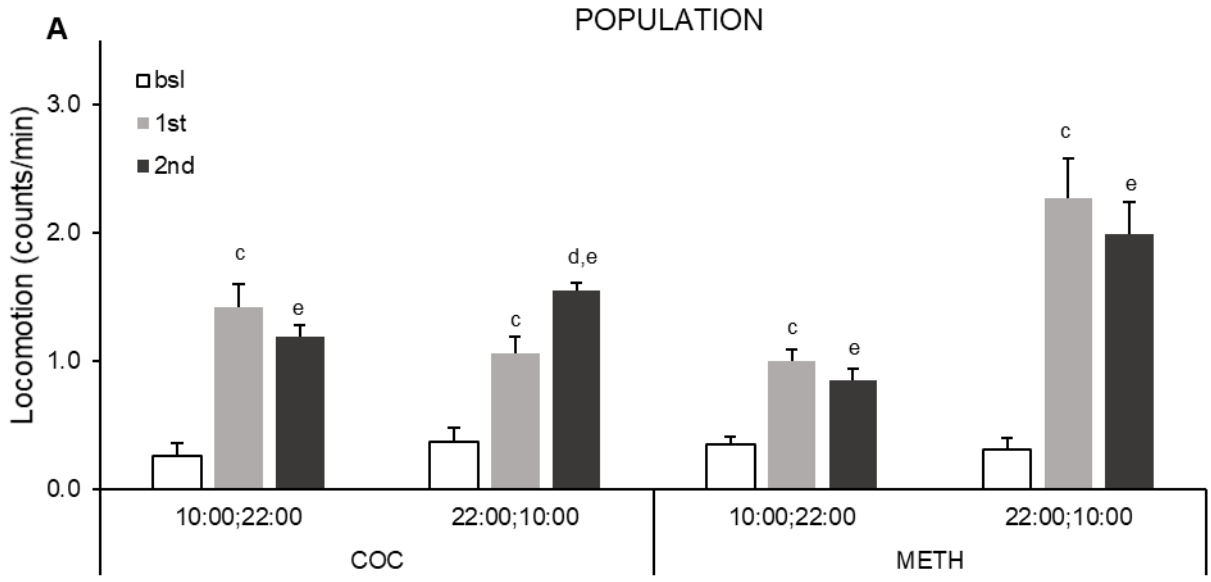


Figure 23. Multiple exposures to vCOC and vMETH lead to LS in population response, while individual SENS remains constant. A) Histogram of mean locomotor activity ± SEM for groups of flies (n=32) both before (bsl) and after administration to volatilized COC (45 µg) and METH (25 µg), firstly at 09:00 (1st), secondly at 19:00 (2nd) and thirdly on the next day at 09:00 (3rd). Statistical significance was p≤0.017 within groups; *-comparison of baseline to after 1st, 2nd and 3rd administration and e- after 1st to after 3rd (using ANOVA for repeated measurements with Bonferroni post-hoc test). **B)** Percentage of individual flies that showed sensitivity to a single exposure (SENS) and locomotor sensitization to three exposures by increasing locomotor activity to each consecutive exposure (LS) in groups exposed

to volatilized COC (COC 45 µg) or METH (METH 25 µg). Data are plotted as histograms showing the mean value of five tests ± SEM. Kruskal-Wallis H-test for nonparametric analysis of independent multiple samples (SENS on 1st, SENS on 2nd and SENS on 3rd) was used with Dunn's post-hoc test. There was no statistical significant difference for COC and METH.

To test if sensitivity or locomotor sensitization is influenced by circadian modulation, we administered two doses of vCOC and vMETH with a 12-hour interval. These experiments were performed in constant darkness to remove the confounding factor of light and to demonstrate if the source of modulation is the activity of the endogenous circadian clock.

Sensitivity to the acute vCOC dose did not vary as a function of the circadian time at population level (Figure 24A), or individual level as the percentage of flies that responded with increased activity at 10:00 did not vary significantly from 22:00 as shown by Mann-Whitney U-test test (Figure 24B). Interestingly, fewer flies showed an increased response to the second dose (relative to the first), when it was given at 22:00, which consequently resulted in a weaker locomotor sensitization. Thus, expression of locomotor sensitization is weaker during the subjective night, than during the subjective day. This shows that circadian clock modulates cocaine induced neuronal plasticity, but not sensitivity to cocaine.



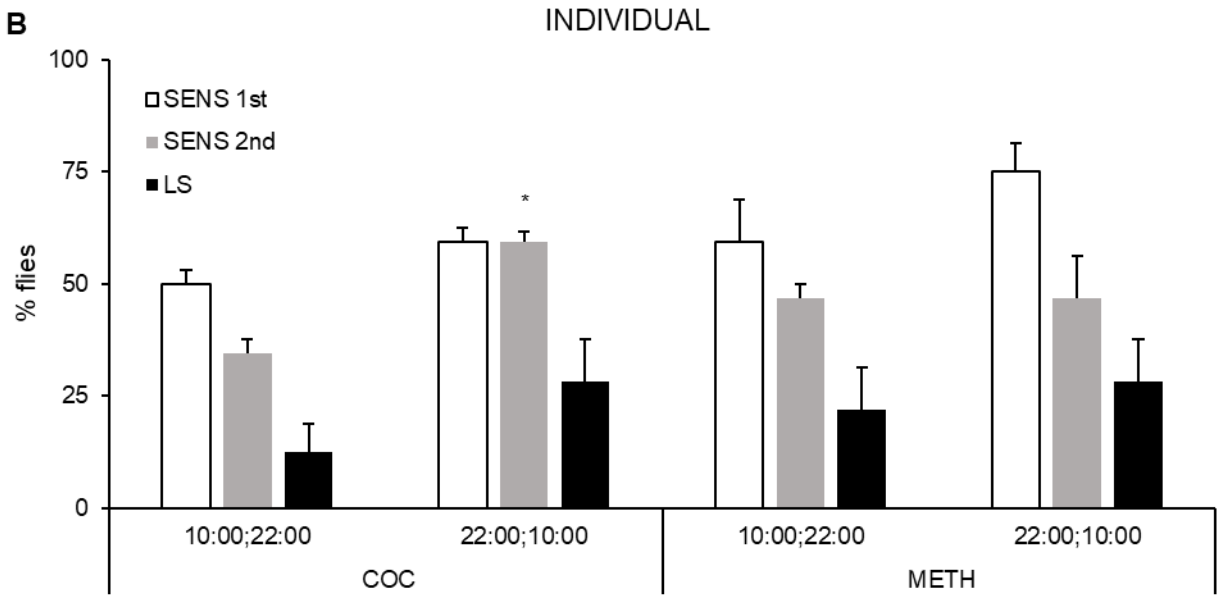


Figure 24. Sensitivity to the first dose is independent of the time of day when the first dose is applied, while intensity of LS depends on the time of the day. A) Average locomotor activity (counts/min) during baseline, 5 minutes before drug exposures (bsl), 5 minutes after the first exposure (1^{st}) and 5 minutes after the second (2^{nd}) exposure to 75 µg of volatilized COC. Average locomotor activity (counts/min) during baseline, 10 minutes before drug exposures (bsl), 10 minutes after the first exposure (1^{st}) and 10 minutes after the second (2^{nd}) exposure to 75 µg of volatilized METH. Exposures given 12 hours apart to a population of wild type (*wt*) flies (n=32 for each group), data are plotted as mean value of three experiments ± SEM. Statistical significance was p≤0.017; c-comparison of baseline to after first administration, d- after first to after second and e - baseline to after second administration (all within the group using ANOVA for repeated measurements with Bonferroni post-hoc test). **B)** Percentage of individual flies that showed sensitivity, indicated by an increase in locomotor activity to the first exposure of volatilized COC and METH relative to baseline (SENS 1st), flies showing further increases in locomotor activity to the second exposure 1st vs 2nd (SENS 2nd) and LS (bsl vs 1^{st} vs. 2^{nd}). Data are plotted as histograms showing the mean value of five tests ± SEM. Mann-Whitney U-test for nonparametric analysis of two independent samples showed a statistically significant difference, *p≤0.05 for flies which increased locomotion after second vCOC exposure (SENS 2^{nd}) at 10:00 when compared to the group exposed to vCOC at 22:00.

The vMETH population response to a first dose at 22:00 is higher when compared to one given at 10:00, while the population response to second dose is lower compared to first dose for both cases (Figure 24A). Analysis of individual fly data showed difference (non-significant) in sensitivity to the first dose and no difference to the second doses of vMETH or LS. This shows that METH differently modulates circadian clock than COC.

4.1.2. Screening using FlyBong platform

All screens were performed by applying standard protocols for vCOC and vMETH (Table 2) and individual data analysis (Table 5). Results are separated into three sections. The first section is focused on the testing of SENS and LS to vCOC in circadian mutants, performed to validate the FlyBong method in the mutant flies strains for which was previously known whether they develop LS or not. Influence of the same genes was also tested to vMETH. In the second section, we have applied genetic manipulations to test similarities and possible differences between vCOC and vMETH pharmacodynamics in vesicular monoamine transporter, dopamine transporter and dopamine receptor type 1. We also combined genetic manipulations with pharmacological manipulation in monoamine synthesis. Dopamine was manipulated using 3-iodo tyrosine (3IY), dopamine, serotonin, octopamine was manipulated using reserpine (res), while serotonin and octopamine were modulated using combination of L-DOPA and res (res+L-DOPA). The third section is focused on the potential influence of redox perturbation by pre-treatment with pro- and antioxidants on the development of SENS to acute doses of vCOC or vMETH, and LS on repeated exposures of vCOC or vMETH.

4.1.2.1. Involvement of circadian genes in SENS and LS

Studies in *Drosophila* and mammals have shown that the genes that control circadian rhythmicity are involved in the regulation of motor activating and arousing effects of psychostimulants (Andretic et al. 1999, Andretic et al. 2005). To validate FlyBong we exposed mutant strains for circadian genes per^{01}, tim^{01}, Clk^{Jrk} and cyc^{01} to vCOC. We tested a gene for a neuropeptide pigment dispersing factor (*pdf*), which conveys signals from circadian pacemaker cells in the fly brain to the rest of the brain and body (Renn et al. 1999). PDF-positive neurons also express *dLmo* gene, a regulator of LIM-homeodomain proteins, identified as a regulator of cocaine sensitivity in *Drosophila* (Tsai et al. 2004).

At the population level we confirmed that the *per*, *Clk* and *cyc* genes are required for locomotor sensitization to vCOC, since locomotor activity after first exposure was higher compared to locomotor activity after second exposure (Suppl. Mat. Figure 7A). We also confirmed that tim^{01} and pdf^{01} mutants behave similar to *wt* flies, suggesting that *tim* and *pdf* gene is not required for development of behavioral sensitization (Suppl.

Mat. Figure 7A). Individual analysis confirmed the importance for *per*, *Clk* and *cyc* in vCOC-induced sensitization (Figure 25A). To determine possible origin of *tim* and *pdf* gene mutants phenotype, we analyzed population and individual response of all flies to two exposures of warm air. Over 50% of the per^{01}, cyc^{01}, tim^{01} and pdf^{01} populations showed sensitivity to warm air, while over 30% of $tim^{01}$and pdf^{01} mutant flies develop locomotor sensitization to warm air (Suppl. Mat. Figure 8AB). These data suggest that the putative locomotor sensitization of tim^{01} and pdf^{01} mutants to cocaine has to be re-examined, as at least part of that phenotype is not cocaine-specific. Very low fraction of per^{01}, Clk^{Jrk} and cyc^{01} flies develops locomotor sensitization to vCOC (Figure 25A) as does to warm air (Suppl. Mat. Figure 8B), arguing for impaired mechanisms of neuronal plasticity in these mutants. Individual level analysis of FlyBong locomotor output and use of appropriate controls offers new information about behavioral responses, which would not be obvious when examining only the average population response to vCOC.

All circadian mutants, beside Clk^{Jrk} showed an increase in SENS when compared to *wt* flies, following acute exposure to vMETH (Figure 25B). LS was lower for all tested circadian mutants when compared to *wt* flies, with only Clk^{Jrk} being statistically significant (Figure 25B). From the population-level data, all mutants failed to develop LS since locomotor activity after first exposure to vMETH was higher compared to locomotor activity after second exposure to vMETH (Suppl. Mat. Figure 7B). In control groups, which did not received vMETH, per^{01}, cyc^{01}, tim^{01} and pdf^{01} showed higher SENS than *wt* flies or Clk^{Jrk} mutants, at both the individual and population levels of analysis (Suppl. Mat. Figure 9A,B). These data suggest that the putative locomotor sensitization of per^{01}, tim^{01} and pdf^{01} mutants to METH has to be re-examined, as at least part of that phenotype is not methamphetamine-specific.

It is important to emphasize that Clk^{Jrk}, of all the mutants tested, was the least sensitive to either vCOC, vMETH or warm air, when studying at both the individual and population-levels of analysis. The pdf^{01} mutants, meanwhile, showed almost the same response to vCOC and vMETH on individual and population level as *wt* flies.

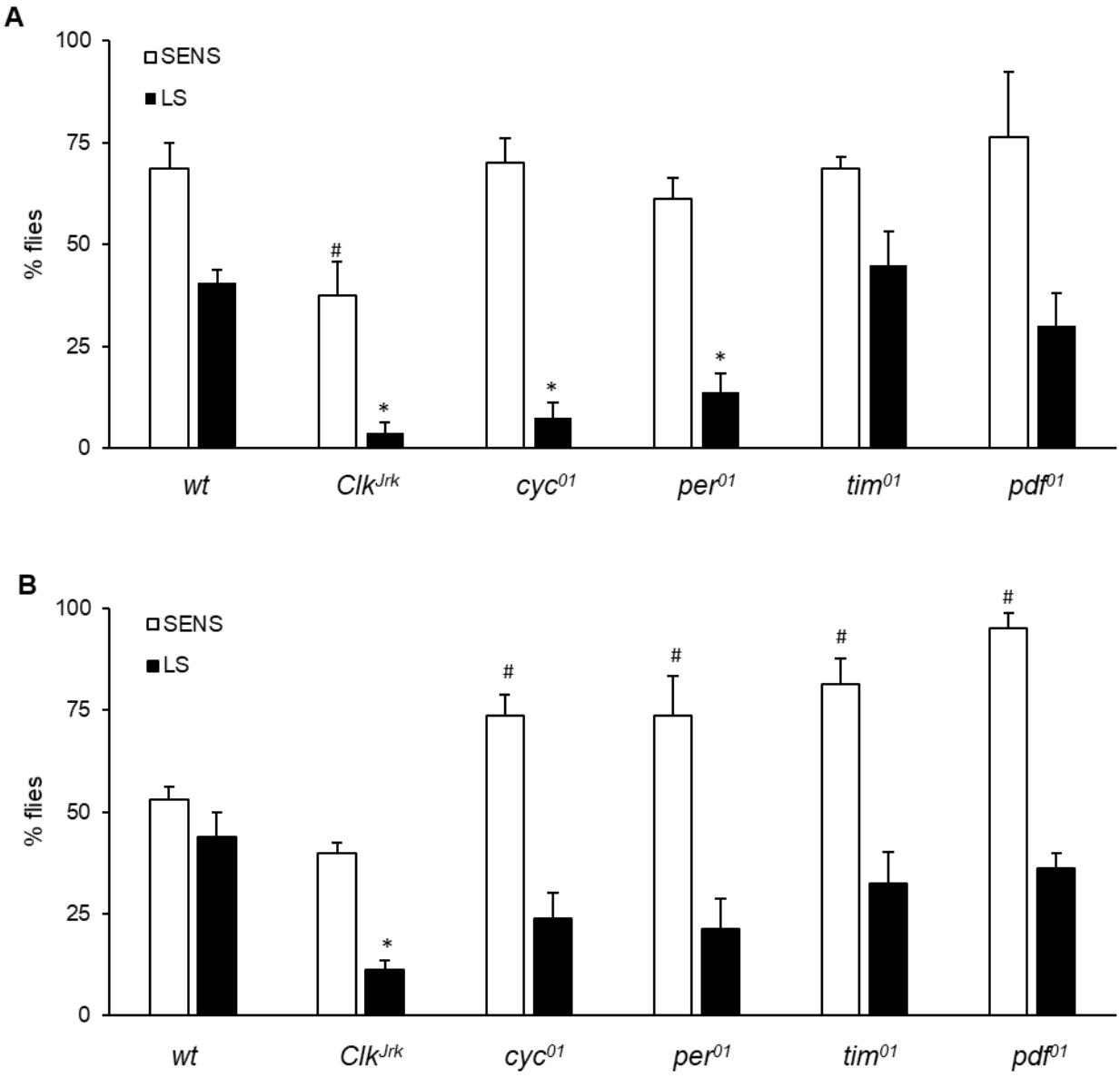


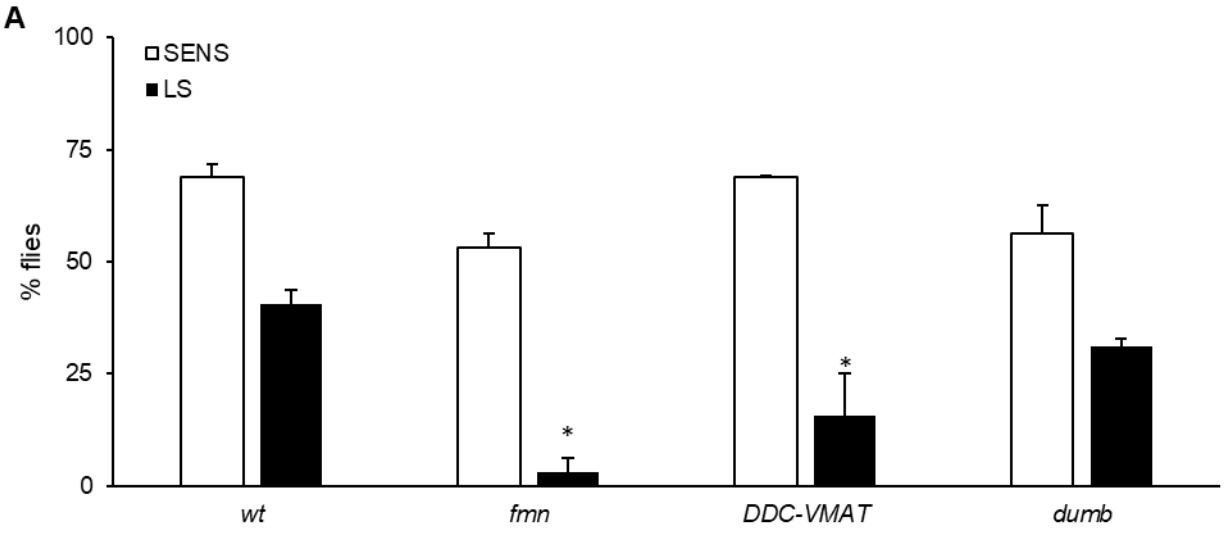
Figure 25. Circadian genes are involved in the development of LS, but not SENS in male flies to A) vCOC and B) vMETH. The flies were divided into groups according to their genotype: *wt*- wild type, *per*^{01}- mutant for the *period* gene, *Clk*^{Jrk} - mutant for the *Clock* gene, *cyc*^{01} - mutant for the *cycle* gene, *tim*^{01} - mutant for the *timeless* gene and *pdf*^{01} - mutant for the *pigment-dispersing factor* gene. The protocol for SENS and LS was performed according to Table 2. All tests were performed on n=32 flies and repeated five times. Results are reported as a mean of five experiments ± SEM. Kruskal-Wallis H-test for nonparametric analysis of independent multiple samples was used with Dunn's post-hoc test $^{\#}p<0.05$ SENS of *wt* flies vscircadian mutants and $^{*}p<0.05$ LS of *wt* flies vs circadian mutants

4.1.2.2. Aminergic modulation of SENS and LS

a) Genetic manipulation of vesicular monoamine transporter, dopamine transporter and dopamine receptor

METH and COC have similar pharmacological and behavioral profiles. However, COC and METH exhibit differences in mechanisms of action and pharmacokinetics. COC binds to DAT on the presynaptic neuron and reduces levels of DR on the postsynaptic neuron, while METH is entering neurons and reversing function of DAT and VMAT. To further validate our platform and to show differences between molecular mechanism in motor-activating effects of vCOC and vMETH, we have used mutant strains of dopamine transporter (*fmn*) and dopamine receptor type 1 (*dumb*). For testing VMAT we have used transgenic flies with DOPA decarboxylase (DDC) Gal4 driver controlling the expression of UAS VMAT RNAi to reduce the level of VMAT in all dopaminergic and serotoninergic neurons.

No tested strains affected the response to an acute dose of vCOC when compared to *wt* flies (Figure 26A), while vMETH exposure induced higher sensitivity in DDC-VMAT transgene flies and *dumb* mutants (Figure 26B). Repeated vCOC exposures significantly reduced LS for the *fmn* mutants and DDC-VAMT transgene flies, while *dumb* mutants had a LS response that was almost the same as for *wt* flies (Figure 26A). Repeated vMETH exposures statistically significantly reduced LS in DDC-VMAT transgene, *dumb* and *fmn* mutant flies when compared to wt flies (Figure 26B).



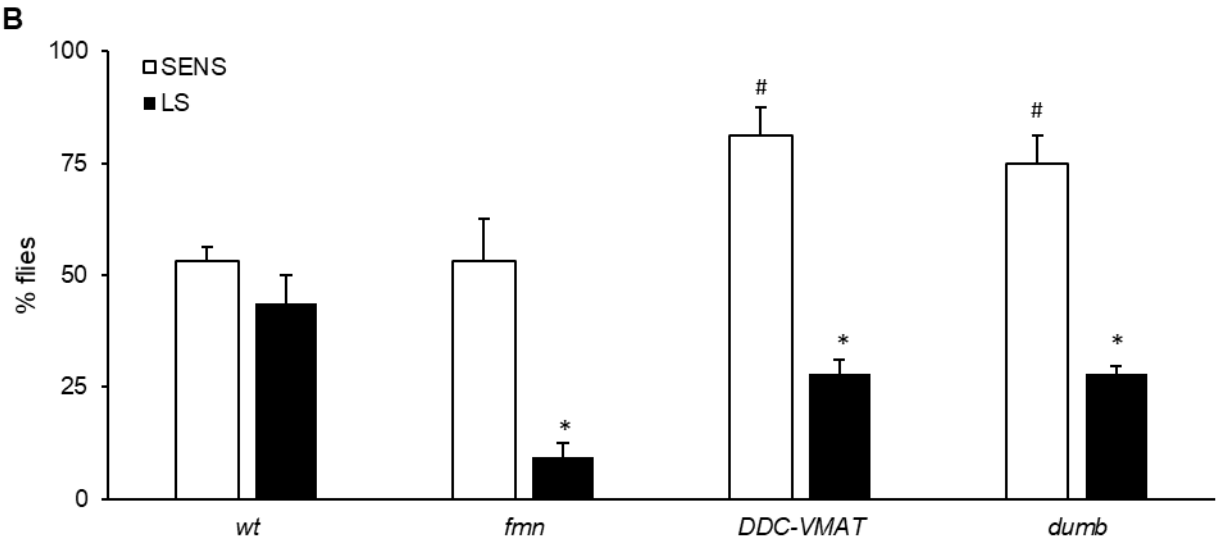


Figure 26. Genetic manipulations of dopaminergic system affect development of LS, but do not affect SENS to A) vCOC and B) vMETH. The flies were divided into groups according to their genotype: *wt*- wild type, dopamine transporter mutant (*fmn*), transgenic flies with DOPA decarboxylase (DDC) Gal4 driver and UAS VMAT RNAi (DDC-VMAT) and dopamine receptor type 1 mutant (*dumb*). The protocol for studying SENS and LS was according to Table 2. All tests were performed on n=32 flies and repeated five times. Results are reported as a mean of five experiments ± SEM. Kruskal-Wallis H-test for nonparametric analysis of independent multiple samples was used with Dunn's post-hoc test. $^{\#}p \leq 0.05$ SENS in *wt* flies vs mutants and * $p \leq 0.05$ LS in *wt* flies vs mutants, following PS exposures.

At the level of population, all mutants responded to acute vCOC exposure (Suppl. Mat. Figure 10A), in agreement with the observed individual-level data. Based on the population data (Suppl. Mat. Figure 10B), only *fmn* mutants did not respond to acute vMETH exposure, while DDC-VMAT and *dumb* showed increased population locomotor activity, in agreement with the observed individual-level data. At the population level, all mutants responded with less locomotor activity to second exposure when compared to activity after the first exposure for both vCOC and vMETH (Suppl. Mat. Figure 10A,B).

From the data obtained, we have concluded that both the dopamine transporter (DAT) and dopamine receptor (DR) are important for LS induced by vCOC and vMETH. These results were predicted, as COC and METH bind to DAT on the presynaptic neuron and reduce levels of DR on the postsynaptic neuron. Previously published data in mammals showed that the vesicular monoamine transporter (VMAT) is important for COC- and METH-induced LS, which is in agreement with results obtained for COC,

but not for METH, in our experiments. One possible explanation for this is that we used DOPA decarboxylase (DDC) Gal4 driver, which expressed in all dopaminergic and serotoninergic neurons. Since, it is reported that DA synthesis is under the control of circadian genes, induced during daytime and repressed during nighttime, it is possible that VMAT RNAi is not equally expressed at 15:00, when the second vCOC administration occurs, as at 19:00 when the second vMETH administration occurs. This suggests that VMAT is important for LS induced by COC, but not METH.

b) Pharmacological manipulation of dopamine, serotonin and octopamine

The *wt* flies were pre-treated with a variety of chemicals for 48 hours to manipulate monoamines: 3-iodo tyrosine (3IY) to reduce DA in whole fly body, reserpine (res) to reduce DA, serotonin and octopamine in the brain, or both res and L-DOPA to reduce serotonin and octopamine in the brain. None of the three pre-treatments affected SENS following acute vCOC exposure, at either the individual (Figure 27A) or population level (Suppl. Mat. Figure 11A). The 3IY and res reduced the LS of flies to repeated vCOC exposures, when compared to non-treated *wt* flies, while a similar reduction after treatment with res+L-DOPA was observed when compared to non-treated *wt* flies (Figure 27A). Population data showed that all three treatments led to reduced locomotor activity following the second exposure, when compared to the first exposure to vCOC (Suppl. Mat. Figure 11A).

Acute exposure to vMETH slightly increased SENS following all three pre-treatments (Figure 27B), as was also seen at the population level (Suppl. Mat. Figure 10B). Repeated vMETH exposures reduced LS after all three treatments (Figure 27B). At the population level, a reduction was also seen in locomotion after the second dose, relative to the first dose of vMETH (Suppl. Mat. Figure 11B).

Reduction of levels of DA, serotonin and octopamine using reserpine lowered the LS of flies to vCOC, compared to the reduction of DA levels only using 3IY. Restoring the levels of DA in reserpine-treated flies using L-DOPA surprisingly resulted in lower LS compared to the res and 3IY treatments, as levels of DA should be restored. A possible explanation is that the L-DOPA used to restore levels of DA can act as an antioxidant and lower the LS response to vCOC, as has been reported for TEMPOL and similar antioxidants (see below).

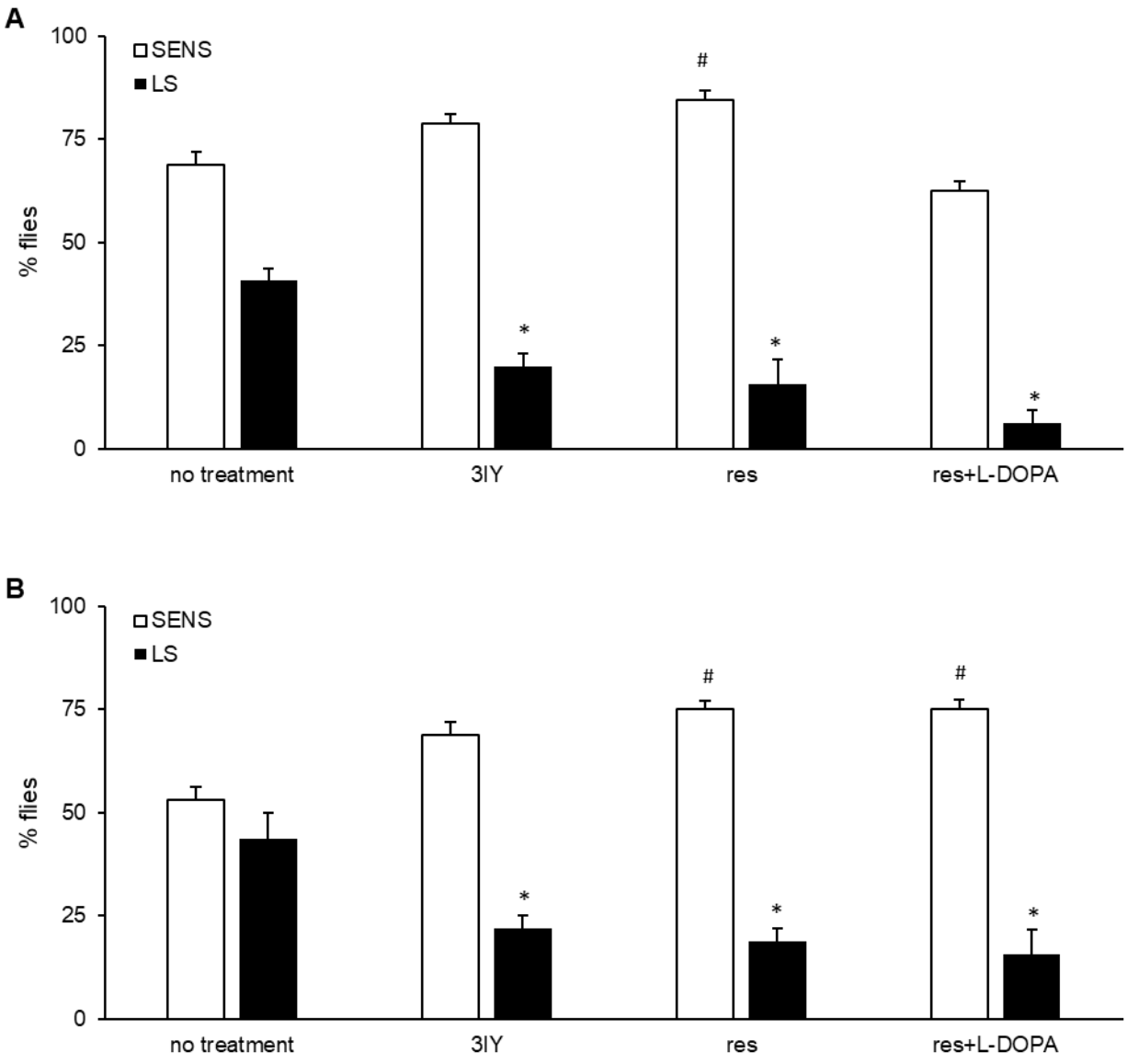


Figure 27. LS, but not SENS depends on dopamine, serotonin and octopamine to A) vCOC and B) vMETH. The flies were divided into groups according to the treatment given: no treatment- wild type flies without treatment, 3IY - *wt* flies pre-treated with 3-iodo tyrosine, res- *wt* flies pre-treated with reserpine, and res+L-DOPA - *wt* flies pre-treated with both reserpine and L-DOPA. The protocol for studying SENS and LS was performed as stated in Table 2, while pre-treatment was performed according to Table 1. All tests were performed on n=32 flies and repeated five times. Results are reported as a mean of five experiments ± SEM. Kruskal-Wallis H-test for nonparametric analysis of independent multiple samples was used with Dunn's post-hoc test. #p≤0.05 SENS for no treatment vs different pre-treatments and *p≤0.05 LS for no treatment vs different pre-treatments, following PS exposures.

4.1.2.3. Antioxidant and prooxidant modulation of SENS and LS

Several antioxidants were used to pre-treat male *wt* flies: quercetin (QUE), tyrosol (TYR) and TEMPOL, and prooxidants hydrogen peroxide (H_2O_2) and paraquat (PQ). We find that the antioxidants QUE, TYR and H_2O_2 significantly lowered SENS to acute vCOC exposure, while TEMPOL and PQ did not affect SENS (Figure 28A). In the population data, the same effect of acute exposure to vCOC can be seen (Suppl.

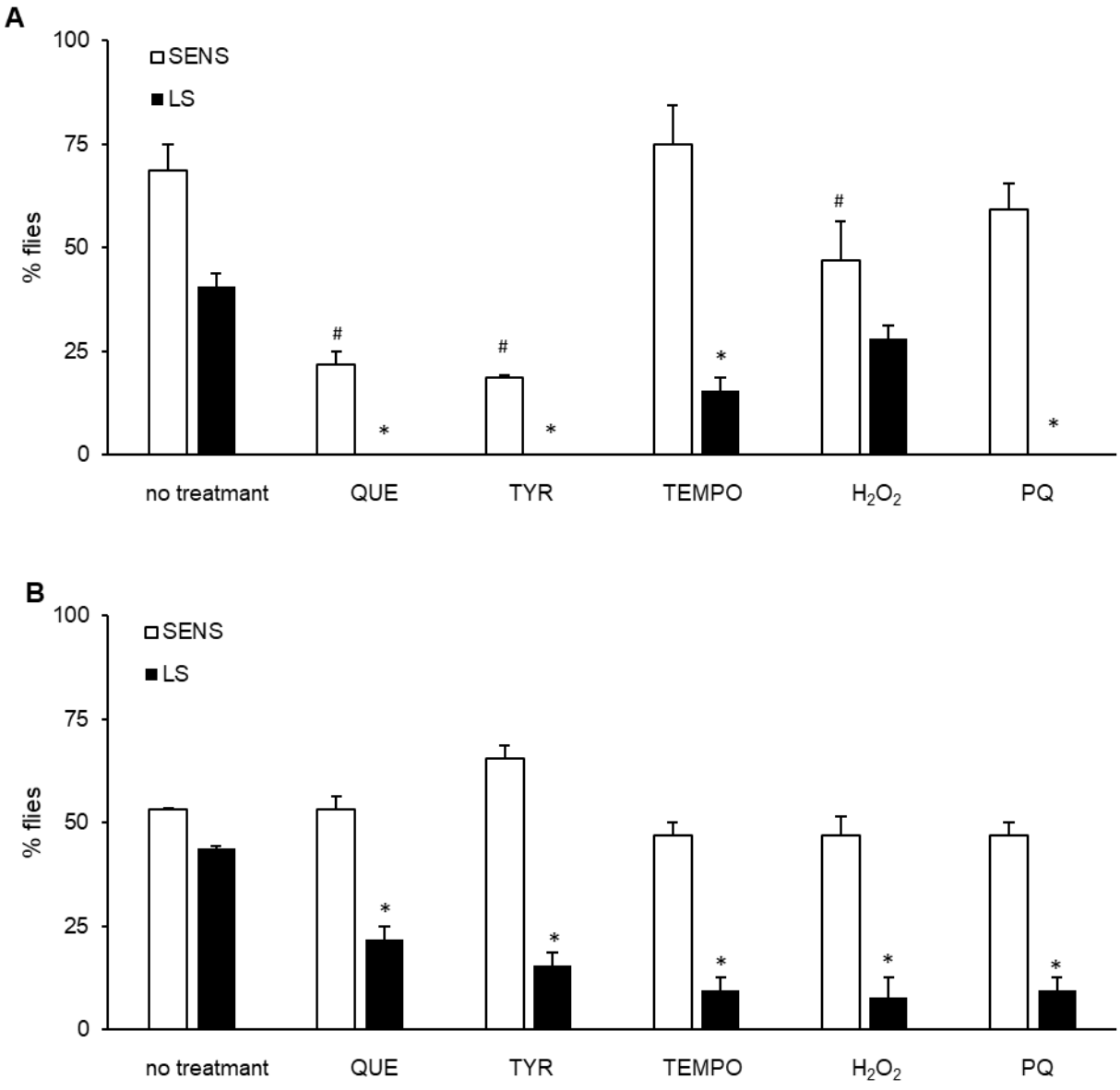
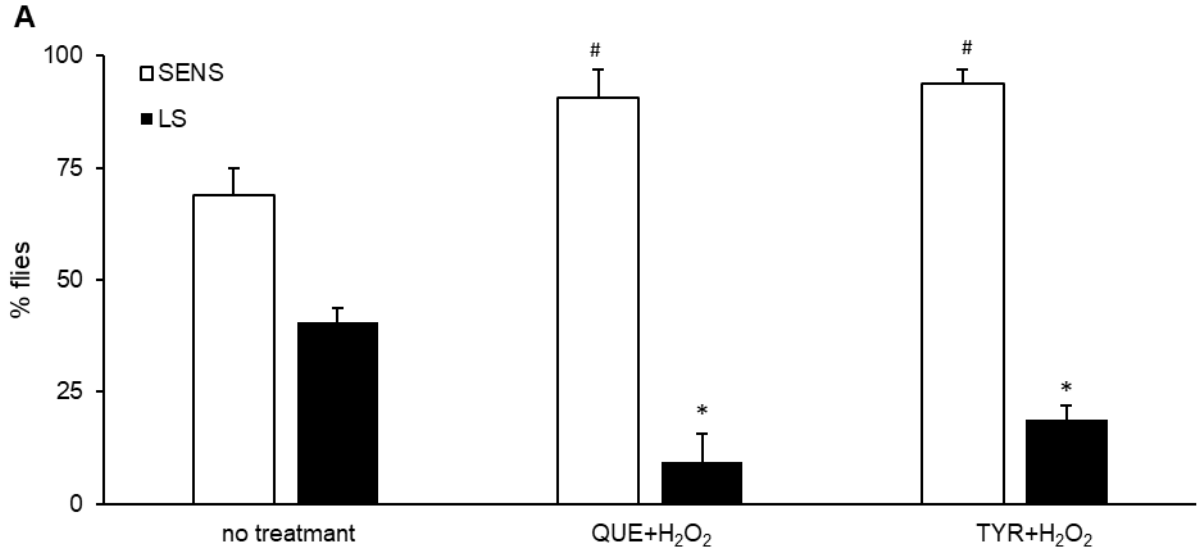


Figure 28. Effect of antioxidants and prooxidants on SENS, and LS to: vCOC A) and to vMETH B). The flies were divided into groups according to their treatment: no treatment- wild type flies without treatment, QUE - *wt* flies pre-treated with quercetin, TYR- *wt* flies pre-treated with tyrosol, TEMPOL- *wt* flies pre-treated with TEMPOL, H_2O_2- *wt* flies pre-treated with hydrogen peroxide, and PQ - *wt* flies pre-treated with paraquat. The protocol for studying SENS and LS was according to Table 2, while the pre-treatment was according to Table 1. All tests were performed on n=32 flies and repeated five times.

Results are reported as a mean of five experiments ± SEM. Kruskal-Wallis H-test for nonparametric analysis of independent multiple samples was used with Dunn's post-hoc test. #p≤0.05 SENS for no treatment vs different pre-treatments and *p≤0.05 t LS for no treatment vs different pre-treatments, following PS exposures.

Mat. Figure 12.A). QUE, TYR and PQ completely abolished LS after repeated exposures to vCOC, while TEMPOL reduced LS, and H_2O_2 did not affect it (Figure 28A). The same result was seen at the population-level (Suppl. Mat. Figure 12A). Antioxidant and prooxidant treatments did not affect SENS to acute vMETH exposure (Figure 28B), at either individual or population level (Suppl. Mat. Figure 12B). All treatments lowered LS, while TEMPOL, H_2O_2 and PQ caused a statistically significant reduction in LS compared to the non-treated group (Figure 28B). The same result of treatments on LS was seen at the population level (Suppl. Mat. Figure 12B).

In an attempt to restore SENS and LS through antioxidant treatments, we combined quercetin and tyrosol with hydrogen peroxide and exposed flies to QUE+H_2O_2 and TYR+H_2O_2. Both combinations not only restored, but also increased SENS compared to the non-treated group following acute vCOC exposure (Figure 29A). At the population level, an increase in locomotion after acute vCOC exposure was also seen in both treated groups compared to non-treated groups (Suppl. Mat. Figure 13A).



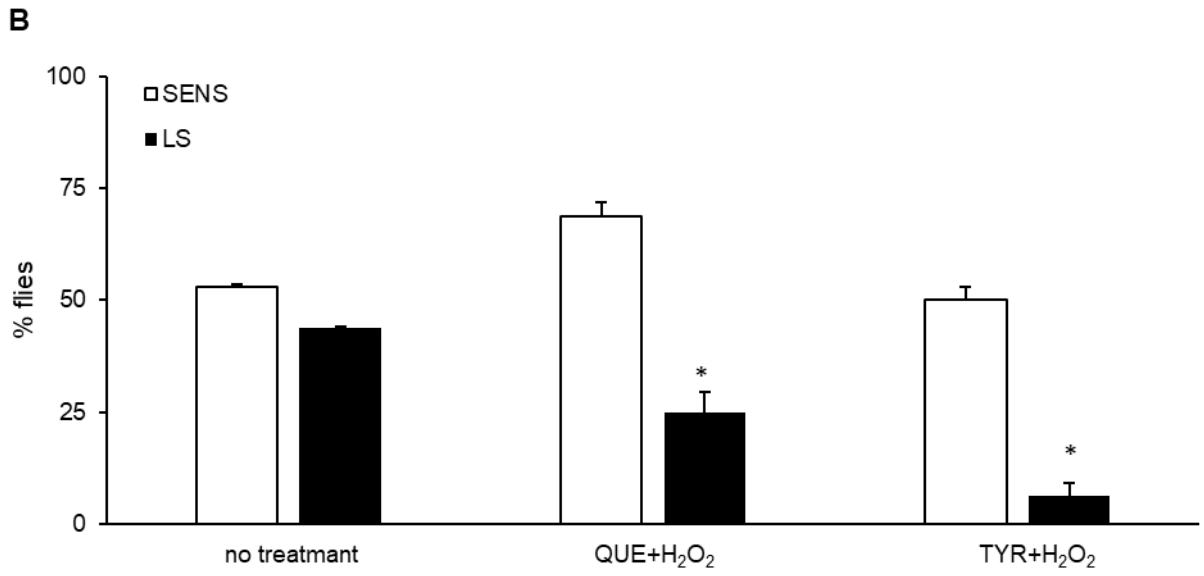


Figure 29. Combination of quercetin and tyrosol with hydrogen peroxide restore SENS and partially LS to: vCOC A), while reduce LS by not effecting SENS to B) vMETH in *wt* male flies. The flies were divided into groups according to the treatment: no treatment- wild type flies without treatment, QUE+ H_2O_2- *wt* flies pre-treated with quercetin and hydrogen peroxide, and TYR+ H_2O_2- *wt* flies pre-treated with tyrosol and hydrogen peroxide. Protocol for SENS and LS was done according Table 2, while pre-treatment according Table 1. All test are performed on n=32 and repeated five times. Results are reported as mean of five experiments ± SEM. Kruskal-Wallis H-test for nonparametric analysis of independent multiple samples was used with Dunn's post-hoc test. $^{\#}p \leq 0.05$ SENS of no treatment vs different pre-treatments and $*p \leq 0.05$ LS of no treatment vs different pre-treatments, following PS exposures.

Same as in the case with the antioxidant and prooxidant administered alone, the combination treatments did not affect SENS to acute vMETH exposure (Figure 29B), as can been seen in the population data (Suppl. Mat. Figure 13B). Flies after QUE+H_2O_2 treatment showed lower LS compared to the non-treated group (Figure 29B), but this LS was comparable to treatment with QUE alone (Figure 28B). Treatment with TYR+H_2O_2 statistically significantly lowered LS compared to LS of the non-treated group or to LS of the group treated with TYR alone. The observed effect of the combined treatment was also seen in the population-level data, where the second vMETH exposure increased locomotion relative to the first vMETH exposure, although this was not statistically significant (Suppl. Mat. Figure 13B).

Redox modulation induced by exogenous pro- and antioxidants has an effect on SENS and LS after both vCOC and vMETH administration. Both QUE and TYR

reduce SENS and abolish LS to vCOC, while H_2O_2 does not have an influence on this parameters. In contrast, combination of QUE and H_2O_2 or TYR and H_2O_2 is restoring LS to vCOC. QUE, TYR and H_2O_2 lower LS to vMETH, while combination of QUE and H_2O_2 or TYR and H_2O_2 additionally lower LS to vMETH. This data suggests an opposite role of H_2O_2 on neuronal modulation in vCOC- and vMETH-induced neuronal plasticity, which further suggests its dependence on change in the redox status.

4.2. Rewarding effect of PS

To test if flies will voluntary self-administer COC and METH, which would indicate rewarding effect of PS in flies, we have used modified CAFÉ assay. Every 24 hours amount of liquid food containing sucrose and food that in addition contained different concentrations of COC and METH was measured. Data are presented as preference index (PI) calculated as difference between amount of consumed food with the drug and amount of consumed food without the drug, divided with total food consumption every 24 hours. Positive PI value indicates that drug is appetitive to flies and that they consume more food with the drug compared to food without the drug, whereas negative PI values indicate that food with drug is aversive to flies and they have lower intake of food with the drug compared to food without the drug.

4.2.1. CAFÉ assay optimization

As part of optimization, we have established that when the capillaries are changed every 24 hours only 6 flies per chamber can be used. Humidity inside of the chamber is important and we optimized it using a cotton soaked with 1 mL of tap water. In this section, we have tested preferential consumption of COC and METH in naive flies, and established optimal COC and METH concentrations, which increase PI over consecutive days. We also investigated influence of a visual cue location in respect to capillary containing drug on preferential consumption.

4.2.1.1. Preferential PS consumption and concentration optimization

To define an optimal concentration that will lead to greatest preferential consumption we exposed naive flies to 0.05, 0.10, 0.15, 0.20, 0.50, 1.00 and 1.50 mg/mL of COC mixed with 100 mM sucrose solution and 0.10, 0.20, 0.30 and 0.40 mg/mL of METH mixed with 100 mM sucrose solution. Flies could choose between two capillaries containing only sucrose solution and two capillaries containing sucrose mixed with drug in different concentrations. We used clear feeding lid without any markings on the pipette tip, capillary or lid itself. Locations of capillaries with or without the drug were the same over 5 consecutive days (Figure 10). Amounts of food consumption were measured every 24 hours. From the daily consumption of food with and without the drug , PI values were calculated and plotted as average PI value from 5 days. Our data show that COC concentrations starting from 0.05 to 0.15 mg/mL are

appetitive to flies in dose dependent way, while doses higher than 0.20 mg/mL are aversive (Figure 30A).

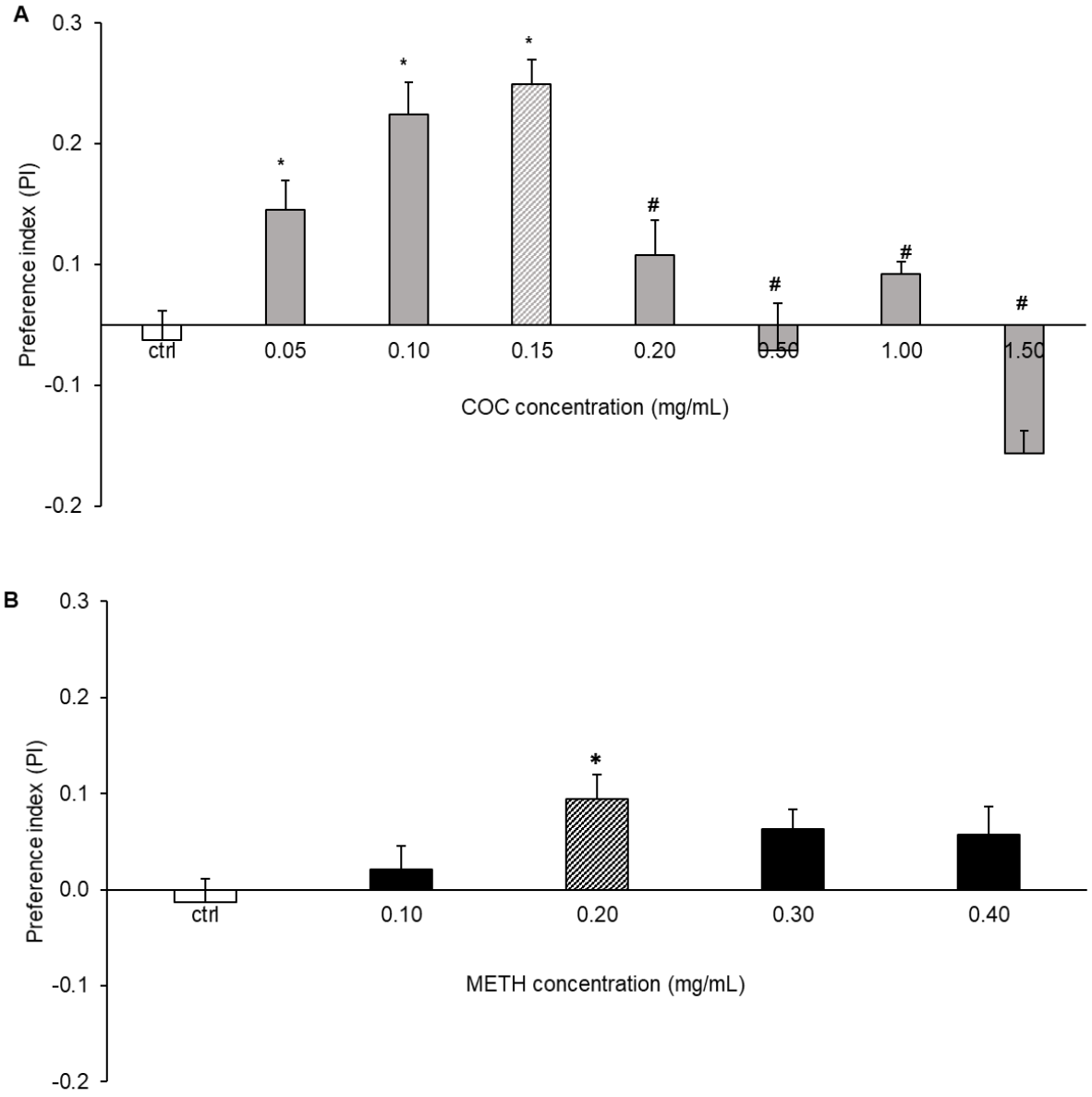


Figure 30. Preferential consumption of COC and METH is dose-dependent. Flies were exposed to two capillary containing non-drug and two capillary containing food with drugs in different concentrations over period of 5 days. The experiments were repeated 2 times with 2 tubes each containing 6 flies (n=24). From daily consumption of food with and without the drug, daily PI values were calculated and plotted as histogram of the mean value of 5 days ± SEM. Control group was exposed only to the food without the drug and PI was calculated regarding position of drug capillary in test groups. *p <0.05 in relation to the control group, #p <0.05 compared to concentrations of 0.10 mg/mL and 0.15 mg/mL (One-way ANOVA, Tukey's multiple comparison). **A)** COC concentration range 0.05, 0.10, 0.15, 0.20, 0.50, 1.00 and 1.50 mg/mL **B)** METH concentration range 0.10, 0.20, 0.30, and 0.40 mg/mL.

The dose of 0.15 mg/mL was chosen as the optimal COC concentration based on the highest average PI value for period of 5 days, and that concentration was used for further tests. In METH self-administration experiments, same as in COC, lower doses are appetitive to flies, in dose-dependent way starting from 0.10 to 0.20 mg/mL, while higher doses starting from 0.30 mg/mL are aversive (Figure 30B). The dose of 0.20 mg/mL was chosen as the optimal METH concentration based on highest average PI value for period of 5 days, and that concentration was used in further tests. In all assays control group did not show any preference to certain capillaries, indicating that flies exposed to drug were attracted to content of the drug inside of capillary, but not to capillary location or some other possible cue from environment.

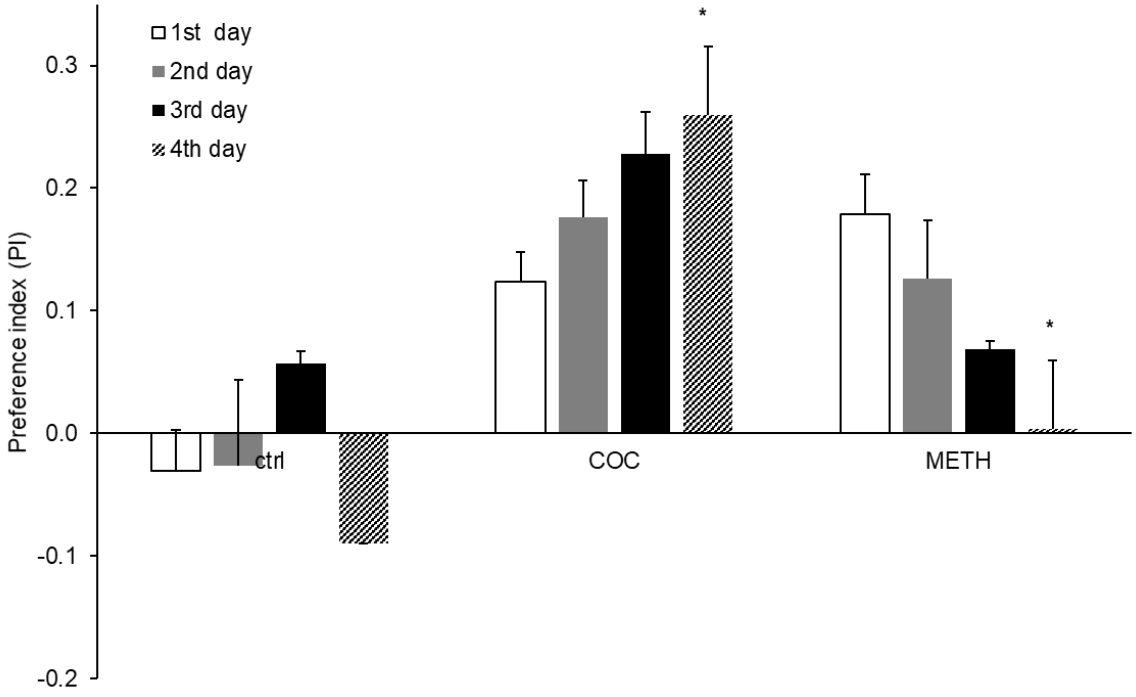


Figure 31. Preferential consumption is increasing for COC and decreasing for METH over consecutive days. Males aged 3-5 days were fed in the control group with aqueous solution of sucrose in all four capillaries, and in the test groups two capillaries contained a sucrose solution and in the other two aqueous solutions of COC at concentration of 0.15 mg/mL and METH at a concentration of 0.20 mg/mL. The volume of consumed food in each capillary was measured every 24 hours and was converted to the preference index (PI). The experiment was repeated 2 times with 2 tubes each containing 6 flies (n=24). Data are plotted as histograms of mean value ± SEM. To determine the difference between the preference index between days, ANOVA for repeated measurements was used followed by Tukey's multiple comparison; *p <0.05.

For better understanding of the preferential consumption of COC and METH, we have monitored PI value over 4 days in row (Figure 31). Flies showed positive PI values for 0.15 mg/mL COC starting from first day. Positive PI values were present during all 4 days of testing, with increasing trend. Positive PI values on the first day of exposure were observed with 0.05 mg/mL and 1.00 mg/mL of COC (Suppl. Mat. Figure 14A). Over consecutive days, preference for lower COC concentration (0.05 mg/mL) was present and it was increasing, compared to higher dosage (1.00 mg/mL) for which preference was decreasing (Suppl. Mat. Figure 14A). PI value for 0.20 mg/mL METH was positive for all 4 days, but compared to 0.15 mg/mL COC it was decreasing over consecutive days. For 0.10 mg/mL or 0.30 mg/mL of METH the preference was decreasing (Suppl. Mat. Figure 14B). Since control group did not show preference to certain side or capillary, we concluded that preference to COC and METH is based on rewarding effect of drug, rather than capillary location or environmental cue. Rewarding effects of COC seems to be stronger than rewarding effects of METH.

4.2.1.1. Influence of cue and drug location on preferential consumption

We have shown that flies self-administer COC and METH, and that PI is not due to environmental effect or location of capillary with the drug. To test if the flies will perform higher PI when capillary containing dug is associated with the cue, we have used colored tips in which drug containing capillary was black. The assay was performed according to the scheme shown in the Figure 10, for 0.15 mg/mL COC and 0.20 mg/mL METH, with (+Q) cue and without (-Q) cue. The results showed (Figure 32) that flies that had cue (+Q) associated with COC showed higher PI values compared to group without cue on pipette tips (-Q). PI in +Q group was increasing over consecutive days, while in -Q group trend was present, but it was not as significant as for +Q group. Preferential consumption of METH was also under influence of cue, but effect was weaker compared to that observed in COC +Q group. The -Q METH group showed high PI value on the first day, but over the days, that value was decreasing. In +Q METH group same negative trend was observed, but PI values were decreasing slower than in -Q METH group. Since both COC and METH shoved better stability of PI over consecutive days when cue is present, we have decided to use black color as a cue in subsequent experiments.

These data suggest that flies associate color cue with the location of the drug. This indicated that as in mammals, drug consumption affects the mechanisms of learning and memory. This effect is more pronounced for COC, which seems to have higher rewarding effect than METH.

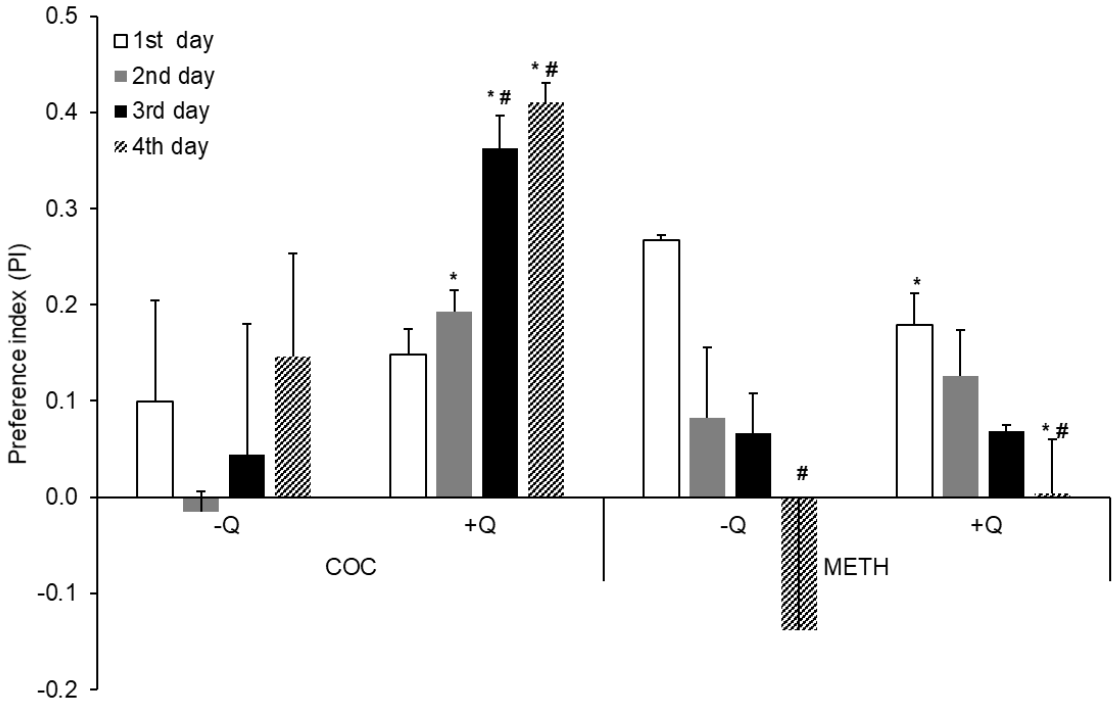


Figure 32. Preferential consumption depends on cue associated with capillary containing COC and METH. In the experiment, males aged 3-5 days were fed in -Q groups with two capillaries contained a sucrose solution and in the other two aqueous solutions of COC at concentration of 0.15 mg/mL and METH at a concentration of 0.20 mg/mL. Flies in +Q groups were exposed to the same procedure as -Q group, but drug containing pipette tips were colored black. The volume of consumed food in each capillary was measured every 24 hours and was converted to the preference index. The experiment was repeated 2 times with 2 tubes each containing 6 flies (n=24). Data are plotted as histograms of mean value ± SEM. To determine the difference between the preference index between days in -Q and +Q group, t-test for independent samples were performed (*$p<0.05$), ANOVA for repeated measurements was used with Tukey's multiple comparison ($^{\#}p<0.05$) in case of determining difference between the preference index between days within the group.

Since the effect of cue was stronger in COC-exposed flies, we tested influence of fixed cue location while changing the location of capillary with drug over period of three days. All groups received COC in concentration of 0.15 mg/mL. One group had fix cue and capillary location (Figure 11) over consecutive days. In second group, cue location was fixed, but location of capillary containing drug was changed, every day on

different side (Figure 12). Third group had fixed cue location but capillaries were switched diagonally every day (Figure 13). If the cue and capillary location are fixed over course of consecutive days, PI was increasing (Figure 33). For the second group, where capillary location was every day on a different side, PI was alternating over the days. Diagonal rotation of capillary with COC leads to decreased PI, as flies were not able to identify capillary with the drug. Based on these results, we used fixed cue location and fixed drug-containing capillary for all further experiments. These results confirm the involvement of learning and memory in preferential consumption of COC.

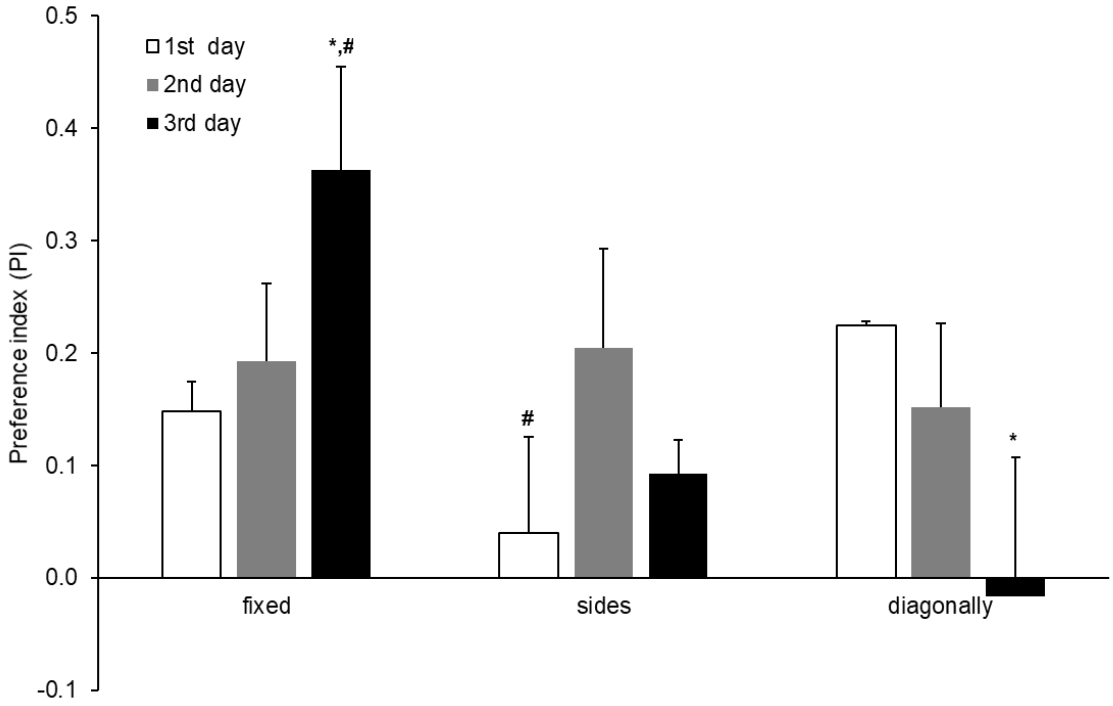


Figure 33. Preferential COC consumption over the course of consecutive days depends on the location of the drug capillary. All groups were exposed with two capillaries with a solution of sucrose and two with a 0.15 mg/mL COC solution. Group “fixed” received daily COC containing capillary at the same location, to "sides" group COC was given every day on the other side of the chamber, and for the "diagonally" group COC location was changed in diagonals. The experiment was repeated 2 times with 3 tubes each containing 6 flies (n=36). Data are plotted as histograms of mean value± SEM. To determine the difference between the preference index between days in fixed, sides and diagonally groups one-way ANOVA was used with Tukey's multiple comparison (*$p < 0.05$), ANOVA for repeated measurements was used with Tukey's multiple comparison ($^{\#}p < 0.05$) in case of determining difference between the preference index between days within the group.

4.2.2. Features of addiction in *Drosophila*

In order to examine whether flies show similar patterns of addictive behavior as humans, we have investigated two experimental manipulations: consumption against negative consequences and deprivation effect. Flies find bitter taste of quinine aversive (Devineni and Heberlein 2009), so we tested whether the flies will self-administer PS against bitter taste, and if flies will consume same or higher amount of drug after period of deprivation. All assays were performed with fix cue location associated with PS over period of 4 or 5 days in a row and concentrations of COC 0.15 mg/mL and METH 0.20 mg/mL.

4.2.2.1. Relapse induced by deprivation

We started experiment with standard self-administration protocol, in both deprived and non-deprived group. Drug containing capillaries were associated with cue. After two days we divided flies in two groups. One group proceeded with the standard protocol (non-deprived group) while in deprived group drug containing capillaries were removed for two days. On the days of deprivation in all four capillaries there was a sucrose solution and PI was calculated for the capillaries at sites where there was a drug solution on the previous days, so it does not represent drug preference but preference for the position of the capillary.

Relapse is usually induced in animal models trough deprivation from the drug, or with a period when the drug is absent from animal environment. After a period of abstinence, animals are again exposed to the drug showing increased or the same drug consumption compared to preference before abstinence. After two-day deprivation, (Figure 34A), COC preference index in deprived group was the same as on day before deprivation, but it was lower than in non-deprived group (Figure 34A). Same protocol applied to METH consumption resulted in an increase in PI when compared to day before deprivation and when compared to non-deprived group (Figure 34B). These results suggest that, as is the case for ethanol self-administration, flies show some elements of relapse after PS withdrawal.

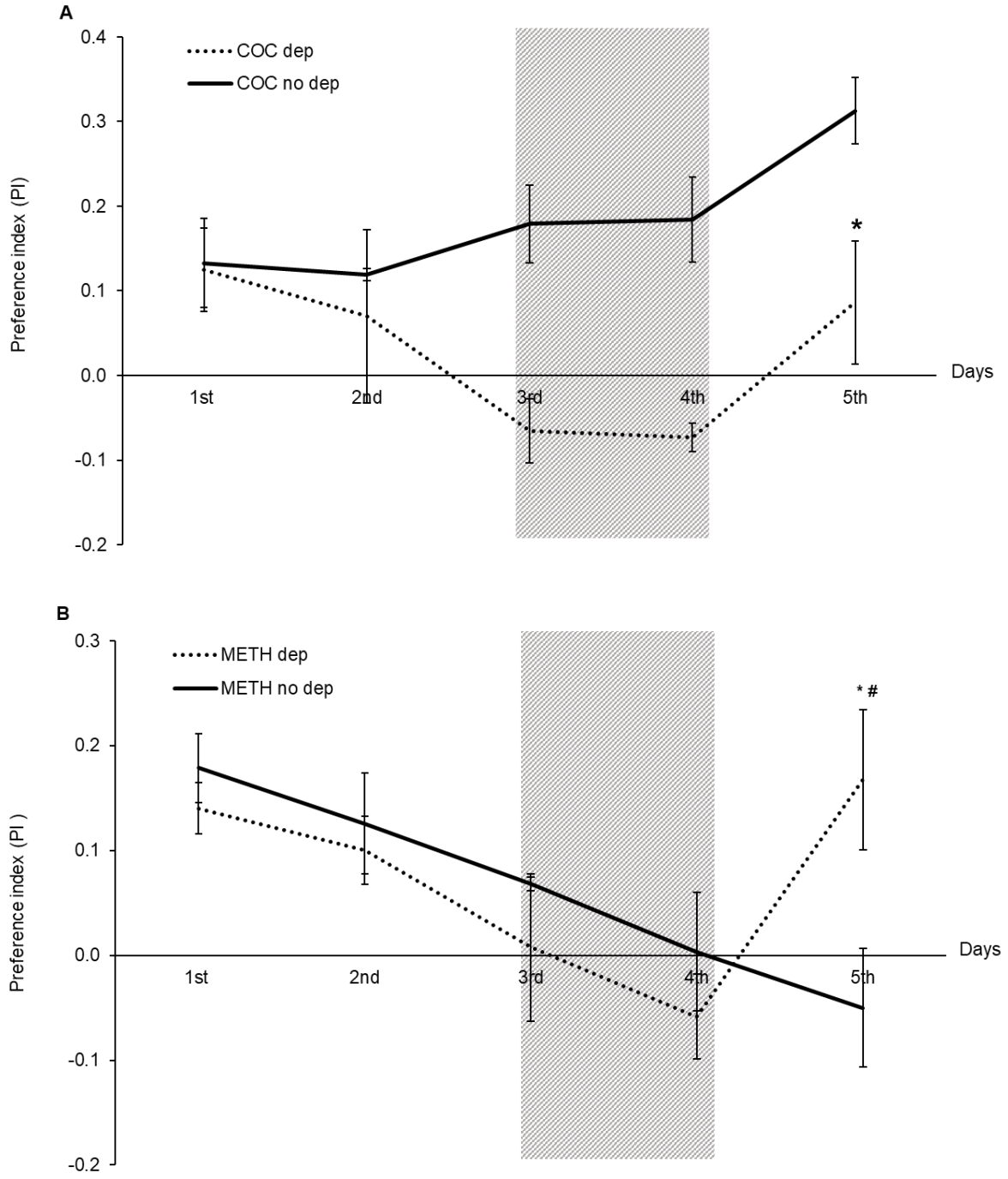


Figure 34. Influence of deprivation on preferential COC and METH consumption. During the 5 days the amount of food with COC 0.15 mg/mL and METH 0.20 mg/mL was measured against the pure sucrose solution and the results were converted to the COC and METH preference index. The experiments were repeated 2 times with 3 tubes each containing 6 flies (n=36). The results are presented as mean ± SEM. *p <0.05 PI of the deprived group vs non-deprived group at same 5th administration day (Student t-test for independent samples) and $^{\#}$p <0.05 PI on second vs fifth day in the deprived group (Student t-test for dependent samples). Figure **A)** shows a comparison of groups that each day had a choice between COC solution and pure sucrose solution (without deprivation) and groups that did not have access to COC on days 3 and 4. Shaded gray panel represents days of

deprivation. Figure **B)** shows a comparison of non-deprivation group and group that did not have access to cocaine on days 3 and 4 for METH. Shaded gray panel represents days of deprivation.

4.2.2.2. Consumption against negative consequence

To test the consumption against negative consequences, we conducted an experiment with 3 groups. One group had a choice between sucrose solution and sucrose solution containing 300 µM quinine (QIN), the second group had a choice between 0.15 mg/mL COC solution or sucrose solution, and the third group had a choice between solution of 0.15 mg/mL COC mixed with 300 µM quinine and sucrose solution.

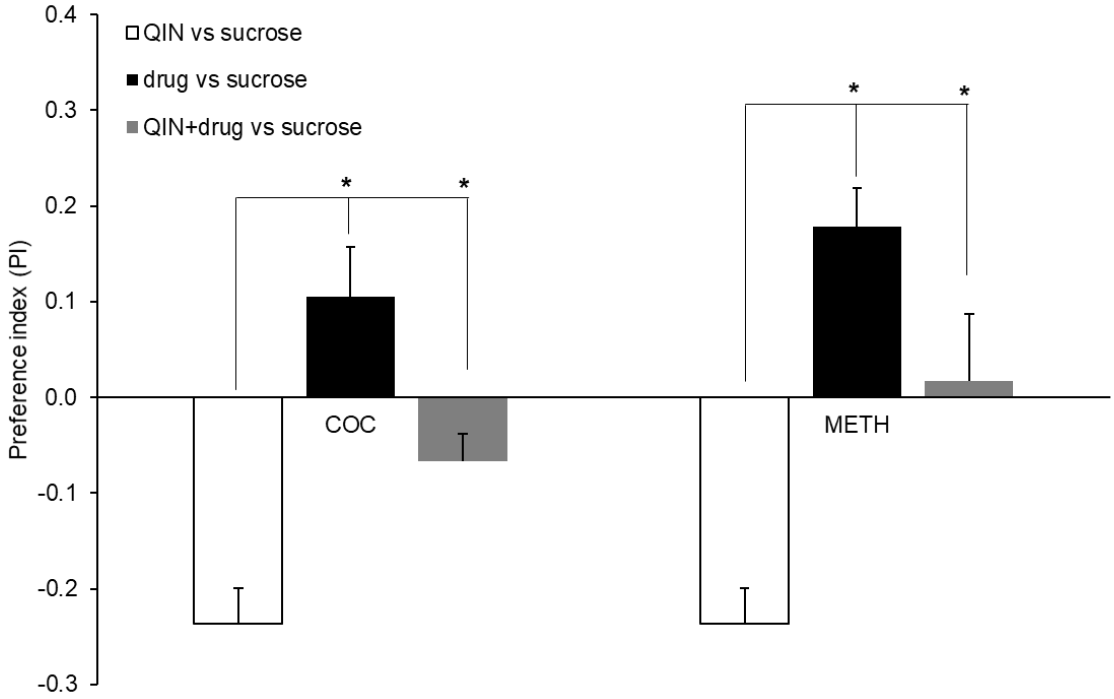


Figure 35. Flies self-administered COC and METH despite the bitter taste of quinine. Flies were divided into three groups: 300 µM QIN solution versus sucrose solution, drug solution (COC 0.15 mg/mL and METH 0.20 mg/mL) versus sucrose solution and 300 µM QIN and drug (COC 0.15 mg/mL and METH 0.20 mg/mL) solution versus sucrose solution. After each of 4 days, the amount of consumed solutions was measured and PI was calculated for each of the solutions versus sucrose solution. Data are presented as histogram of mean PI ±SEM over 4 days in row. The experiment was repeated 2 times with 3 tubes each containing 6 flies (n=36). The differences between the groups were determined by one-way ANOVA and Tukey's multiple comparison, *$p < 0.05$.

Our control group was offered sucrose versus COC and we confirmed that flies prefer COC to sucrose solution (average PI of 0.11). When the choice between sucrose

and QIN solution was offered, flies avoided QIN capillaries (average PI of -0.24). Negative PI (average PI -0.07), was also observed when flies had a choice between COC mixed with QIN versus sucrose solution, but this PI was statistically higher compared to PI to QIN solution alone (Figure 35). Flies showed negative PI for QIN during all four days, while PI increased to positive values for COC mixed with QIN (Suppl. Mat. Figure 15A).

The same experiment was repeated with a solution of METH in concentration of 0.20 mg/mL. Flies showed preference for a solution with METH compared to sucrose (average PI 0.18), and avoided QIN solution (control equal to the one from COC experiment, mean PI -0.24). For METH and QIN solution there was positive average PI of 0.02. Flies showed negative PI for QIN during all four days, while PI increased to positive value for QIN mixed with METH (Suppl. Mat. Figure 15B).

4.2.3. Gene screens using CAFÉ assay

For following screens, we used optimized protocol (Table 6) with fixed cue and capillary locations (Figure 10).

4.2.3.1. Circadian genes

Since the circadian genes *period*, *clock* and *cycle* were reported to be associated with locomotor sensitization to COC (Andretić et al. 1999), and we confirmed that using FlyBong, we wanted to examine the role that circadian genes play in preferential consumption of COC and METH. Average PI for COC (Figure 36) for all circadian mutants was lower than the preference index of *wt* flies , but only per^{01} mutant showed statistically significantly lower PI than *wt* flies. Lower average PI in circadian mutants is the consequence of decreasing PI trend over consecutive days (Suppl. Mat. Figure 16A). PI on a first day in all mutants is equal to PI of *wt* flies, but decreased over several days.

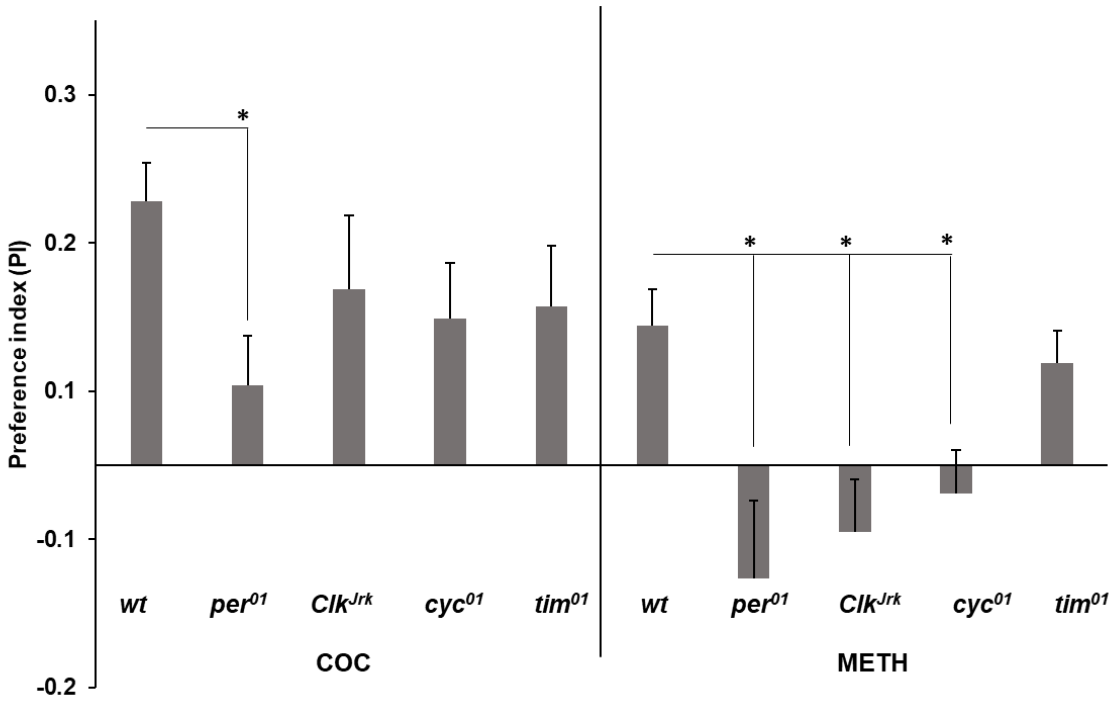


Figure 36. Circadian mutants have different influence on preference for COC and METH. During four days, flies had a choice between drug solutions of cocaine (COC), 0.15 mg/mL (left panel) and methamphetamine (METH), 0.20 mg/mL (right panel) and the sucrose solution. Every 24 hours the volume consumed was measured and converted to the Preference Index (PI). Data are presented as histogram of mean PI ± SEM over 4 days in row. The experiment was repeated 2 times with 3 tubes

each containing 6 flies (n=36). The graph shows the average PI during 4 days for following genotypes: wild type (*wt*), null mutation in period gene (per^{01}), Jrk allele of *Clock* gene (Clk^{Jrk}), and null alleles in *cycle* and *timeless* gene (cyc^{01}) (tim^{01}). One-way ANOVA and Tukey's multiple comparison (*p <0.05) were used to determine the difference between the groups.

Both processes of neuronal plasticity induced by COC, preferential consumption and sensitization, are dependent on the same circadian gene per^{01} suggesting potential involvement of this gene in dopamine release associated with rewarding effect induced by COC. However, long-term memory formation in this mutant is lower compared to *wt* flies. Since we have established that preference for drugs depends on the capillary location and cue, indicating involvement of learning and memory, it is possible that self-administration performance is lower in per^{01} due to learning and memory deficits.

Average PI for METH (Figure 36) of all circadian mutants was lower than the preference index of *wt* flies, while per^{01}, Clk^{Jrk} and cyc^{01} mutants showed statistically significantly lower PI than *wt* flies. Lower average PI in circadian mutants is the consequence of difference in PI for METH in all circadian mutants during the days compared to *wt* flies (Suppl. Mat. Figure 16B). On the first day PI for METH in per^{01} mutants was low and it was increasing over the days, while Clk^{Jrk} mutant shown same negative PI over the days indicating avoidance of capillary containing METH. In contrast to that, PI for METH of cyc^{01} and tim^{01} was positive on the first day, but it was decreasing over the days.

Both processes of neuronal plasticity induced by METH, preferential consumption and sensitization, are depending on the same circadian gene Clk^{Jrk}. This could suggest involvement of this gene in the dopamine release associated with rewarding effect induced by METH. However, Clk^{Jrk} flies have defect in feeding and visual behavior. This is important since flies orally self-administer METH, and the location of capillary containing METH is associated with the cue.

4.2.3.2. Aminergic modulation

Dopamine is the major neurotransmitter involved in the mechanism of COC and METH pharmacodynamics. Therefore, we wanted to check whether the vesicular monoamine transporter, dopamine transporter and dopamine receptor type 1 mutants

had a different preference for COC and METH from *wt* flies. We used mutant in dopamine transporter fumin (*fmn*), *dumb* mutant in dopamine receptor type 1 and transgenic flies with DOPA decarboxylase (DDC) Gal4 driver and UAS VMAT RNAi to reduce the level of VMAT in all dopaminergic and serotoninergic neurons.

Since dopamine transporter is a key target for COC and METH, we have expected that *fmn* mutants will have a different preference for COC and METH compared to *wt* flies. The *fmn* flies perform lower PI for COC (Figure 37), as a consequence of increasing PI in *fmn* mutants over the days, which start from really high negative PI values to almost positive PI values (Suppl. Mat. Figure 17A). This indicates that other neurotransmitters besides dopamine are important for COC induced reward effect. PI for METH in *fmn* mutants was statistically lower in comparison to *wt* flies (Figure 37), as a consequence of negative PI values for *fmn* mutants during all days (Suppl. Mat. Figure 17B).

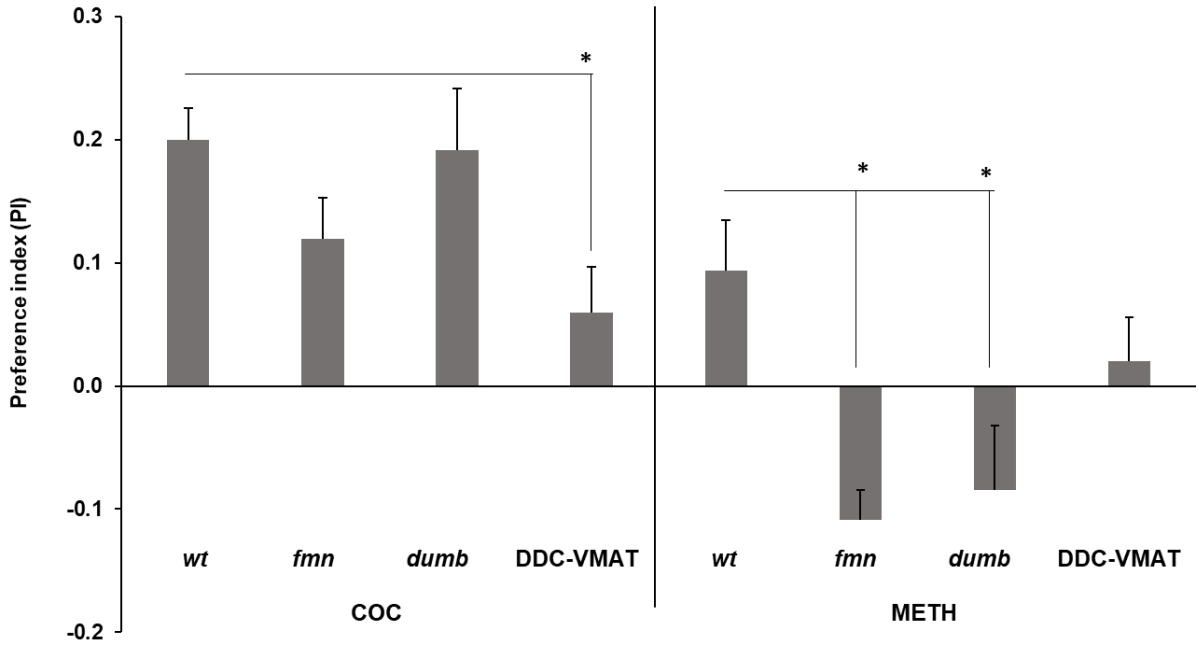


Figure 37. Manipulations in monoaminergic system affect preferential preference for COC and METH. Flies were divided into groups according to the genotype: *wt* - wild type, *fmn* - fumin, dopamine transporter mutant, *dumb*- mutant in dopamine-like receptor 1 and DDC-VMAT - transgene flies with DOPA decarboxylase (DDC) Gal4 driver and UAS VMAT RNAi to reduce the level of VMAT in all dopaminergic and serotoninergic neurons. During four days, flies had a choice between the COC 0.15 mg/mL or METH 0.20 mg/mL and the pure solution of sucrose. Every 24 hours, the amount of consumed liquids was measured and converted to the preference index (PI). Data are plotted as histograms of four days average PI. The experiment was repeated 2 times with 2 tubes each containing 6 flies (n=24). Left

panel **COC** shows the mean index of preferences ± SEM for COC during 4 administrations for each genotype, while right panel **METH** shows the 4 days mean preference index ± SEM for METH and each genotype. One-way ANOVA and Tukey's multiple comparison were used to determine the differences between the groups (*p <0.05).

The *dumb* mutants show same PI for COC as *wt* flies, while in case of METH PI is statistically lower than for *wt* flies (Figure 37). PI for COC in *dumb* mutants is increasing over the days, while PI for METH is decreasing, similar as for *wt* flies (Suppl. Mat. Figure 17A,B). Postsynaptic dopamine receptor type 1 is important for locomotor sensitization (Figure 26A,B) to vCOC and vMETH, but in the case of rewarding effect it is only important for METH. This data suggests that DA availability via DAT and DA action through DR1 are important for METH pharmacodynamics, while COC effect involves other monoamines as well.

Manipulation in dopaminergic and serotoninergic vesicular monoamine transporters using RNAi, showed that locomotor sensitization (Figure 26A,B) and preference to COC and METH depend on functional VMAT (Figure 37). This data suggests that VMAT is important for both locomotor and rewarding effect of psychostimulant-induced neuronal plasticity. By following PI over the days in DDC-VMAT transgene flies, it can been seen that PI for COC and METH is increasing over the days, starting from negative PI on the first day to almost positive on the last day of experiment (Suppl. Mat. Figure 17A,B). We conclude that METH rewarding effect is more correlated with dopaminergic activity, since all three proteins DAT, VMAT and DR1 are important for METH preference. COC-induced rewarding effect depends on not only dopamine, but also on other monoamines, based on evidence of statistically lower PI for COC when VMAT is silenced in dopaminergic and serotonergic using RNAi.

4.3. Biochemical measurements

Biochemical measurement results are divided into three sections. The first section focuses on the influence of COC and METH on redox status in male *wt* flies. The second section provides information on the redox status of *wt* flies pre-treated with antioxidants and hydrogen peroxide. Influence of COC and METH on redox status of antioxidants and hydrogen peroxide pre-treated flies is also discussed in the second section. Third section focuses on antioxidant capacity of all tested antioxidants and possible hydrogen peroxide scavenging capacity, based on observed behavioral response to vCOC and vMETH.

4.3.1. Effect of PS on redox status

We have measured effects of vCOC and vMETH on activity of antioxidant enzymes CAT and SOD, and highly reactive species ROS and H_2O_2 production after single or double exposures, and compared that to the levels in the untreated flies. All assays were performed on whole body extracts of *wt* male flies 3-5 days old.

4.3.1.1. Influence on antioxidant enzymes

Both vCOC and vMETH administrations decrease SOD activity after acute (1st) and repeated (2nd) exposures, compared to SOD activity before exposure (Figure 38).

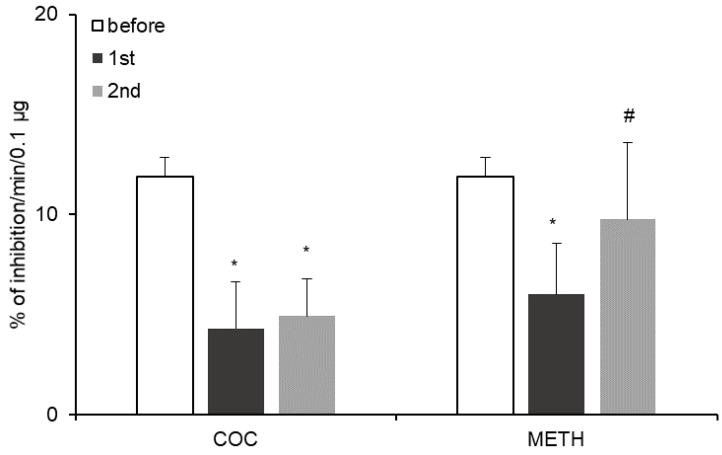


Figure 38. Acute and repeated exposures to vCOC and vMETH decreased total SOD enzyme activity. SOD enzyme activity was quantified in extracts of whole flies as percentage to which whole body extract inhibits the oxidation of quercetin in the presence of TEMED. Data are plotted as histograms of mean ± SEM of triplicates. Statistically significant differences ($p<0.05$) were determined between:

before and after first (1st), before and after second (2nd) administration (*) and between first (1st) and second (2nd) administration (#) using one-way ANOVA and Tukey's multiple comparison.

Both single and double exposures to vCOC and vMETH led to increase in CAT activity. CAT activity increased stepwise after vCOC exposures, while initial increase after single vMETH exposure decreases after second vMETH exposure (Figure 39).

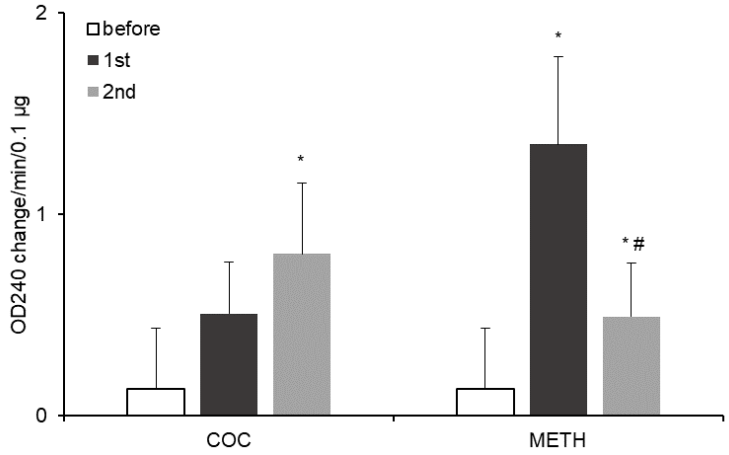


Figure 39. Catalase enzyme activity is increased after acute and repeated vCOC and vMETH exposures. Catalase enzyme activity was quantitated in extracts of whole flies before, and plotted as histogram of mean ± SEM of triplicates. Statistically significant differences ($p < 0.05$) were determined between: before and after first (1st), before and after second (2nd) administration (*) and between first (1st) and second (2nd) administration (#) using one-way ANOVA and Tukey's multiple comparison.

4.3.1.2. Influence on ROS and H_2O_2 production

To see if the change in the activity of antioxidant enzymes is correlated with the change of the amount of free radicals, we measured the amount of ROS and H_2O_2. We observed that first exposure to vCOC and vMETH did not increase ROS production, while second exposure increased ROS after vCOC, but not after vMETH exposure (Figure 40). First exposure decreased H_2O_2 after vCOC and vMETH compared to baseline (Figure 41). Second vCOC exposure increased H_2O_2 compared to baseline and to the first exposure. Repeated vMETH exposures did not increase H_2O_2 compared to first exposure, but the level of H_2O_2 was lower compared to before vMETH exposure.

It is unexpected that METH did not lead to the increased ROS, but METH even reduced H_2O_2, mostly because of a stronger effect of METH on DA release compared to COC. In the case of vCOC, first dose induced increase in ROS and H_2O_2, controlled by antioxidant enzymes, while after second exposure vCOC is not efficient anymore.

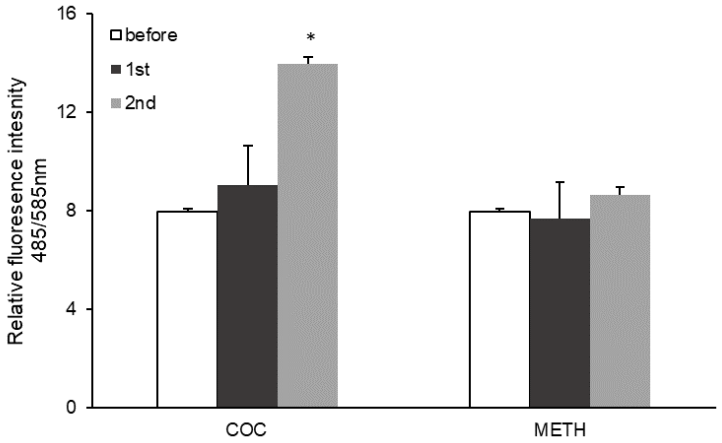


Figure 40. vCOC and vMETH lead to different induction of ROS production. Amount of ROS production was determined by measuring DHE fluorescence at 485/585 nm from whole body extracts, data are plotted as mean ± SEM of triplicates. Statistically significant differences ($p<0.05$) were determined between: before and after first (1st), before and after second (2nd) administration (*) and between first (1st) and second (2nd) administration (#) using one-way ANOVA and Tukey's multiple comparison.

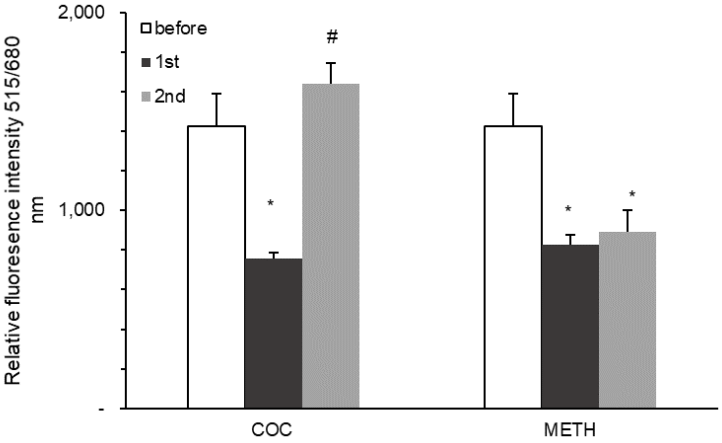


Figure 41. H_2O_2 production after single and double vCOC and vMETH exposure. H_2O_2 production was measured as H_2DCF fluorescence at 515/680 nm from whole body extracts. Statistically significant differences ($p<0.05$) were determined between: before and after first (1st), before and after second (2nd) administration (*) and between first (1st) and second (2nd) administration (#) using one-way ANOVA and Tukey's multiple comparison.

4.3.1.3. Correlation between parameters

SOD and CAT are endogenous enzyme systems that catalyze free radicals and ROS neutralization reactions. Superoxide ($O_2^{\bullet -}$) is the major precursor for the production of other ROS species generated due to the action of various cellular mechanisms. SOD converts $O_2^{\bullet -}$ to H_2O_2, which can be neutralized through the action

of CAT. To maintain redox balance, cells increase SOD and CAT activity if levels of ROS are elevated. Similarly, elevated H_2O_2 will increase CAT activity. Inhibition of one antioxidant enzyme in this chain, or over-production of ROS and H_2O_2 induced by PS administration could lead to cell oxidative stress. From the correlations in the case of vCOC redox status biomarkers, it can be seen (Table 8) that all parameters are positively correlated, with exception of SOD and ROS, and SOD and CAT, which are negatively correlated. Significant correlations after vCOC exposures are: positive correlation in increases of CAT and ROS, and negative correlation between decreased SOD and increased CAT. This data suggest that vCOC inhibits SOD activity leading to increased ROS amounts, while CAT activation could be the consequence of enhanced hydrogen peroxide production resulting from DA and COC metabolism.

Table 8. Correlation between vCOC determined parameters of redox status. Positive correlation is indicated as (+) and negative as (-). Statistically significant Pearson correlation coefficient ($p<0.05$) is colored in gray.

	H_2O_2	ROS	CAT	SOD
H_2O_2				
ROS	+			
CAT	+	+		
SOD	+	-	-	

The vMETH correlations are negative, with exception of SOD and H_2O_2, and SOD and ROS, which are positive (Table 9). The correlation between CAT and H_2O_2 is negative and statistically significant, indicating that as CAT is increasing, H_2O_2 production is decreasing. Similar outcome can be seen for SOD and CAT correlation, with increasing CAT and decreasing SOD. Overall, vMETH does not induce ROS production or increase SOD activity, and amount of H_2O_2 is decreased, likely because of increased CAT activity.

Both vCOC and vMETH inhibit SOD and induce CAT activity, leading to negative correlation between these two enzymes. In case of vCOC, both ROS and H_2O_2 are increased, while in case of vMETH, ROS and H_2O_2 levels remain the same. Differences between vCOC and vMETH are possible due to PS-activated metabolic pathways and differences in monoamines involved in PS specific processes of neuronal plasticity. Additionally, redox biomarkers for vCOC were measured at 09:00

and 15:00, while for vMETH were measured at 09:00 and 19:00 h based on behavioral endophenotype of sensitization. It is thus possible that some of the observed effects on redox balance could be a consequence of different metabolism due to the time of the day.

Table 9. Correlation between vMETH determined parameters of redox status. Positive correlation is indicated as (+) and negative as (-). Statistically significant Pearson correlation coefficient ($p<0.05$) is colored in gray.

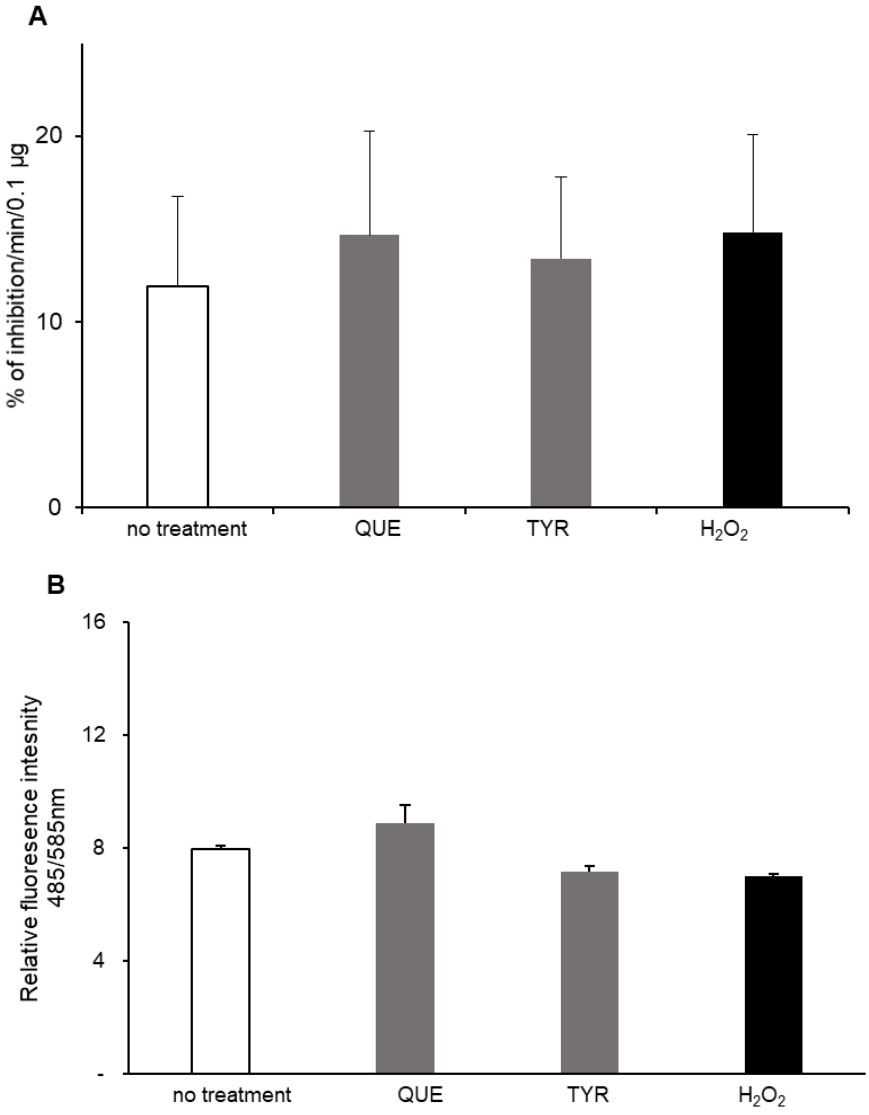
	H_2O_2	ROS	CAT	SOD
H_2O_2				
ROS	-			
CAT	-	-		
SOD	+	+	-	

4.3.2. Exogenous antioxidant and prooxidant treatment

Based on the hypothesis that antioxidant pre-treatment will abolish LS and oxidative stress by reducing ROS and H_2O_2 production after vCOC and vMETH exposure, we have quantified redox parameters in *wt* flies exposed to 18 hour of antioxidants (QUE and TYR) and prooxidant H_2O_2 and compared it to non-treated group.

4.3.2.1. Influence on pre-treatment on oxidative status of flies

Pro- and antioxidant pre-treatments did not affect SOD activity (Figure 42A) and ROS production (Figure 42B). All pre-treatments decreased H_2O_2 (Figure 42C), while antioxidants increased CAT compared to non-treated group and prooxidant-treated group (figure 42D).



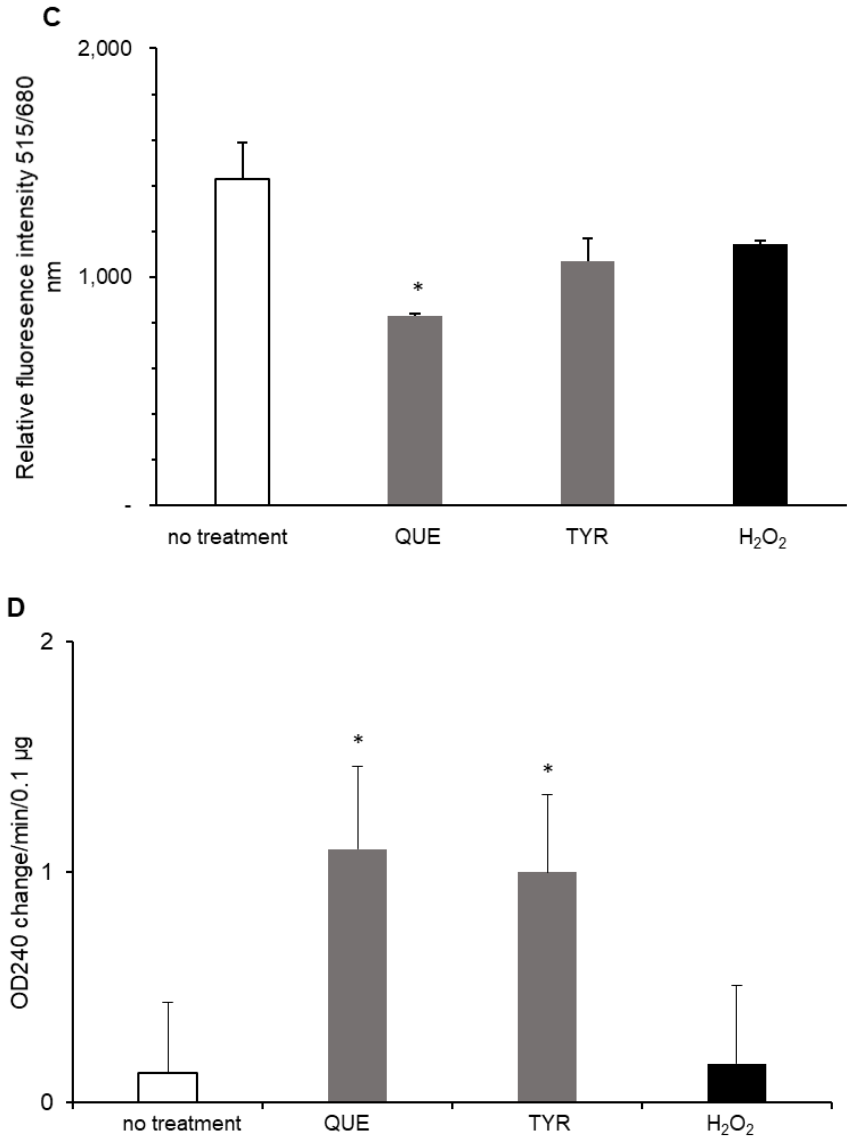


Figure 42. Influence of anti- and prooxidant pre-treatment on a redox biomarkers. Data are plotted as histograms of mean ± SEM of triplicates. Statistically significant differences (*p<0.05) were determined between non-treated and pre-treated groups with QUE, TYR and H_2O_2 using one-way ANOVA and Tukey's multiple comparison. **A)** SOD enzyme activity was quantitated in extracts of whole flies as percentage to which whole body extract inhibits the oxidation of quercetin in the presence of TEMED. **B)** Amount of ROS production was determined by measuring DHE fluorescence at 485/585 nm. **C)** H_2O_2 production was measured as H_2DCF fluorescence at 515/680 nm from hole body extracts. **D)** Catalase enzyme activity was quantitated in extracts of whole flies.

4.3.2.2. Oxidative status of pre-treated flies exposed to PS

a) Superoxide dismutase

As we have shown previously, pre-treatment with either pro- or antioxidants did not change SOD activity. Consistent with previous results of vCOC and vMETH exposures in non-treated flies, pre-treated flies had decreased SOD activity after both vCOC and vMETH administration (Figure 43A,B). This indicates that pro- and antioxidant have negligible effect on SOD activity.

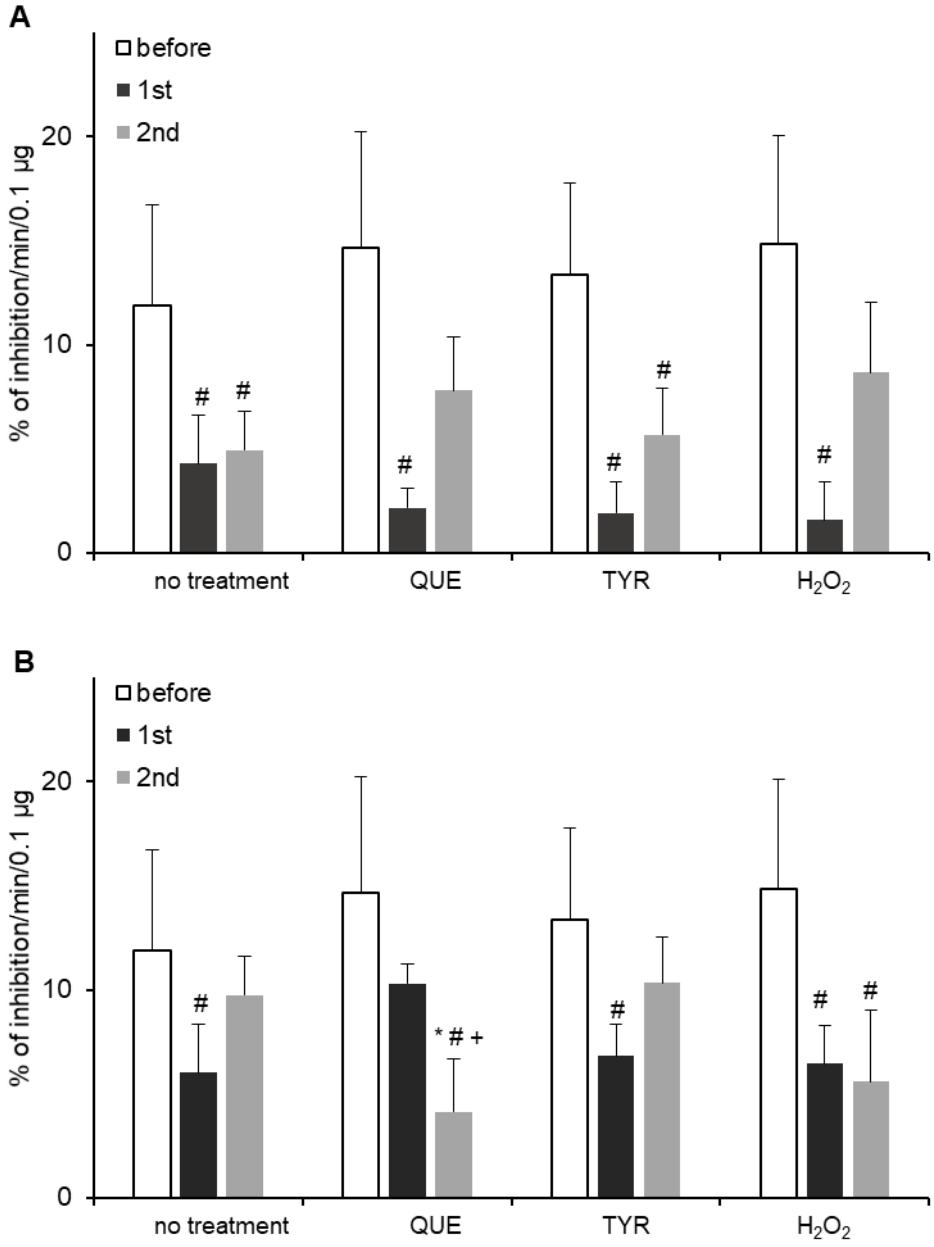


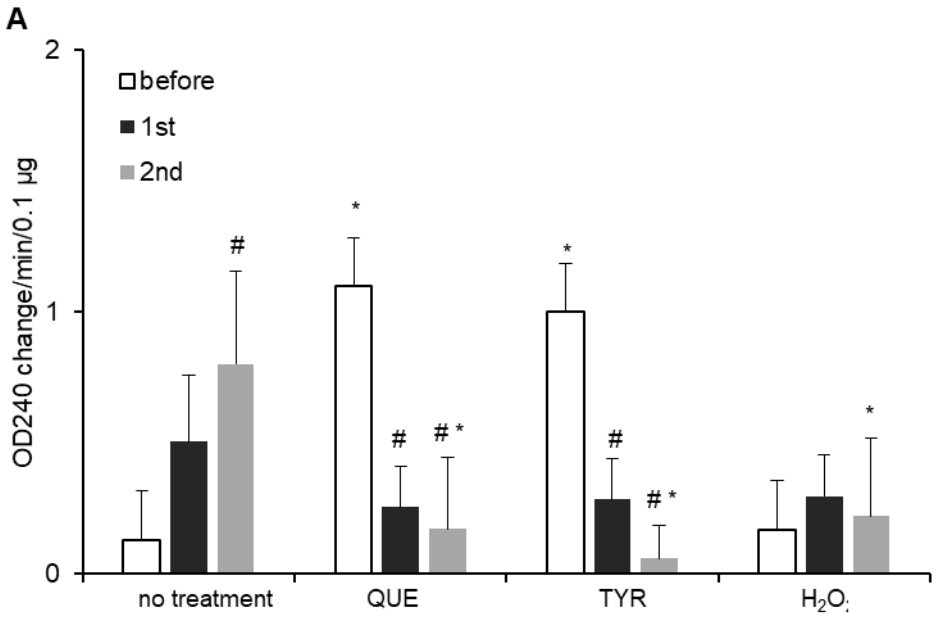
Figure 43. Change in SOD activity induced by COC or METH is not modulated by anti- and prooxidant pre-treatment. SOD enzyme activity was quantitated in extracts of whole flies as

percentage to which whole body extract inhibits the oxidation of quercetin in the presence of TEMED. Data are plotted as histograms of mean ± SEM of triplicates. Statistically significant differences ($p<0.05$) were determined between: before and after first (1^{st}), before and after second (2^{nd}) administration ($^{\#}$) and between first (1^{st}) and second (2^{nd}) administration ($^{+}$) within groups using one-way ANOVA and Tukey's multiple comparison. Statistical differences between non-treated group and all treated groups QUE, TYR and H_2O_2 before, after 1^{st} and after 2^{nd} exposure (*) were evaluated using one-way ANOVA and Tukey's multiple comparison ($p<0.05$).

b) Catalase

Antioxidant treatment had significant effect on CAT activity in vCOC-exposed flies (Figure 44A). First, treatment with QUE and TYR significantly increased CAT before exposure to vCOC (Figure 42D). Second, after exposure to vCOC, catalase levels significantly decreased. H_2O_2 pre-treatment did not change basal level of CAT activity, but prevented the vCOC-induced increase in CAT activity (Figure 44A). Compared to vCOC, METH administration had opposite effect on CAT activity and led to the further induction of CAT activity particularly after first exposure (Figure 44B). H_2O_2 pre-treatment similarly led to increase in CAT activity after vMETH exposure.

Therefore, it seems that CAT activity was more susceptible to change as consequence of pro-, antioxidants and drug treatment. Effects of COC and METH on pre-treated flies were opposite.



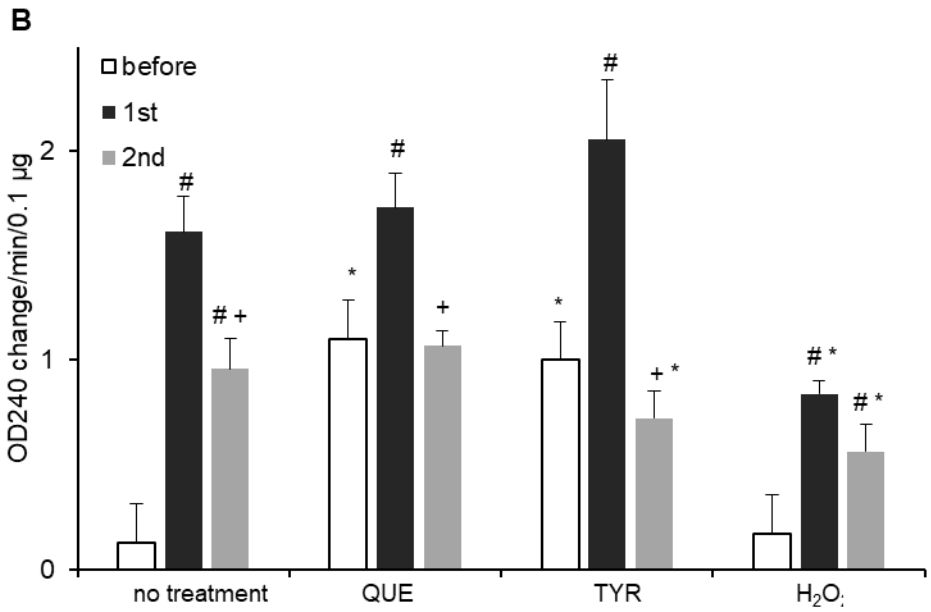


Figure 44. Catalase enzyme activity changes as consequence of antioxidant treatment and COC A) and METH exposure B). Catalase enzyme activity was quantified in the extracts of whole flies, and plotted as histogram of mean ± SEM of triplicates. Statistically significant differences ($p<0.05$) between: before and after first (1^{st}), before and after second (2^{nd}) administration ($^{\#}$) and between first (1^{st}) and second (2^{nd}) administration ($^{+}$) within groups were evaluated using one-way ANOVA and Tukey's multiple comparison. Statistical differences between non-treated group and all treated groups QUE, TYR and H_2O_2 before, after 1^{st} and after 2^{nd} exposure (*) were evaluated using one-way ANOVA and Tukey's multiple comparison ($p <0.05$).

c) Reactive oxygen species

The vCOC increases ROS amount only after second exposure, and that increase is diminished in antioxidant pre-treated groups (Figure 45A). Surprisingly, prooxidant H_2O_2 also decreases vCOC-induced ROS production. METH does not lead to increased ROS production, and antioxidant treatment lead to decreased ROS levels (Figure 45B). Again, H_2O_2 had similar effect to antioxidants.

Both vCOC and vMETH inhibit SOD and increase CAT activity in untreated flies. In antioxidant pre-treated flies, exposure to vCOC decreased activity of SOD and CAT, while ROS production is increased only after 2^{nd} exposure. In antioxidant pre-treated flies exposure to vMETH did not influence CAT and SOD activity and ROS production, compared to non-treated flies. This suggests that antioxidants induce CAT through metabolic pathways of antioxidants degradation. CAT up-regulation then suppresses CAT activity after vCOC exposure, but increases CAT activity after vMETH

administration. All this suggests that vMETH leads to lower exogenous redox perturbation compared to vCOC. It is unexpected that pro- and antioxidants have similar effects on ROS.

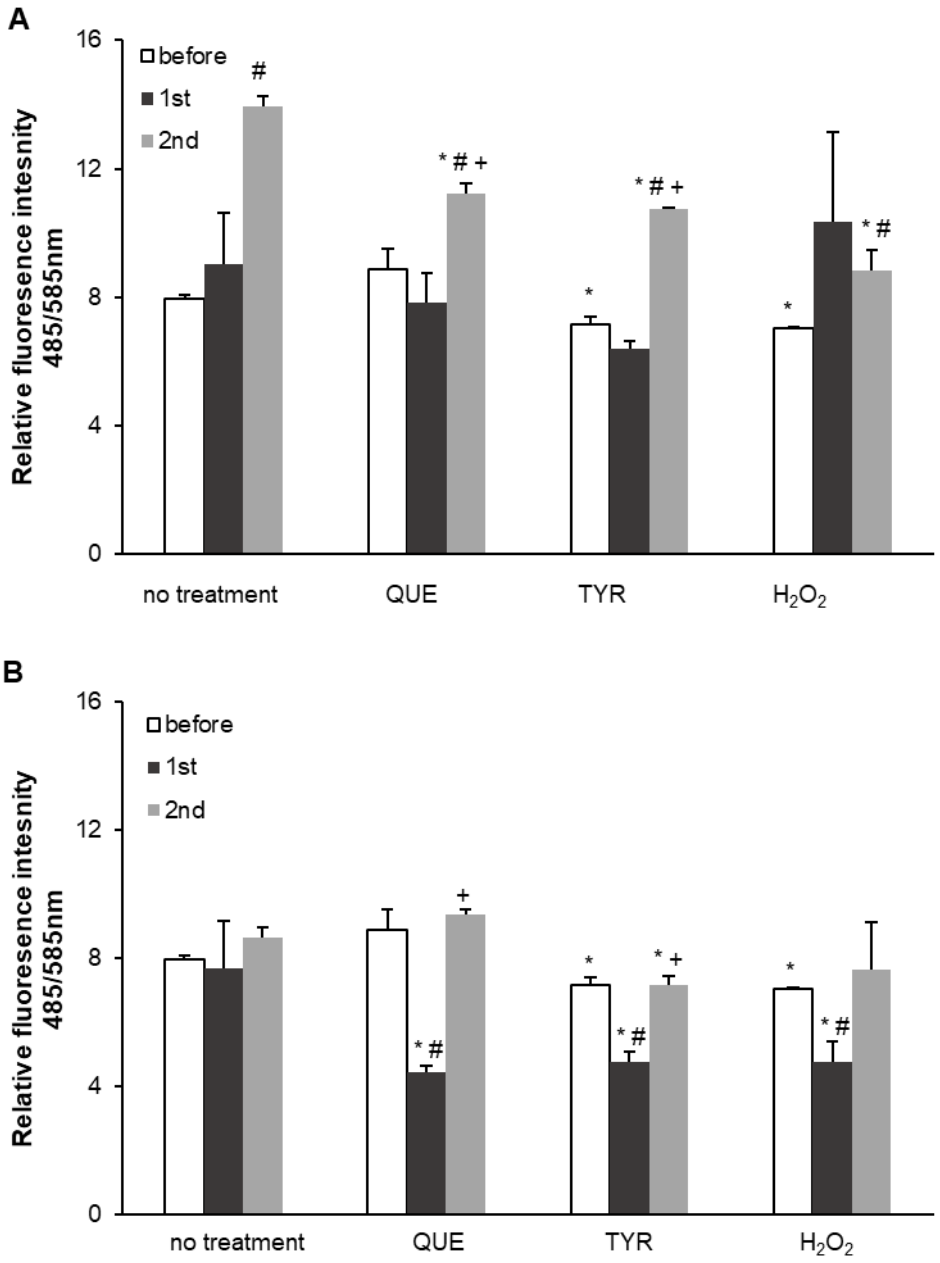


Figure 45. COC A) and METH B) have opposite effects on ROS production. Amount of ROS production was determined by measuring DHE fluorescence at 485/585 nm from whole body extracts. Data are plotted as mean ± SEM of triplicates. Statistically significant differences ($p<0.05$) were determined between: before and after first (1^{st}), before and after second (2^{nd}) administration ($^{\#}$) and between first (1^{st}) and second (2^{nd}) administration ($^{+}$) within groups using one-way ANOVA and Tukey's multiple comparison. Statistical differences between non-treated group and all treated groups QUE, TYR and H_2O_2 before, after 1^{st} and after 2^{nd} exposure (*) were evaluated using one-way ANOVA and Tukey's multiple comparison ($p<0.05$).

d) Hydrogen peroxide

Further support for lower susceptibility to exogenous redox perturbation response after vMETH exposure is evident from measurement of H_2O_2. H_2O_2 quantity decreases in pre-treated flies after vMETH exposure, similar to the situation in non-treated flies (Figure 46B). In the antioxidant pre-treated flies that decrease is smaller, possibly because endogenous antioxidants are less activated in the presence of exogenously supplied antioxidants.

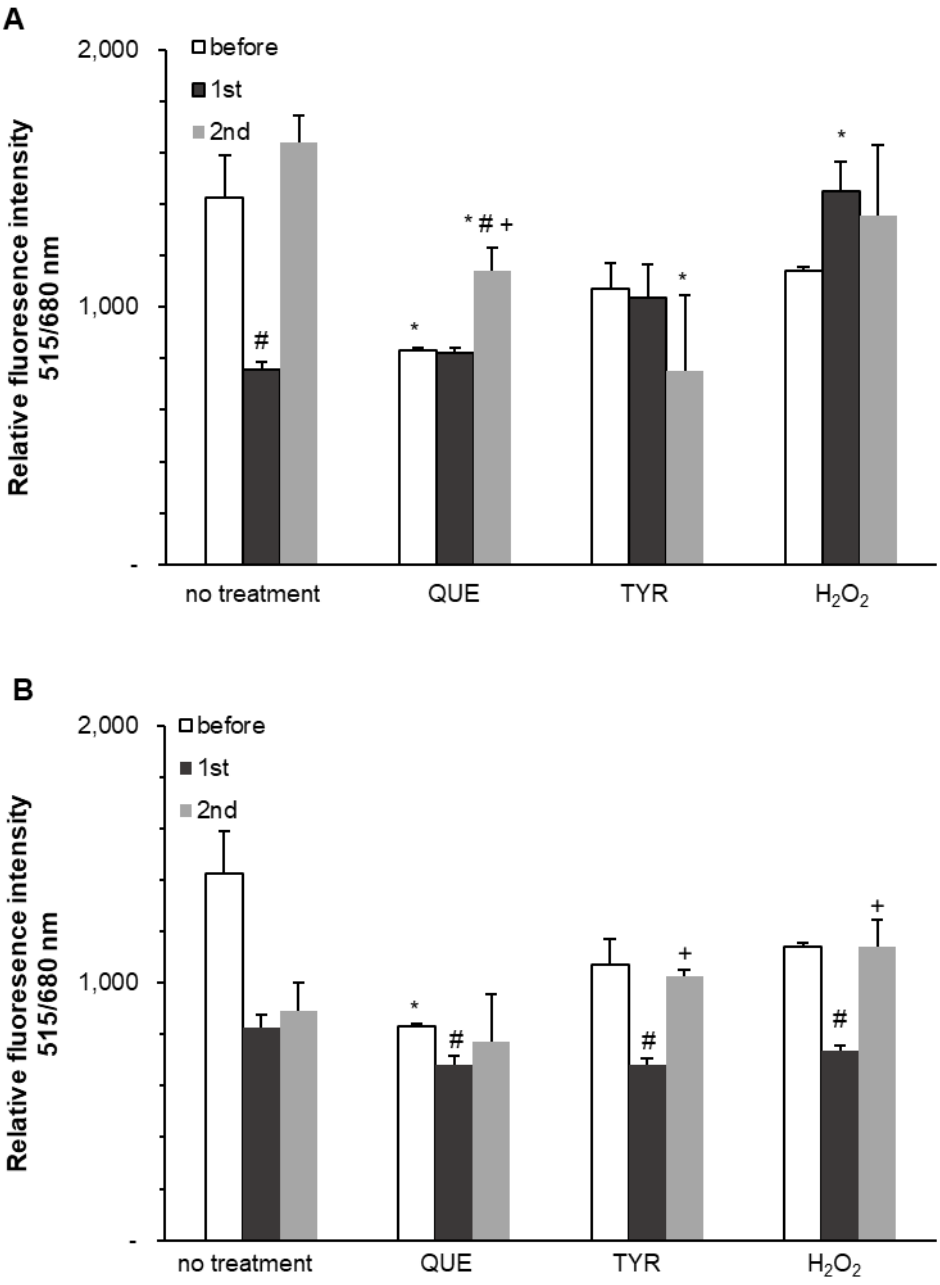


Figure 46. Antioxidant effect on H_2O_2 is more pronounced in A) COC than B) METH exposed flies. H_2O_2 production was measured as H_2DCF fluorescence at 515/680 nm from whole body extracts. Statistically significant differences ($p<0.05$) were determined between: before and after first (1st), before

and after second (2^{nd}) administration ($^{\#}$) and between first (1^{st}) and second (2^{nd}) administration ($^{+}$) within groups using one-way ANOVA and Tukey's multiple comparison. Statistical differences between non-treated group and all treated groups QUE, TYR and H_2O_2 before, after 1^{st} and after 2^{nd} exposure (*) were evaluated using one-way ANOVA and Tukey's multiple comparison ($p<0.05$).

Again, vCOC exposure lowered H_2O_2 production, but after second vCOC exposure H_2O_2 levels were higher than after METH treatment. However, antioxidant treatment helped in reducing H_2O_2 spike after second exposure, as it can be predicted from the antioxidant effect of exogenously applied antioxidants.

4.3.2.3. Correlation between parameters

The vCOC exposure in antioxidant treated flies reverses the correlation between CAT and ROS, which is now negative (Table 10) compared to untreated group (Table 8), where it was positive. Similarly, correlation between SOD and CAT in antioxidant treated flies is positive, while in non-treated group exposed to vCOC was negative. There are other differences between groups, but they are not statistically significant. Overall, major difference between non-treated and antioxidant-treated groups is lower CAT activity in antioxidant treated groups.

Table 10. Correlation between vCOC determined parameters of redox status in antioxidant pre-treated groups (QUE/TYR). Positive correlation is indicated as (+) and negative as (-). Statistically significant Pearson correlation coefficient ($p<0.05$) is colored in gray. Major differences between correlations in non-treated (Table 8.) and anti-oxidant treated groups were colored in black.

	H_2O_2	ROS	CAT	SOD
H_2O_2				
ROS	+/-			
CAT	-/+	-/-		
SOD	-/+	+/-	+/+	

Prooxidant-treated and non-treated group exposed to COC have all identical correlations, with exception of negative correlation between SOD and H_2O_2 in prooxidant-treated group (Table 11), which was positive in non-treated group (Table 8). This is consequence of lower CAT activity as it was the case for antioxidant-treated group.

Table 11. Correlation between vCOC determined parameters of redox status in prooxidant pre-treated group. Positive correlation is indicated as (+) and negative as (-). Statistically significant Pearson correlation coefficient ($p<0.05$) is colored in gray. Black colored are Major differences between correlations in non-treated (Table 8.) and prooxidant-treated group were colored in black.

	H_2O_2	ROS	CAT	SOD
H_2O_2				
ROS	+			
CAT	+	+		
SOD	-	-	-	

The vMETH exposure in antioxidant-treated flies reverses correlation between ROS and H_2O_2, so that it becomes positive (Table 12), in comparison to untreated

group (Table 9), where it was negative. Other differences between groups are not statistically significant. Major differences between untreated and antioxidant-treated groups is higher CAT activity in antioxidant-treated groups.

Table 12. Correlation between vMETH determined parameters of redox status in antioxidant pre-treated groups (QUE/TYR). Positive correlation is indicated as (+) and negative as (-). Statistically significant Pearson correlation coefficient ($p<0.05$) is colored in gray. Major differences between correlations in non-treated (Table 9.) and anti-oxidant-treated groups are colored in black.

	H_2O_2	ROS	CAT	SOD
H_2O_2				
ROS	+/+			
CAT	-/-	-/-		
SOD	+/+	-/+	+/-	

Prooxidant-treated and untreated group exposed to METH have similar correlations, with exception of positive correlation between ROS and H_2O_2 in prooxidant-treated group (Table 13), which was negative in non-treated group (Table 9). This is consequence of lower CAT activity compared to antioxidant-treated group.

Table 13. Correlation between vMETH determined parameters of redox status in prooxidant pre-treated group. Positive correlation is indicated as (+) and negative as (-). Statistically significant Pearson correlation coefficient ($p<0.05$) is colored in gray. Major differences between correlations in non-treated (Table 9.) and prooxidant-treated group are colored in black.

	H_2O_2	ROS	CAT	SOD
H_2O_2				
ROS	+			
CAT	-	-		
SOD	+	+	-	

Antioxidant treatment modulated CAT activity differently for vCOC- and vMETH-treated groups when compared to non-treated group, which can be the consequence of up-regulation of the redox sensitive enzyme mechanism. It is interesting that feeding flies with H_2O_2 as prooxidant did not induce expected increase in redox biomarkers such as CAT. Possible explanations can be that H_2O_2 pre-exposure was too short, concentration was not optimized, and H_2O_2 might have decomposed in the food before ingestion. This could ultimately resulted in beneficial rather than negative consequences for organism. We base this conclusion on our results where H_2O_2 pre-

treatment lowers CAT activity, and is not increasing it, as it would be expected. Additionally, all other measured redox biomarkers in H_2O_2 pre-treated flies were lower for both vCOC and vMETH groups, compared to non-treated and antioxidant-treated groups.

Overall, we have summarized all behavior phenotypes and their genetic and biochemical characteristics in mammals and *Drosophila* (Table 14 and Table 15).

Table 14. COC induced behaviour phenotypes and their genetic and biochemical characteristics in mammals and *Drosophila*. + behaviour endophenotype can be induced and quantify, ↑ increment, ↓ decrement, ↔ same, **#** endophenotype is different than *wt* endophenotype, * endophenotype is same as *wt* endophenotype, - not been published jet, grey difference between *Drosophila* and mammals. *Drosophila* data are from this work.

COCAINE			*Drosophila melanogaster*	Mammals
Behaviour endophenotype	Sensitivity		+	+
	Locomotor sensitization		+	+
	Self-administration		+	+
Redox biomarker	CAT activity		↑	↓ (Macedo et al. 2005)
	SOD activity		↓	↑(Dietrich et al. 2005)
	ROS production		↑	↑(Dietrich et al. 2005)
	H_2O_2		↑	↑(Dietrich et al. 2005)
Circadian genes	Locomotor sensitization	*per*	#	#(Abarca et al. 2002)
		Clk	#	* (Abarca et al. 2002)
		cyc	#	-
		tim	*	-
		pdf	*	-
	Self-administration	*per*	#	#(Abarca et al. 2002)
		Clk	*	* (Abarca et al. 2002)
		cyc	*	-
		tim	*	-
Dopamine transporters and receptors	Locomotor sensitization	DAT	#	# (Hall et al. 2009)
		VMAT	#	# (Wang et al.1997)
		DopR	#	# (Lebestky et al. 2009)
	Self-administration	DAT	*	* (Rocha et al. 1998, Sora et al. 1998)
		VMAT	#	*(Takahashi et al. 1997)

		DopR	*	# (Bergman et al. 1990)
Locomotor sensitization	Monoamines	dopamine	#	#
		serotonin	#	#
		octopamine	#	-
		other trace monoamine	#	#
	Antioxidant influence		#	#(Jang et al. 2015)
	Prooxidants influence		#	-

Table 15. METH induced behaviour phenotypes and their genetic and biochemical characteristics in mammals and *Drosophila*. + behaviour endophenotype can be induced and quantify, ↑ increment, ↓ decrement, ↔ same, # endophenotype is different than *wt* endophenotype, * endophenotype is same as *wt* endophenotype , - not been published jet, grey difference between *Drosophila* and mammals. *Drosophila* data are from this work.

METAMPHETANINE			*Drosophila melanogaster*	Mammals
Behaviour endophenotype	Sensitivity		+	+
	Locomotor sensitization		+	+
	Self-administration		+	+
Redox biomarker	CAT		↑	↑(Koriem et al. 2012)
	SOD		↓	↓(Frenzilli et al. 2007)
	ROS		↔	↑(Kita et al. 2009)
	H_2O_2		↓	↑(Moszczynska 2017)
Circadian genes	Locomotor sensitization	*per*	*	-
		Clk	#	-
		cyc	*	-
		tim	*	-
		pdf	*	-
	Self-administration	*per*	#	#(Abarca et al. 2002)
		Clk	#	* (Abarca et al. 2002)
		cyc	#	-
		tim	*	-
Dopamine transporters and receptors	Locomotor sensitization	DAT	#	# (Xua et al. 2000)
		VMAT	#	# (Wang et al.1997)
		DopR	#	# (Tella 1994)
	Self-administration	DAT	#	# (Brennan et al. 2009)
		VMAT	#	*(Takahashi et al. 1997)
		DopR	#	# (Bergman et al. 1990)
	Monoamines	dopamine	#	#

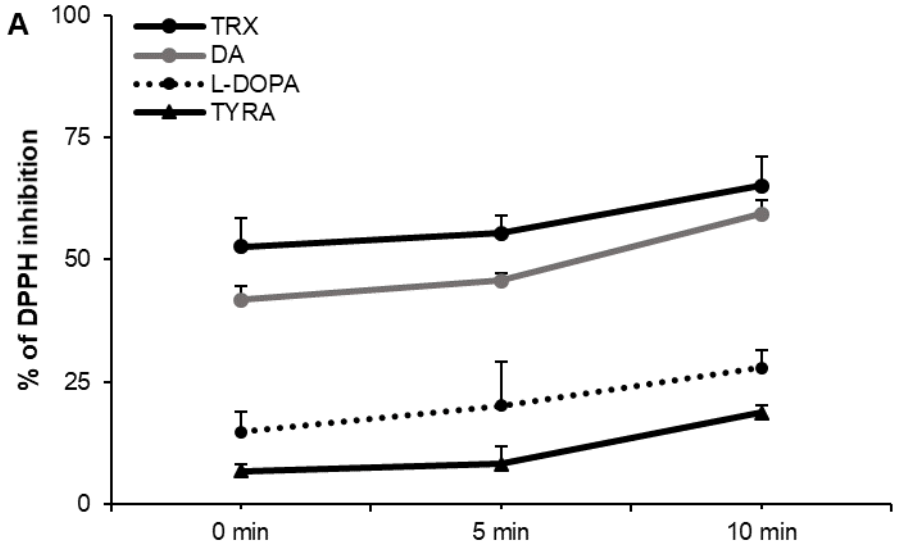
Locomotor sensitization		serotonin	#	#
		octopamine	#	-
		other trace monoamine	#	#
	Antioxidant influence		#	#(Jang et al. 2017)
	Prooxidants influence		#	-

4.3.3. Antioxidant and H_2O_2 scavenging capacity

In order to determine the antioxidant properties of chemical substances that we have used in our experiments, we have performed DPPH assay. We also measured the antioxidant capacity of major monoamines that we investigated and manipulated in previous experiments, and their precursors. To verify their oxidative effects, we also measured antioxidants quercetin (QUE), tyrosol (TYR) and TEMPOL.

We show that L-DOPA has surprisingly high antioxidant capacity relative to Trolox standard. This is in agreement with chemical structure of monoamines, since molecules with more easily accessible protons show better antioxidant properties in DPPH test. Based on this, order of antioxidant potency is dopamine > L-DOPA > tyramine. This prediction agrees with our results from DPPH test (Figure 47A). Relative to the number of hydroxyl groups bounded to the benzene ring and alkyl groups in *para*- orientation relative to hydroxyl group, we have proposed DPPH antioxidant capacity with following order of potency QUE>TYR>TEMPOL, which was experimentally confirmed (Figure 47B).

Additionally, we have tested antioxidant properties of quinine, bitter substance added to COC and METH solution during testing of preference consumption against negative consequences using CAFÉ assay. Reason for this testing was to confirm that lower PI for COC mixed with QIN (Figure 35) is not due to possible



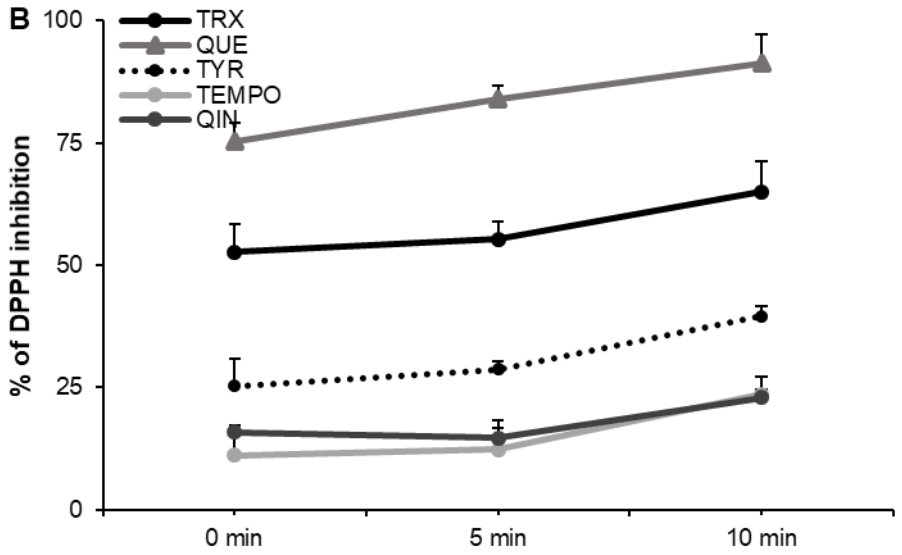


Figure 47. Antioxidative capacity of A) DA-dopamine, L-DOPA, TYRA-tyramine and B) QUE-quercetin, TYR–tyrosol, TEMPOL and QIN-quinine measured in methanol by DPPH assay. The results are presented as a percentage of inhibition of the absorbance at 515 nm (absorption max for DPPH) in time points of 0, 5 and 10 minutes for 0.09 mM concentration of all tested molecules. Data are plotted as average of triplicates.

antioxidant effect of quinine. We show that quinine has lower antioxidant capacity compared to Trolox (Figure 47B), in one proton reaction, and that in the DPPH assay it is not a strong antioxidant.

We have suspected that H_2O_2 pre-treatment does not induce redox enzymes because it is scavenged through monoamines. Unfortunately, we could not test the hydrogen peroxide scavenging properties of dopamine, L-DOPA, tyramine, octopamine and tryptophan due to the very close wavelengths of the absorbance maximum in the UV-VIS spectrum, of proposed monoamines and hydrogen peroxide (Suppl. Mat. Figure 18A). Additionally, we wanted to check if exogenous QUE, TYR, TEMPOL could scavenge hydrogen peroxide and thus lower CAT activity, as we previously proposed, but because of the same wavelength of absorbance maximum in UV-VIS spectra for QUE, TYR, TEMPOL and hydrogen peroxide it was not possible to test this hypothesis (Suppl. Mat. Figure 19B).

5. DISSCUSION

The main aim of this thesis is to identify molecular mechanisms of neuronal plasticity induced by psychostimulants in forms of two endophenotypes that are relevant for the development of addiction: locomotor sensitization and rewarding effects. Neural plasticity is within both studied endophenotypes because it takes a while to develop endophenotypes, indicating gene activation and changes in neural networks. In the data presented, neural plasticity was not directly measured and proven, but it was reported as indirect influence on gene modulation.

We have developed a new method for induction and quantification of locomotor activity after acute and repeated volatilized PS administration in *Drosophila*, that we named FlyBong. We use a version of previously published method for drug delivery (McClung and Hirsh 1998, Bainton et al. 2000, Lease and Hirsh 2005, Heberlein et al. 2009, Gakamsky et al. 2013), while locomotor activity was quantified using a commercially available platform (TriKinetics). Optimized FlyBong protocol: a) induces minimal animal handling, b) objectively quantifies the locomotor activity before and after drug administration , c) delivers the same amount of drug to individual flies, d) allows data analysis either at single fly or the population level, e) allows simultaneous monitoring of locomotor activity of 32 flies. FlyBong can be easily scaled up, and it is suitable for genetic screens enabling testing of large number of flies at the same time. These high throughput objective and reproducible features of the FlyBong represent significant improvement over previously developed methods.

Previously published assays were based on transferring flies before and after drug administration, leading to high amount of animal handling, which could lead to low reproducibility between experiments (McClung and Hirsh 1998, Gakamsky et al. 2013). Flies in FlyBong are not moved or manipulated during experiment, thus lowering experimental error due to animal manipulation and increasing reproducibility. To achieve this, we placed flies into recording tubes one day before the testing in order to habituate them to the novelty of environment. Flies were placed in recording tubes with food to avoid starvation, and humidified incubator with light:dark cycles to avoid dehydration, temperature and light fluctuations. Overall, we have tried to control environmental factors, which ultimately led to robust and reproducible method.

Delivering a precise drug amount to the individual flies within a population had not been achieved using previously published methods (McClung and Hirsh 1998, Bainton et al. 2000, 2005). Attempts to deliver a known amount of drug via injection or an airbrush were not simple and required sophisticated animal handling (Dimitrijevic et al. 2004, Lease and Hirsh 2005). In a FlyBog, COC and METH are volatilized in a flask and delivered to the individual flies in recording tubes. Based on the volume of the flask, tubing and dispenser, proportion of vCOC and vMETH condenses on their surfaces. We have therefore measured residual of PS on the surface of the recording tubes and on the flies, by which we have shown that less than 10% of the volatilized PS reaches the individual tube and around 3% of PS reaches the fly. Here we have shown that the drug amount received by each fly is equivalent, as the amount of drug per tube is independent of recording tube location within the monitor, and does not vary significantly between experiments.

The most important improvement of FlyBong, relative to previous systems, is that it provides both population and individual measures of locomotor activity. We have shown that there is individual variability in flies sensitivity to motor-activating effects of vMETH and vCOC, and that only a fraction of these sensitive flies go on to develop locomotor sensitization. Our findings are in agreement with previously published studies in mammals where it was shown that not all *wt* animals responded on acute or repeated drug administrations (Gulley et al. 2003, Kamenes et al. 2004). Therefore, this suggests that FlyBong can be used for the selective breeding of flies that are either sensitive and develop locomotor sensitization versus those that do not, in order to identify the potential genetic contributions for these behaviors, as an alternative to a genetic screen.

We have several results that support conclusion that different neural mechanisms govern sensitivity and regulate locomotor sensitization. First, three consecutive exposures to vCOC and vMETH each led to similar percentage of flies showing sensitivity, while the percentage of the flies that develop locomotor sensitization to the three exposures is much lower. Second, sensitivity does not vary as a function of circadian time, while locomotor sensitization does. Third, although sensitivity of *Clk* mutant flies is similar to that of *wt* flies, they fail to develop locomotor sensitization.

The reason that vCOC was initially selected as a drug of choice for *Drosophila* was that it led to dramatic and concentration-dependent changes in motor behaviour, as exemplified by the appearance of uncoordinated, uncommon or stereotypical behaviours (McClung and Hirsh 1998). Increases in forward locomotion were observed at lower doses, and have been quantified using methods based on oral administration of cocaine or its injection into the abdomen (Lebestky et al. 2009, Dimitrijevic et al. 2004). The 75 µg dose of vCOC, used in our experiments, is the same as dose used in other experiments in which it was delivered from a heated filament positioned in the close proximity to a group of ten flies in a vial (McClung and Hirsh 1998, McClung and Hirsh 1999, Andretic et al. 1999, Li et al. 2000, Park et al. 2000). Transition from increased locomotion into uncoordinated or stereotypical behavior is dose-dependent. As we have used a one IR beam DAM monitor for quantifying locomotor activity, we are aware of these limitations and have identified cocaine concentrations that lead to dose-dependent change in locomotor activity, but not in other uncoordinated behaviors. We show that only 3% of 75 µg of vCOC is sufficient to induce increase in locomotor activity. This disadvantage of the system can be improved by applying multiple IR beam monitors which than can record activity trough whole DAM tube not only in the middle, as we have proposed, since activity occurs also on the left and right side of the recording tube. Lack of response with female flies could be addressed by testing lower and shorter strengths of airflows compared to males, or by applying free base COC which has lower volatilization temperature which than would lower environmental factors of airflow and dehydration. Absence of a wider ranging dose-response curve using FlyBong argues for the appearance of uncoordinated behaviors at higher doses, which in our system could not be recorded as an increase in locomotion. Further work with FlyBong could be useful in drug research of other easily volatilized substances and genetic screens for new candidate genes underlying behavior associated with those drugs.

Population data can sometime differ from individual data and other way around. Difference between population and individual results is present when in population response increment in locomotion is consequence of small amount of the flies which are somewhere around the middle of the tube at time of the exposure, and after drug administration they start to increase their locomotion elevating population average, but not individual response. Difference between individual and population results, can be

the consequence of slight increment of individual fly locomotion, which is not obvious in population results, but when analysed at individual level, the effect is stronger.

Using FlyBong we wanted to induce increment in locomotion on acute and repeated exposures to vCOC and vMETH. We have shown that *wt* male flies are sensitive to acute vCOC and vMETH exposure (McClung and Hirsch 1998, Andretic et al. 2005), and that flies can develop locomotor sensitization to repeated vCOC exposures, as it was previously reported for vCOC (McClung and Hirsch 1998). Repeated vMETH exposures can also lead to LS, but this was not previously tested in flies. Based on the differences in pharmacodynamics and pharmacokinetics between COC and METH we have established that minimal time between two exposures for inducing locomotor sensitization is 6 hours for vCOC, and 10 hours for vMETH. Additionally we have established that females could not be tested for a motor-activating effect of PS using the protocol for delivery of volatilized drugs, as environmental perturbation (hot air for volatilization) causes significant increase in their locomotion. We believe that sensitivity and locomotor sensitization endophenotype can be induced and quantified in females, but it would require developing a different mode of drug delivery.

METH and COC exhibit some differences and similarities in mechanisms of action. By applying optimized method, we have tested the involvement of the vesicular monoamine transporter, dopamine receptor type 1 and dopamine transporter in the processes of sensitivity and locomotor sensitization to vCOC and vMETH. We have shown that, all three proteins must be functional, in order to induce locomotor sensitization to volatilized COC and METH, what is in line with behavior observed in mammals (Hall et al. 2009, Fukushima et al. 2007). COC binds to DAT and blocks uptake of dopamine from synaptic cleft (German et al. 2015), while *fmn* mutants have elevated dopamine prior to vCOC administration (Faville et al. 2015). We believe that acute vCOC administration does not increase population locomotion, since dopamine is already elevated, while repeated vCOC exposure cannot induce additional increment in locomotion possibly due to oxidative elimination of dopamine induced by COC (Meiser et al. 2013). METH reverses function of DAT, which then pumps dopamine from cytosol of the presynaptic neuron to the synaptic cleft. Same as for COC, we believe that acute vMETH dose does not increase population locomotion

since dopamine levels are elevated in *fmn* mutants, while repeated vMETH exposure cannot induce additional increment in locomotion (Kita et al. 2009).

Dopamine like receptor type 1 mutant (*dumb*) showed response on acute vCOC and vMETH exposures at population and individual level. Repeated exposures to vCOC and vMETH have not influenced population response, but they lowered individual response compared to *wt* flies. Feeding *dumb* mutants with COC has not induced motor-activating effect as it was case in *wt* flies (Lebestky et al. 2009). We have shown that vCOC induces around 25% of flies to gradually increase their locomotion in comparison to 40% in *wt* flies. This can be the consequence of higher sensitivity of *dumb* mutants to the warm airflow, rather than to PS (Lebestky et al. 2009). In addition, we cannot exclude that 75 µg of COC is too high concentration for *dumb* mutants.

Mammal mutants in dopamine receptor type 1 have lower sensitivity to METH (Xue et al. 2000), while in our experiments, sensitivity is the same as in *wt* flies, at population and individual level after acute vMETH exposure. The *dumb* mutants have lower level of dopamine prior to drug administration (Faville et al. 2015), but after COC and METH acute exposures dopamine levels should be elevated inducing increment in locomotion as it was observed from population and individual data. Repeated drug exposures have not induced sensitization possible due to lower basal level of dopamine in *dumb* mutants. Vesicular monoamine transporter (VMAT) RNAi was expressed in serotoninergic and dopaminergic neurons under DDC driver using UAS VMAT - DDC Gal 4 binary expression system. This genetic manipulation has not influenced sensitivity, but lowered sensitization to vCOC and vMETH at population and individual level compared to *wt* flies. VMAT2 mutant heterozygote mice shows sensitivity to acute COC exposure, while no sensitization was observed after repeated exposures (Wang et al. 1997), what is in line with our population and individual data. Using VMAT2 mutant heterozygote mice it was reported that sensitization to METH was delayed and that sensitivity was the same as in *wt* mice (Fukushima et al. 2007), which agrees with our results. Additionally, we show that VMAT RNAi mutants have lower sensitization to vCOC compared to vMETH suggesting importance for DDC promotor activity in sensitization to vCOC. VMAT RNAi synthesis is under influence of DDC promotor and circadian genes (Ishida et al. 2002.). Second administration to

vCOC occurs at 15:00 when serotonin and dopamine synthesis is high, leading to higher VMAT RNAi concentration, in comparison to second administration of vMETH at 19:00 when concentration of VMAT RNAi is lower, since dopamine and serotonin synthesis is silenced.

The potential involvement of monoamines in the development of sensitivity and sensitization to vCOC and vMETH was tested by pharmacological reduction of dopamine using 3-iodo tyrosine (3IY), and reduction of dopamine, serotonin and octopamine was investigated using administration of reserpine (res). We have shown that pre-treatment with 3IY and res reduce, but do not abolish, locomotor sensitization to COC and METH, without effecting sensitivity. Our results are in agreement with previously published results reporting that 3IY and res reduce sensitization on COC in flies (Bainton et al. 2000). These results indicate involvement of other trace monoamines, such as tyramine, which involvement in the neuronal plasticity induced by the drugs was reported (McClug and Hirsh 1998). In attempt to restore dopamine, we fed reserpine-pre-treated flies with L-DOPA (Riemensperger et al. 2011). These flies have shown sensitivity to acute vCOC and vMETH dose at population and individual level, but sensitization was lower for both tested drugs at population and individual level in comparison to res-treated flies. We explain this result with potential antioxidant properties of L-DOPA (Gow-Chin and Chiu-Luan 1997). We based this interpretation on reports demonstrating that pre-treatment of mammals with antioxidants lowers sensitization to COC (Jang et al. 2015) and METH (Jang et al. 2017), and on results from DPPH assay, which showed high antioxidant properties of L-DOPA (Figure 47A). L-DOPA-pre-treatment can restore dopamine in flies (Riemensperger et al. 2011), presumably through conversion to DA, but antioxidant effects of L-DOPA alone on behavior are not excluded.

Using FlyBong platform, we have confirmed that per^{01}, cyc^{01} and Clk^{Jrk} mutants are sensitive to the acute dose of vCOC (Andretic et al. 1999), but they do not develop locomotor sensitization to the repeated vCOC exposures, at population and individual levels. Sensitivity and sensitization in tim^{01} and pdf^{01} mutants are the same as in *wt* flies (Andretic et al. 1999, Heberlein et al. 2009, Tsai et al. 2004). This suggests separate molecular mechanisms involved in the regulation of behavioral phenotype to the acute versus multiple exposures of vCOC, which can be precisely measured using

FlyBong. Individual analysis identified an increased sensitivity to vCOC in *pdf*^{01} and *tim*^{01} mutants, which is in accordance with the role of *pdf* in regulating light-mediated arousal (Shimada et al. 2016, Renn et al.1999) and promoting of locomotor activity by light in *tim*^{01} mutants (Lu et al. 2008). However, locomotor sensitization to vCOC is not completely cocaine-specific, but it is also consequence of sensitization to the warm airflow. Thus, *pdf*^{01} and *tim*^{01} flies will require further study in order to determine the role that these genes play in neuronal plasticity. Since both mutants have increased sensitivity to the warm air and develop locomotor sensitization to the warm air, future studies will have to use an alternate mode of delivering COC. Our results from population level analysis of *pdf*^{01} mutants, show normal sensitivity to vCOC, similar to normal sensitivity measured as negative geotaxis of a population of *pdf*^{01} mutants to vCOC (Tsai et al. 2004). However, our analysis of individual flies defined new phenotypes that were not immediately obvious in a population. Thus, analysis of the locomotor activity of individual flies, as well as their respective controls, significantly aids in differentiating cocaine-specific from non-specific effects.

METH sensitivity and sensitization depend on functional CLK protein, while other circadian mutants *per*^{01}, *cyc*^{01}, *tim*^{01} and *pdf*^{01} have shown lower sensitization, without effect on sensitivity. This data indicates that COC- and METH-induced sensitization endophenotype depends on functional CLK protein, while the role the other circadian genes in drug-induced neuroplasticity is drug-dependent. Circadian genes modulate the amount of dopamine synthesis and degradation (Golombek et al. 2014), possibly through direct binding to the promotor region of genes coding for dopamine synthesis and degradation enzymes. Drugs induce enhanced production of circadian genes (Krishnan et al. 2008, Miyazaki and Asanuma 2008), possibly due to the dopaminergic loss by oxidative degradation and increased reactive oxygen species (ROS) production. It was shown that circadian genes could sense redox changes through the PAS domain contained in their protein structure (Möglich et al. 2009).

The vCOC administration induces larger redox perturbation and depends on multiple circadian genes, while vMETH induces lower redox perturbation and depends on smaller number of circadian genes. This hypothesis was confirmed by measuring ROS and H_2O_2 production and activity of CAT and SOD enzymes in *wt* flies exposed to acute and repeated vCOC and vMETH. In *wt* flies, COC induced higher ROS and

H_2O_2 production compared to values before exposures, as well as increased CAT and lowered SOD activity. Studies in mammals show that COC lowers CAT activity (Macedo et al. 2005), and increases SOD activity, ROS and hydrogen peroxide production (Dietrich et al. 2005). Difference between results obtained on mammals and our results in CAT and SOD activity could be due to the different pathway of dopamine degradation in the flies compared to mammals, since flies do not have MAO and COMT enzymes (Paxon et al. 2005). Other possible explanation is that CAT and SOD activity were measured in whole body extracts, not only in heads and/or brains of vCOC-exposed flies, so data on redox perturbation in flies exposed to vCOC is systemic and not neuron-specific.

The vMETH sensitization depends only on *Clk* circadian protein, and shows lower susceptibility to exogenous redox perturbation and low amount of ROS production. The vMETH induced same amount of ROS but lowered H_2O_2 production compared to values before exposures, and led to increased CAT and lower SOD activity. In mammals, METH induces ROS generation (Kita et al. 2009, Kita et al. 2003, Moszczynska 2017), but it was shown that ROS generation in METH-exposed animals is dopamine dependent (Larsen et al. 2002). Our results are in line with studies in mammals, where increased CAT activity (Koriem et al. 2012), and decreased SOD activity was reported (Frenzilli et al. 2007). In mammals, METH decreases dopamine metabolism catalyzed by MAO and induces dopamine autoxidation leading to increases in ROS. Since we have measured the same increase in ROS and decrease in H_2O_2 production in whole body extract it is possible that systemic effect of METH is less toxic for flies than effect of vCOC. Furthermore, the second administration of vCOC was given at 15:00, while second administration of vMETH was at 19:00. These time differences can than influence measured redox markers due to changes of metabolic activity, since metabolic activity is higher during day-time and lower at night-time.

The influence of exogenous pre-treatment with pro- and antioxidant was tested on behavioral response to acute and repeated vCOC and vMETH exposure. We have shown that both anti- and prooxidants abolish locomotor sensitization, lower sensitivity to vCOC, and lower locomotor sensitization without effecting sensitivity to vMETH. This data suggests that redox balance is important for vCOC-induced behavior, while less

for vMETH-induced behavior. By comparing antioxidant capacity of antioxidant quercetin and tyrosol, which we used for pre-treatment, we have shown that vMETH sensitization can be explained with quercetin and tyrosol antioxidant capacity. Based on that we propose vMETH response as more sensitive to redox perturbations induced by prooxidant, while COC sensitization as more susceptible to both pro- and antioxidant redox perturbations, which does not depend on antioxidant capacity.

We have measured influence of antioxidants quercetin and tyrosol on redox enzymes before and after vCOC and vMETH exposures, in order to correlate redox perturbations and exposure to the drugs with changes in behavior. We have shown that antioxidant-pre-treated flies have lower SOD, and increased ROS and H_2O_2 production after vCOC exposure, as in non-treated flies, and that pre-treatment lowers CAT activity, in opposite way than in untreated flies. Antioxidant-pre-treated flies have lower SOD, but same ROS and H_2O_2 production after vMETH exposure, as observed in non-treated flies, while CAT activity is lower in contrast to untreated flies. This indicates that CAT activity is sensitive to antioxidant effect on behavior and to vCOC and vMETH. One potential explanation for this is negative up-regulation of CAT through *per* gene (Krishnan et al. 2008), meaning that increased PER, characteristic for exposures to PS, lowers CAT. Another explanation is that antioxidants can non-enzymatically remove COC and METH oxidative metabolites, which then induce lower CAT activity, without effecting SOD activity or ROS and H_2O_2 production. Since we have shown that, unlike vCOC, vMETH behavioral response does not depend on PER protein, it is possible that lower CAT induced by antioxidants interfere with PER protein, which can be behaviorally seen as lower METH and absent COC sensitization.

Hydrogen peroxide pre-treatment in flies did not affect redox status biomarkers, CAT and SOD activity, and ROS and H_2O_2 production, suggesting that hydrogen peroxide does not act as prooxidant. We have shown that hydrogen peroxide pre-treatment lowers sensitization to vMETH, while vCOC sensitivity and sensitization do not depend on hydrogen peroxide pre-treatment. From this data, we hypothesize that beside expected prooxidant hydrogen peroxide influence, hydrogen peroxide can potentially act as neuromodulator. This effect is present more in vMETH- than in vCOC-treated groups, since we proposed that vMETH is more sensitive on redox perturbation. One possible explanation was that exogenous hydrogen peroxide is

metabolized through endogenous monoamines by hydrogen peroxide scavenging, since CAT and SOD have shown no influence of hydrogen peroxide. Unfortunately, we could not test *in vitro* hydrogen peroxide scavenging through monoamines, because of the same wavelength of maximal absorbance for hydrogen peroxide and monoamines, making it difficult to follow hydrogen peroxide concentration without having effect of monoamines. Thus, to test this hypothesis we will have to apply a different approach.

To test the effect of hydrogen peroxide as neuromodulator, we pre-treated flies with hydrogen peroxide and antioxidants for which we have shown to abolish LS to COC and lower LS to METH. Flies pre-treated with quercetin and hydrogen peroxide showed increased response to acute vCOC dose at population and individual level, compared to non-treated group, and group treated with only quercetin or hydrogen peroxide. At population level, there is no sensitization, but at individual level, around 10% of flies have shown sensitization. Effect of tyrosol and hydrogen peroxide, as well as the combination of quercetin and hydrogen peroxide pre-treatment had the same effect on sensitivity and sensitization to vCOC at individual and population level. With tyrosol and hydrogen peroxide pre-treatment, 20% of flies in the population has shown sensitization to repeated vCOC exposures, compared to 10% for quercetin and hydrogen peroxide. This difference can be consequence of higher antioxidant capacity of quercetin compared to tyrosol. Quercetin can than reduce the hydrogen peroxide effect through reactions in fly food before flies consumes it, or by metabolic removal of hydrogen peroxide.

Pre-treatment with antioxidant and hydrogen peroxide did not affect sensitivity to vMETH compared to untreated group, but the sensitization was lower compared to treatment with only one antioxidant. Since we have already shown that vMETH sensitization depends on quercetin and tyrosol antioxidant capacity, and that METH response is more sensitive to redox perturbation induced by prooxidant, lower response to pre-treatment with combination of quercetin and hydrogen peroxide, compared to only quercetin pre-treatment was expected. Similar results were seen for pre-treatment with combination of tyrosol and hydrogen peroxide, compared to pre-treatment with tyrosol alone. Furthermore, by applying combination of hydrogen peroxide and antioxidant through food, the antioxidant effect could have occurred in the food, before ingestion, which can ultimately lead to the different starting hydrogen

peroxide and antioxidant concentrations. At the end, future work on treatment and prevention of drug addiction could be orientated on the adjustment of food supplements, since we have shown that exogenous pre-treatment with anti- and prooxidants can modulate behavioral response.

It was previously reported that flies voluntary self-administer ethanol containing solution (Devineni and Heberlein 2009), when they have been offered to choose between sweet solution with and without ethanol. Here we show for the first time that flies have high preference for COC-containing food, and moderate for METH-containing food.

The measure that was used for determining preferential consumption was the preference index (PI), where positive PI values are associated with the higher drug food consumption indicating that drug is appetitive to the flies. Negative PI values are associated with the higher consumption of food without the drug, indicating that drug is repulsive to the flies. We optimized protocol for COC- and METH self-administration by using 100 mM sucrose solution offered to flies through two capillaries, while METH and COC were added to other two capillaries. We have adapted capillary feeder (CAFÉ) assay used by Devineni and Heberlein in 2009, by optimizing the number of flies in the each vial to six, and by controlling humidity.

By applying optimized assay we have established that flies preferentially self-administer COC and METH over sugar solutions in a dose-dependent way. Preference for COC was increasing over the consecutive days, in the way as it was reported for mammals (Bernnan et al. 1990), while preference for METH was decreasing, in a way opposite from previous findings in mammals (Bergman et al. 2009). Since in our assay the administration is oral, the concentration of offered drugs plays an important role, due to bitter taste of COC and METH (Amrein and Bray 2003). In general, lower drug concentrations are more appetitive to flies than higher doses. Therefore, it is possible that lower preference to METH in flies is due to the oral administration, since in mammals METH is delivered by injection. Additionally, METH has anorexic effect, which can lead to lower food consumption in flies and smaller PI.

Additional way of testing if preference for PS in flies is influenced by bitter taste of drug can be done by testing Proboscis Extension Response (PER). Flies respond

with PER to appetitive substances like sugars and fatty acids, and ignore aversive ones like bitter or with high salt. Aversive stimuli will also inhibit the PER response elicited by the appetitive substances if applied simultaneously on the proboscis. The probability of a PER response depends on the hedonic value of the stimuli (e.g. concentration of sugar, ratio of bitter:sweet in a mixture) and the internal drive of the fly (e.g. amount of starvation). In addition, it would be necessary to test whether attraction to or avoidance of COC and METH in flies is odor-dependent. Various assays can be used, such as trap assay and T- and Y-maze. Outcomes of such experiments would help in elucidating the mechanisms of PS self-administration in flies.

We have shown that preferential consumption of COC and METH includes processes of learning and memory (Sanchis-Segura and Spanagel 2006), since preference depends on the capillary location and it can be enhanced by adding a visual cue that is associated with the drug (Kaun et al. 2011). These results indicate that flies will self-administer psychostimulants when they have choice between food with drug and food without drug, and that this process might involve action of PS on motivational circuits in the brain that control drug-taking behavior, similar as in mammals.

Relapse is usually induced in animal models by withdrawing the drug and after a period of abstinence, introducing the drug again. Phenotype of relapse is defined by drug consumption that is increased or the same drug consumption as before the period of abstinence. We have established that two days of deprivation from COC result in the same PI as before the period of deprivation, while METH deprivation is resulting in the higher PI. These results indicate that flies show relapse after period of the drug deprivation similar to mammals, and that self-administration is based on the rewarding effect induced by COC and METH consumption.

We have also tested if the flies self-administer COC and METH in spite of negative consequences, and have shown that flies will overcome bitter taste of the quinine in order to self-administer COC and METH. Additionally, we have tested antioxidant capacity of quinine, in order to remove doubts about lower PI for COC and METH mixed with QIN based on possible antioxidant effect of quinine on preferential consumption, since anti- and prooxidant were shown to have influence on locomotor-activating effect of COC and METH. Our results have shown that QIN is weak

antioxidant in DPPH assay and that it is not interfering with preference for COC and METH.

Dopamine transporter is a key target in COC and METH pharmacodynamics. We have shown that *fmn* mutant flies, carrying mutation in dopamine transporter have lower PI for COC and METH. DAT function is dynamically regulated by multiple intracellular and extracellular signaling pathways and several protein-protein interactions. Such homeostatic regulations can explain that DAT mutation in mammals does not affect conditional place preference (CCP) or self-administration of COC (Rocha et al. 1998, Sora et al. 2001). In *fmn* mutant flies, PI for both COC and METH is increasing over the days, indicating that dopamine is not the only neurotransmitter important for COC- and METH-induced rewarding effect in flies (Sora et al. 2001). Indeed, there is abundant evidence that in flies neurotransmitter and neuromodulator octopamine predominately conveys rewarding effect (Scaplen and Kaun 2016, Mizunami et al. 2015, Waddell 2013).

Mutants in dopamine receptor type 1 (*dumb*) showed negative PI for METH, while preference for COC was the same as in *wt* flies. Mutations in D1 and D2 receptor in mammals block self-administration of COC (Bergman et al. 1990), while D1 and D2 receptor antagonists reduce METH self-administration (Bernnan et al. 2009). Our manipulation of vesicular monoamine transporters using RNAi in dopaminergic and serotoninergic neurons has shown that locomotor sensitization and preference to COC and METH is dependent on functional VMAT. VMAT2 heterozygote mice show a reduction in METH rewarding effect, while their COC rewarding effect remains the same (Takahashi et al. 1997). From presented data, we can conclude that METH rewarding effect is more correlated with dopamine levels, since all three mutations *fmn*, *dumb* and DDC-VMAT demonstrated lower preference for METH, while COC preference depends not only on dopamine, but also on other monoamines.

Since the circadian genes *period*, *clock* and *cycle* were reported to be associated with locomotor activating effect of COC (Andretic et al. 1999), we have tested their effect on preferential consumption to COC and METH. Average PI for COC in all circadian mutants was lower than the preference index of *wt* flies, however only for per^{01} mutants PI was significantly lower than in the *wt* flies. This data is in line with mammalian studies demonstrating that *Per1* mutant mice show abolishment of

rewarding effect measured in conditioned place preference assay (Abarca et al. 2002), and that *Clock*Δ*19* mutant mice retained normal preference for COC. Average PI for METH was lower in all tested circadian mutants than the preference index of *wt* flies, and significantly lower in per^{01}, Clk^{Jrk} and cyc^{01} mutants.

This data indicates involvement of circadian genes in both processes of neuronal plasticity induced by drugs, locomotor sensitization and rewarding effect. The COC-induced preferential consumption and sensitization, depending on the same circadian gene *per*, suggests potential importance of this gene in dopamine release associated with rewarding effect. Since we have established that preference to drugs depends on capillary location and cue, indicating involvement of learning and memory, it is possible that self-administration performance is lower in per^{01} mutant due to poorer performance in learning and memory tasks (Sakai et al. 2004). Both locomotor sensitization and rewarding effect of METH depend on the same circadian gene *Clk* suggesting the importance of this gene in dopamine release associated with rewarding effect induced by METH. However Clk^{Jrk} flies have defect in feeding and visual behavior (Xu et al. 2008, Mazzoni et al. 2005), which could affect performance in CAFÉ assay.

We have shown that *Drosophila melanogaster* can be used to study endophenotypes of locomotor sensitization and self-administration. Our data indicate that these two processes of drug-induced neuronal plasticity share common neuronal mechanisms since circadian genes, dopamine transporter and dopamine receptors play a role in regulating *Drosophila* behavior.

6. CONCLUSION

This work resulted in the development of two new objective and high-throughput methods for quantification of motor activating and rewarding effect in *Drosophila melanogaster*, allowing research of the genetic basis of neural plasticity induced by PS. Elucidating COC- and METH-induced circadian/redox feedback loops should help in the understanding of drug-induced mechanisms of neuronal plasticity, which could lead to new discoveries important in the prevention and treatment of addiction. Based on the provided data we have concluded:

- FlyBong induces increment in locomotion following acute exposure to vCOC and vMETH, with minimal time between two exposures for inducing LS of 6 hours for vCOC, and 10 hours for vMETH
- male and female flies differently responded to acute and repeated vCOC and vMETH exposure
- functional dopamine transporter, dopamine receptor and vesicular monoamine transporter are needed for induction of LS to vCOC and vMETH
- reduction of dopamine, serotonin and octopamine, decrease but do not abolish LS to vCOC and vMETH, indicating possible involvement of other trace monoamines
- *per*, *Clk* and *cyc* genes are involved in the development of LS to vCOC, while only *Clk* is important for vMETH-induced LS
- in contrast to vMETH, vCOC increases ROS and H_2O_2 production
- redox balance is important for COC-induced LS, while it is less important for LS induced by METH administration
- vMETH-induced LS depends on the potency of antioxidant and it is more sensitive to prooxidant, whereas LS response to vCOC is equally sensitive to both pro- and antioxidant pre-treatment
- susceptibility to pro- and anti-oxidant pre-treatment is based on correlation between amount of redox production and circadian genes
- H_2O_2 pre-treatment did not affect redox status biomarkers, but lowered LS to vMETH, while vCOC-induced LS was the same as in *wt* flies

- QUE and H_2O_2, or TYR and H_2O_2, restored LS to vCOC, and additionally lowered LS to vMETH indicating the opposite role of H_2O_2 on vCOC- and vMETH-induced neuronal plasticity
- flies self-administer COC and METH over sugar solutions in dose-dependent way
- preferential consumption includes processes of learning and memory
- flies exhibit moderate deprivation effect when drugs were withdrawn
- flies overcame the bitter taste of quinine in order to self-administer COC and METH
- COC preferential consumption and LS depend on the same circadian gene *per,* while METH preferential consumption and LS depend on the circadian gene *Clk*
- rewarding effect of METH is more correlated with dopamine release while COC-induced rewarding response depends not only on dopamine, but also on other monoamines

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8. LIST OF ABBREVIATIONS USED

3IY = 3-iodotyrosine

CAFÉ = Capillary Feeder Assay

CAT = Catalase

Clk = Clock gene

COC = Cocaine

COMT = Catechol-O-amine Transferase

CPP = Conditional Place Preference

cyc = Cycle gene

DA = Dopamine or 4-(2-aminoethyl)benzene-1,2-diol

DAMS = Drosophila Activity Monitoring System

DAT = Dopamine Transporter

DDC = DOPA Decarboxylase

DOPAC =3,4-dihydroxyphenylacetic acid

DOPAL = 3,4-dihydroxyphenylacetaldehyde

DPPH = α,α-diphenyl-β-picrylhydrazyl

DopR1 = Dopamine Receptor Type 1

GAL4 = (Yeast) galactose-responsive transcription factor

HVA= Homovanillic Acid

H_2O_2 = Hydrogen Peroxide

L-DOPA = L-3,4-dihydroxyphenylalanine

LS = Locomotor Sensitization

MAO = Monoamine Oxidase

METH = Methamphetamine

PAS = Per: period; ARNT: aryl hydrocarbon receptor nuclear transporter; Sim: single-minded protein domain

per = Period gene

pdf = Pigment Dispersing Factor gene

PS = Psychostimulant

PQ = Paraquat

QUE = Quercetin or 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-*4H*-chromen-4-one

QIN = Quinine

res = Reserpine

RNAi = Ribonucleic acid interference

ROS = Reactive Oxygen Species

SENS = Sensitivity

SOD = Superoxide Dismutase

TEMPOL = 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl

tim = Timeless gene

TRX = trolox

TYR = Tyrosol or 2-(4-Hydroxyphenyl)ethanol

TYRA = Tyramine

UAS = (Yeast) Upstream Activating Sequence

vCOC = Volatilized Cocaine

VMAT = Vesicular Monoamine Transporter

vMETH = Volatilized Methamphetamine

9. LIST OF FIGURES

10. LIST OF TABELS

11. SUPPLEMETARY MATERIALS

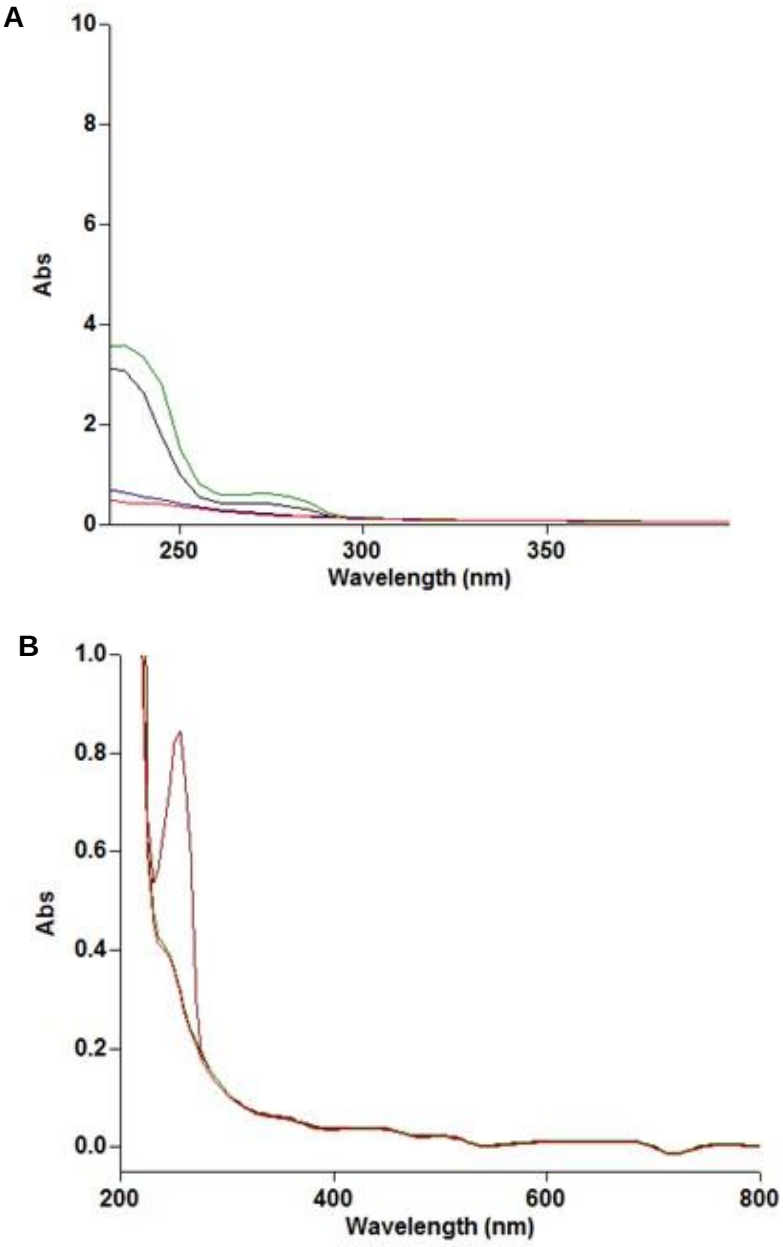


Figure 1. Stability of COC and METH after volatilization and effect of warm airflow duration on locomotor activity in *wt* males. A) UV-VIS spectra of COC in range 400-230 nm dissolved in distilled water using plastic civets. Random samples after COC volatilization (blue and red) and COC in known concentration of 0.3 mg/mL (gray) and 0.5 mg/mL (green) have the same absorption peaks around 230 and 275 nm characteristic for COC maximum absorbance in UV-VIS spectra. Based on overlapping UV-VIS spectra of samples after heating and samples without COC heating we did not see any additional peaks, so we concluded that COC is stabile after volatilization without metabolites and/or heat induced decomposition molecules such as benzoylecgonine, ecgonine and ecgonine methyl ester. **B)** UV-VIS spectra of METH in range 800-200 nm dissolved in distilled water using plastic civets. Random samples after METH volatilization (gray and red) and METH in known concentration of 0.3 mg/mL (pink) have the same absorption peak around 250 nm characteristic for METH maximum absorbance in UV-VIS spectra. Based on overlapping UV-VIS spectra of samples after heating and samples without METH heating, we did not see any additional peaks, so we concluded that METH is stable after volatilization without metabolites and/or heat induced decomposition molecules. **C)** Histogram of different durations of airflow plotted as mean population locomotor activity (32 flies per group), 5 minutes before and 5 minutes after exposure to warm air for COC, and 10 minutes before and 10 minutes after exposure to warm air for METH. A warm airflow of one minute leads to no significant difference between levels of activity before and after exposure in both 5 and 10 minutes, and therefore this time was chosen as the standard duration for COC and METH delivery. Data are plotted as mean activity ± SEM for 32 flies in 5 and 10 minutes resolution (all tested groups). Statistical significant differences ($p \leq 0.05$) are indicated by a: comparison of activity before and after exposure (within the group using t-test for dependent samples) and b: activity in the control group (no airflow) compared to after exposure in groups exposed to warm air flow (one-way ANOVA with Dunnett post-hoc test).

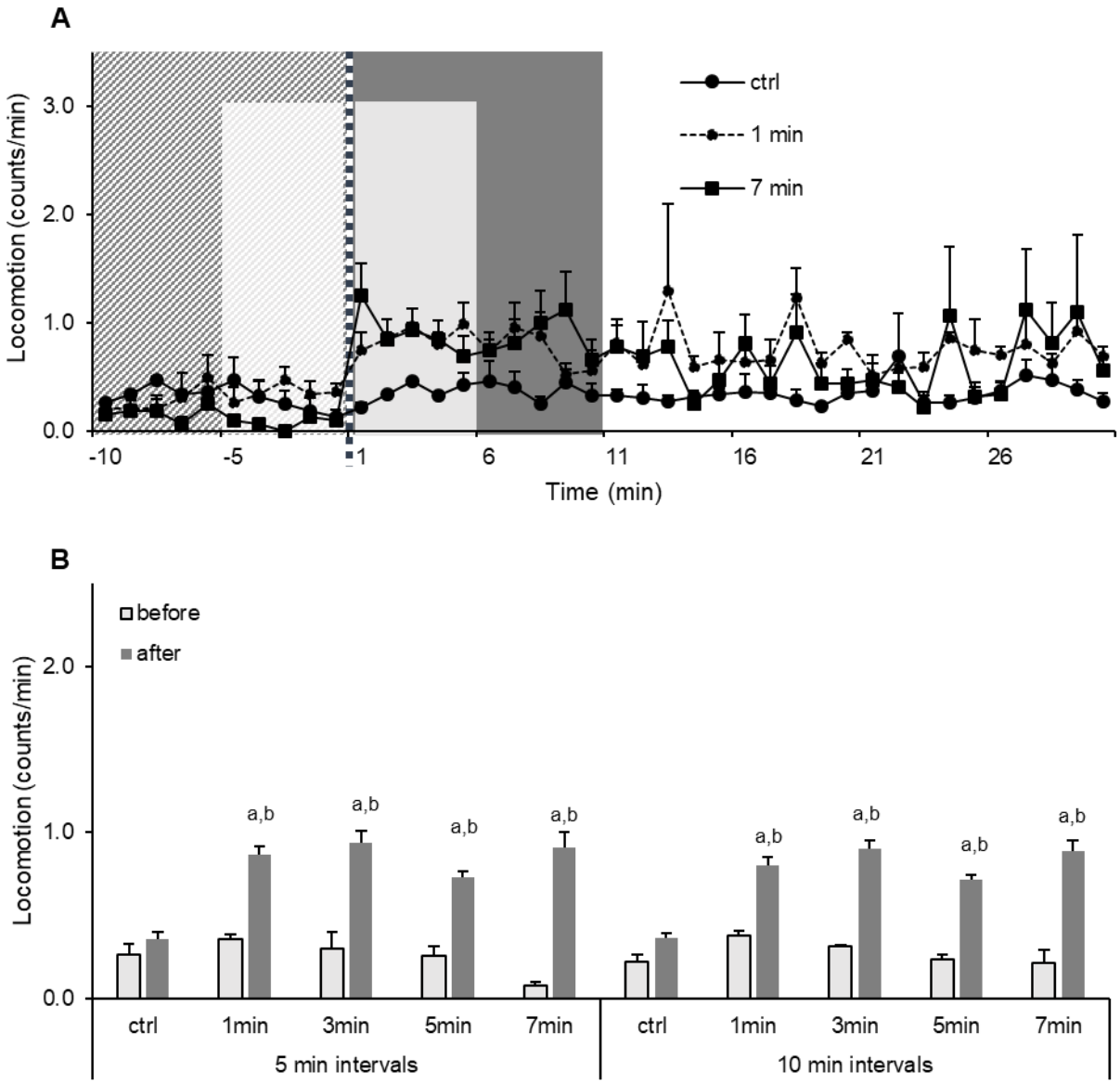
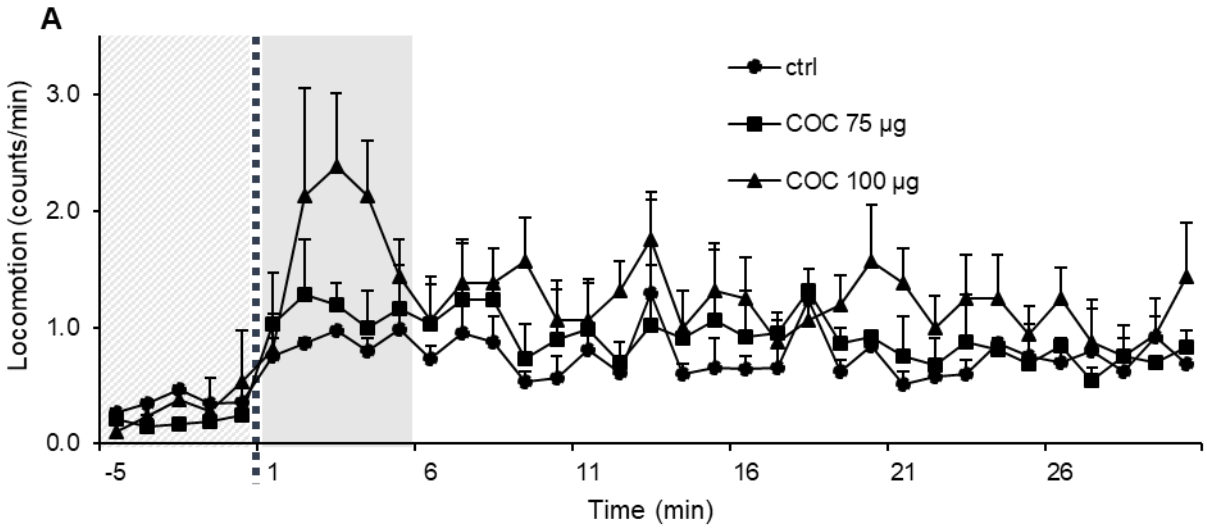
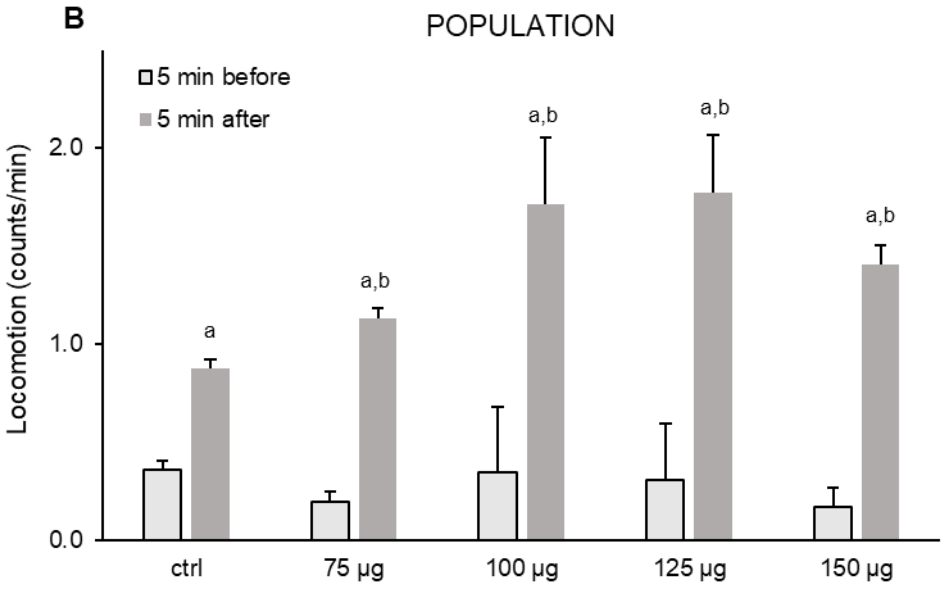


Figure 2. Effect of warm airflow duration on locomotor activity in *wt* females. A) Average activity ± SEM for flies exposed to 1 and 7 minutes duration of 2.5 L/min airflow from a flask heated for 8 minutes, without PS. Kinetic graph shows mean ± SEM for 32 flies per group at a resolution of one minute. Shaded light gray panel represent locomotor activity 5 minutes, shaded dark gray panel 10 minutes before drug exposure, dotted line is time of the exposure, and light gray panel is first 5 minutes and dark gray panel 10 minutes after the exposure. **B)** Histogram of different durations of airflow plotted as mean population locomotor activity (32 flies per group) 5 minutes before and 5 minutes after exposure to warm air for COC, and 10 minutes before and 10 minutes after exposure to warm air for METH. All tested airflows significantly increased population response in female flies. Data are plotted as mean activity ± SEM for 32 flies in 5 and 10 minutes resolution (all tested groups). Statistical significant differences ($p \leq 0.05$) are indicated by a: comparison of activity before and after exposure (within the group using t-test for dependent samples) and b: activity in the control group (no airflow) compared to after exposure in groups exposed to warm airflow (one-way ANOVA with Dunnett post-hoc test).


A
Locomotion (counts/min)
3.0
2.0
1.0
0.0
-5
1
6
11
16
21
26
Time (min)
ctrl
COC 75 µg
COC 100 µg


B
POPULATION
5 min before
5 min after
Locomotion (counts/min)
2.0
1.0
0.0
a
a,b
a,b
a,b
a,b
ctrl
75 µg
100 µg
125 µg
150 µg

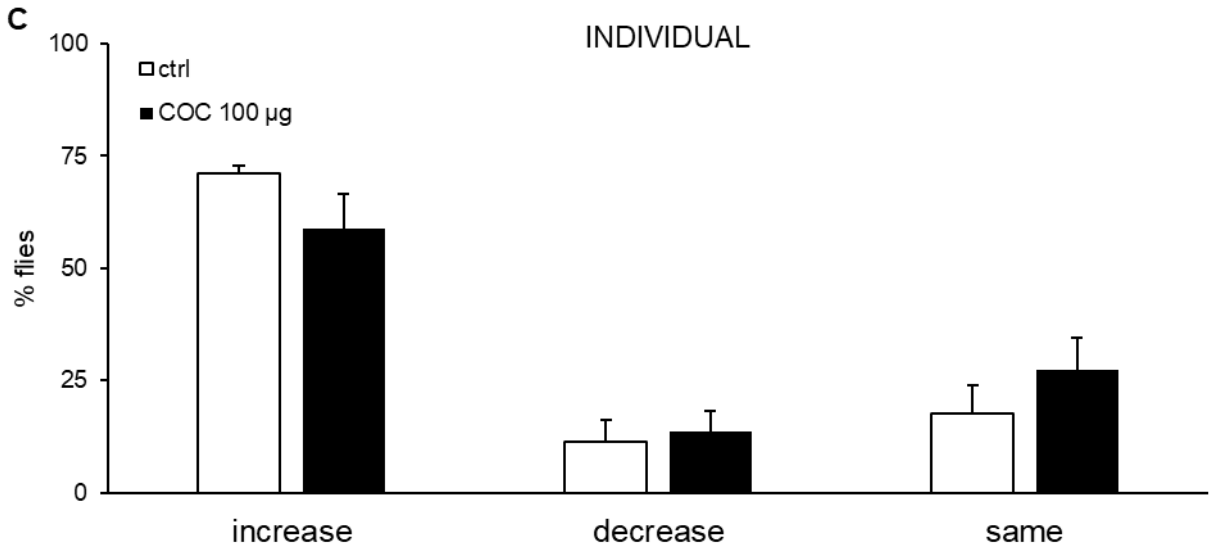
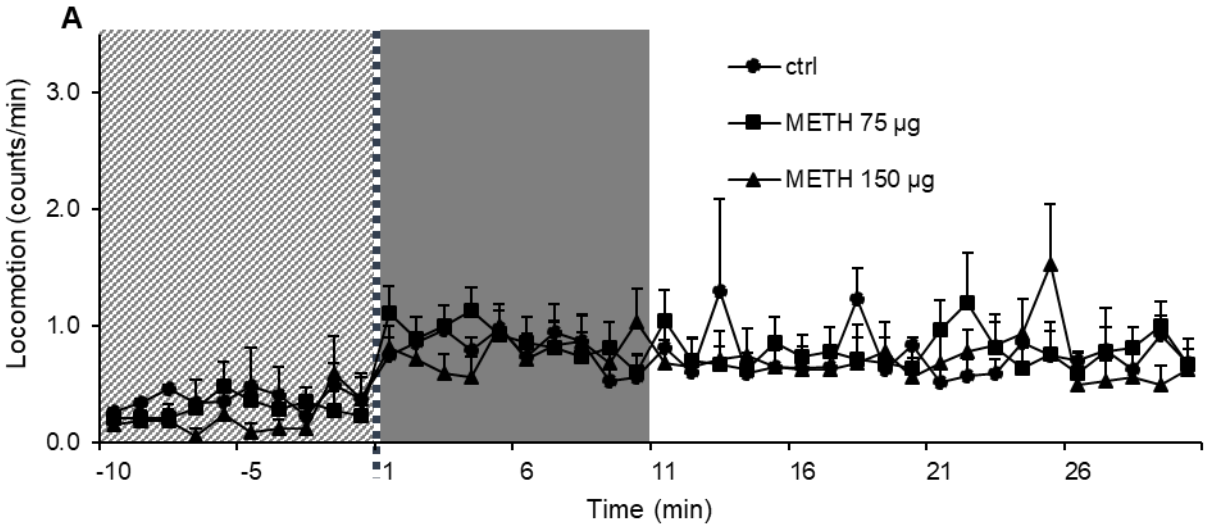
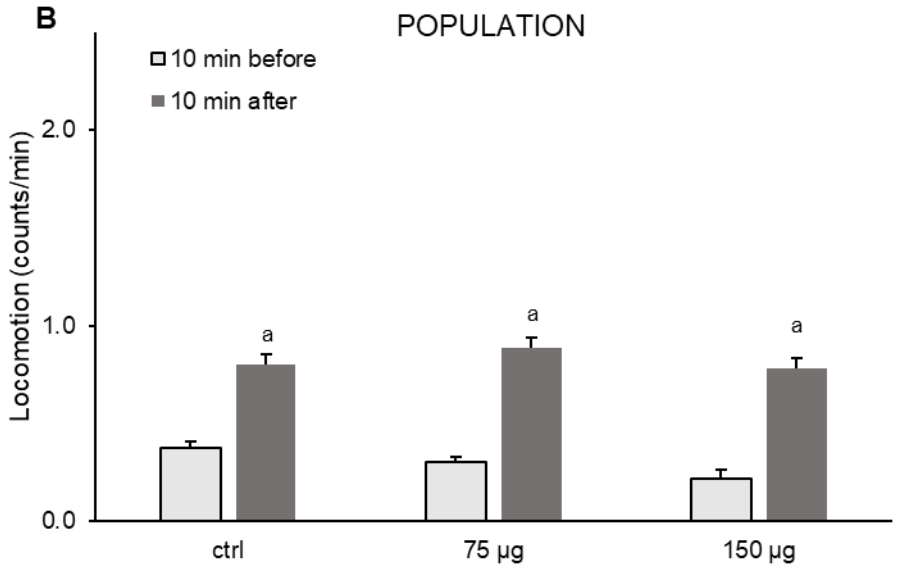

C
INDIVIDUAL
ctrl
COC 100 µg
% flies
100
75
50
25
0
increase
decrease
same

Figure 3. At the individual level, response of female *wt* flies is not COC-specific. A) Kinetic graph of locomotion expressed as number of counts per minute for control group of flies (n=32, exposed to warm air), and test group exposed to 75 µg of volatilized COC (n=32), and 100 µg of volatilized COC (n=32). The shaded light gray panel indicates the 5 minutes immediately prior to exposure, the dotted line is the time of exposure and the light gray panel indicates 5 minutes after exposure. **B)** Histogram of different amounts of volatilized COC (75 to 150 µg) plotted as a mean of the population (32 flies) locomotor activity 5 minutes before and 5 minutes after exposure to COC. Statistical significance ($p \leq 0.05$) is indicated by: a: comparison of activity in the 5 minutes immediately before and after exposure (within the group using t-test for dependent samples); b: activity after exposure, compared between the control group and group exposed to volatilized COC (one-way ANOVA with Dunnett post-hoc test). **C)** Amount of individual fly locomotor activity 5 min before exposure was compared to 5 minute after exposure to 100 µg of volatilized cocaine (n=32) and categorized as an increase, decrease or no change and compared to control group (n=32) that received warm airflow. Data are plotted as histograms showing the mean value of five tests ± SEM. There is no difference in sensitivity between control group and group that received 100 µg of COC tested by Mann-Whitney U-test for nonparametric analysis of two independent samples.


A
Locomotion (counts/min)
3.0
2.0
1.0
0.0
-10
-5
-1
6
11
16
21
26
Time (min)
ctrl
METH 75 µg
METH 150 µg


B
POPULATION
10 min before
10 min after
Locomotion (counts/min)
2.0
1.0
0.0
a
a
a
ctrl
75 µg
150 µg

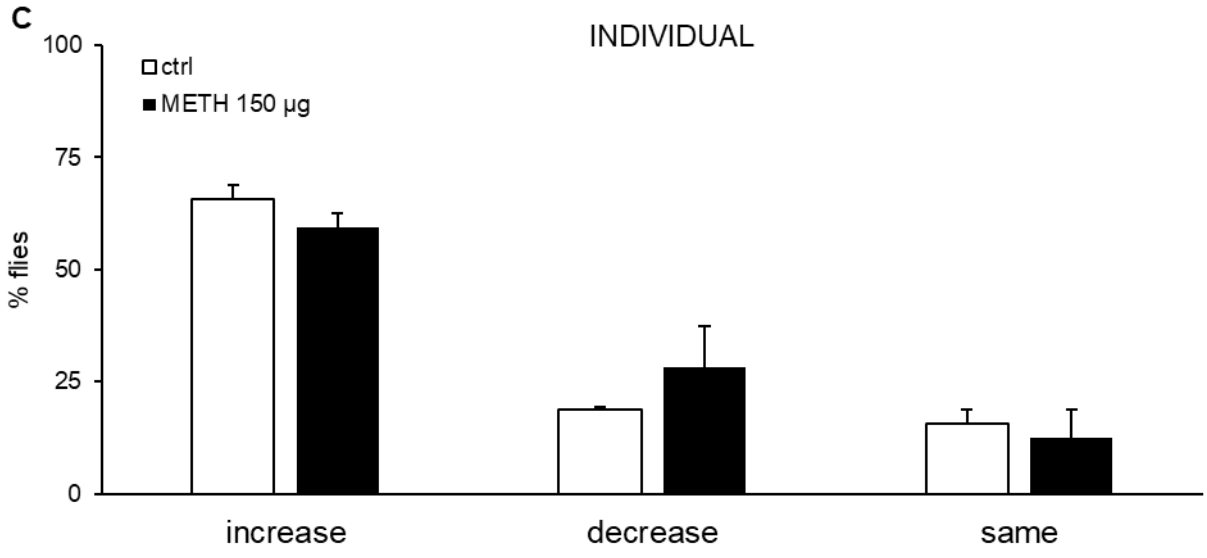

C
INDIVIDUAL
ctrl
METH 150 µg
% flies
100
75
50
25
0
increase
decrease
same

Figure 4. At the individual level, response of female *wt* flies is not METH specific. **A)** Kinetic graph of locomotion expressed as number of counts per minute for control group of flies (n=32, exposed to warm air), and test group exposed to 75 µg of volatilized METH (n=32), and 150 µg of volatilized METH (n=32). The shaded dark gray panel indicates the 10 minutes immediately prior to exposure, the dotted line is the time of exposure and the dark gray panel indicates 10 minutes after exposure. **B)** Histogram of different amounts of volatilized METH (75 and 150 µg) plotted as a mean of the population (32 flies) locomotor activity 10 minutes before and 10 minutes after exposure to METH. Statistical significance ($p \leq 0.05$) is indicated by a: comparison of activity in the 10 minutes immediately before and after exposure (within the group using t-test for dependent samples); b: activity after exposure, compared between the control group and group exposed to volatilized METH (one-way ANOVA with Dunnett post-hoc test). **C)** Amount of individual fly locomotor activity 10 min before exposure was compared to 10 minute after exposure to 150 µg of volatilized METH (n=32) and categorized as an increase, decrease or no change and compared to control group (n=32) that received warm airflow. Data are plotted as histograms showing the mean value of five tests ± SEM. There is no difference in the sensitivity between control group and group that received 150 µg of METH tested by Mann-Whitney U-test for nonparametric analysis of two independent samples.

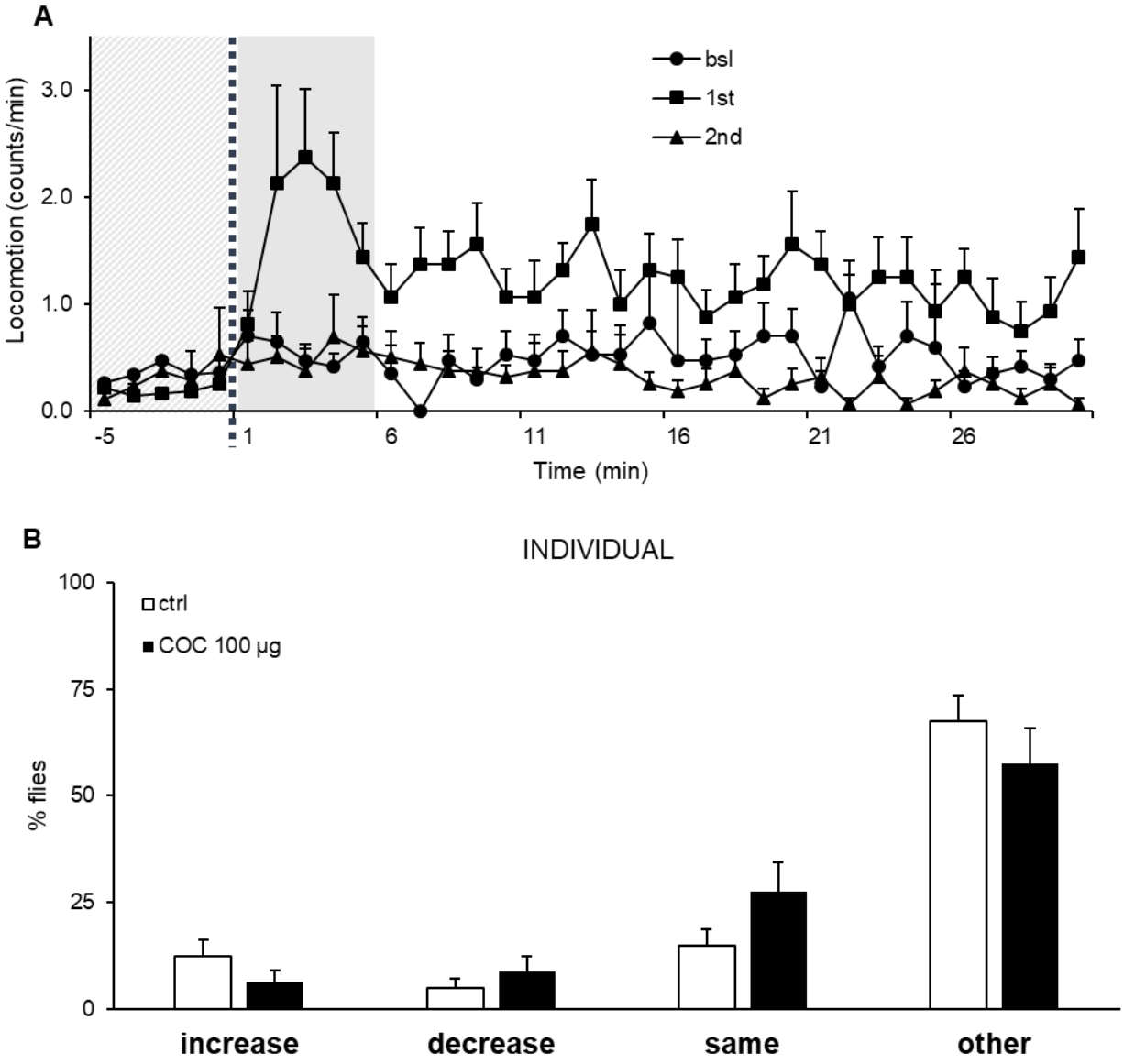


Figure 5. Our standard protocol for COC administration does not induce locomotor sensitization in *wt* female flies. A) Kinetic graph of locomotion expressed as number of counts per minute for group exposed to twice to volatilized 100 µg COC (n=32), once at 09:00 (1^{st}) and then at 15:00 (2^{nd}), along with their baseline (bsl) activity before drug administration. The shaded light gray panel indicates the 5 minutes immediately prior to exposure, the dotted line is the time of exposure and the light gray panel indicates 5 minutes after exposure. Locomotor activity after 2^{nd} exposure is similar to baseline levels. **B)** Amount of individual fly locomotor activity 5 min before exposure was compared to 5 minute after the first and second exposures to 100 µg of volatilized cocaine (n=32). These were categorized as an increase, decrease, no change or other (flies that did not satisfy criteria for previous three groups), when compared the effect on the control group (n=32) that received warm airflow only. Data are plotted as histograms showing the mean value of five tests ± SEM. Percentage of flies in each category is similar between group that received 100 µg of COC and warm air control tested by Mann-Whitney U-test for nonparametric analysis of two independent samples.

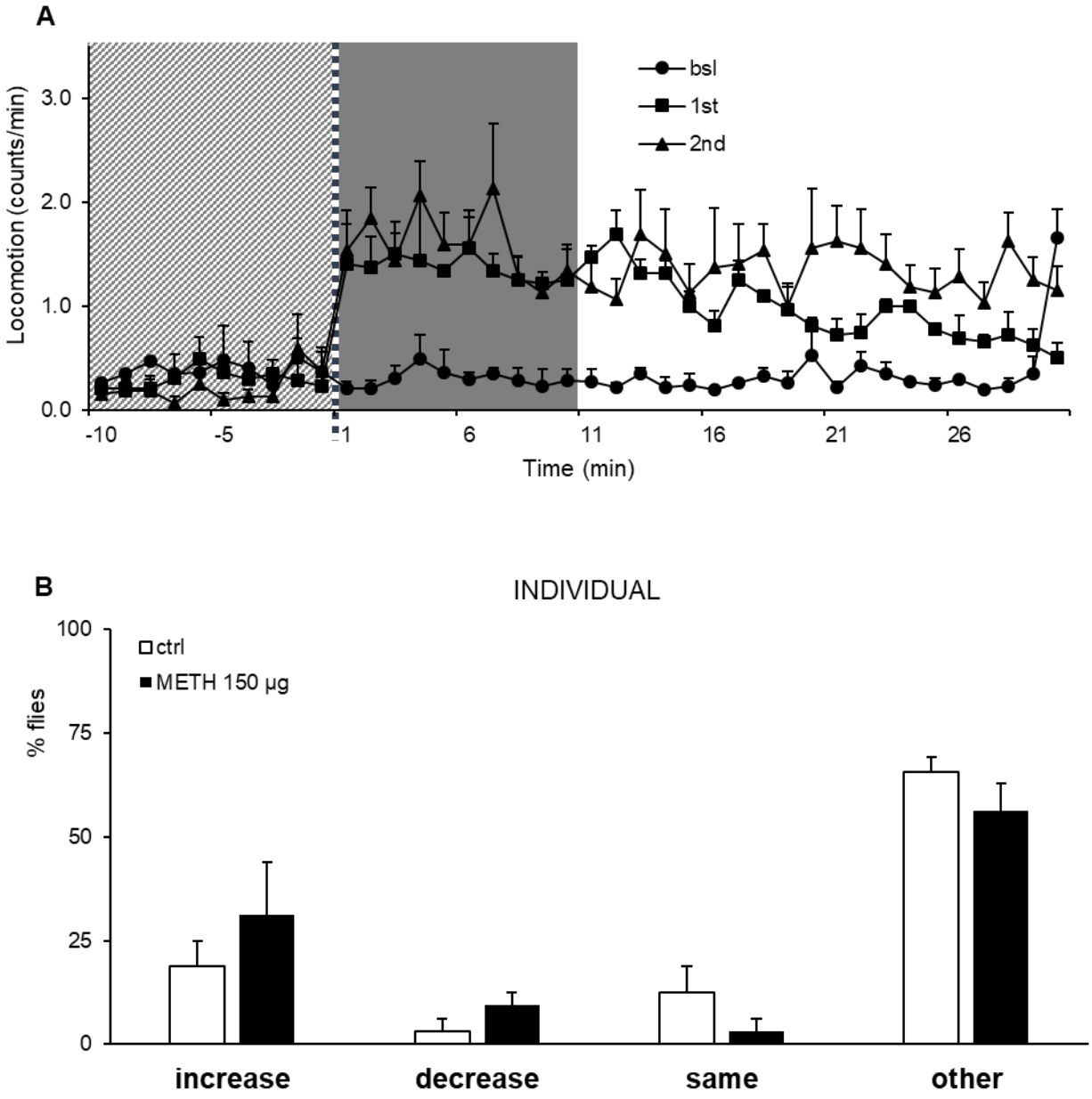


Figure 6. Our standard protocol for METH administration does not induce locomotor sensitization in *wt* female flies. A) Kinetic graph of locomotion expressed as number of counts per minute for group exposed to twice to volatilized 150 µg METH (n=32), once at 09:00 (1st) and then at 19:00 (2nd), along with their baseline (bsl) activity before drug administration. The shaded dark gray panel indicates the 10 minutes immediately prior to exposure, the dotted line is the time of exposure and the dark gray panel indicates 10 minutes after exposure. Locomotor activity after 2nd exposure is similar to 1st exposure levels. **B)** Analysis of individual flies from data in A). Amount of individual fly locomotor activity 10 min before exposure was compared to 10 minute after the first and second exposures to 150 µg of volatilized METH (n=32). Data are plotted as histograms showing the mean value of five tests ± SEM. These were categorized as an increase, decrease, no change or other (flies that did not satisfy criteria for previous three groups), when compared the effect on the control group (n=32) that received warm airflow only. Percentage of flies in each category is similar between group that received 150 µg of

METH and warm air control and statistically non-significant difference was determined by Mann-Whitney U-test for nonparametric analysis of two independent samples.

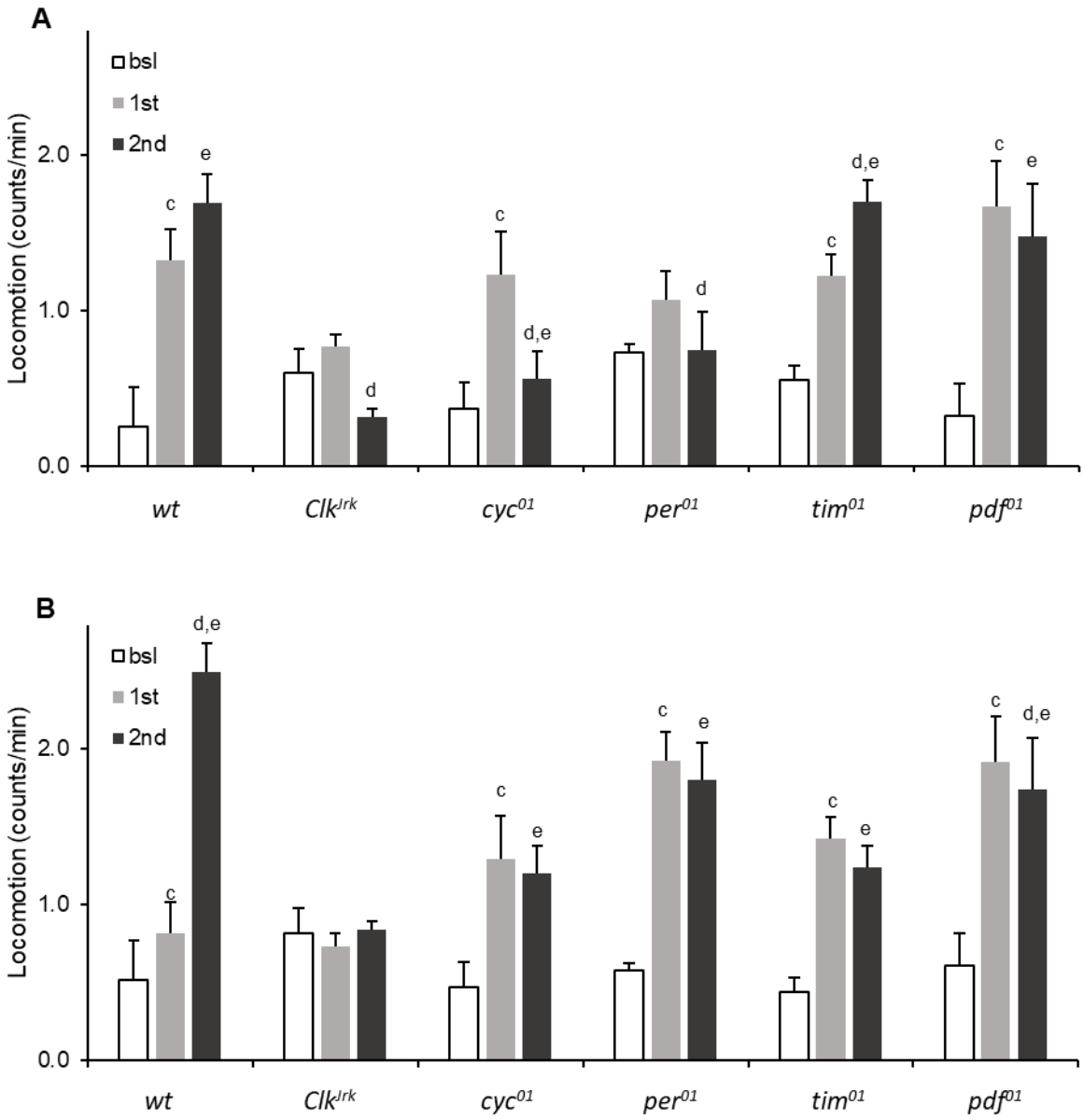


Figure 7. Population data on circadian mutants after PS exposures. Fly populations were either wild type (*wt*) or mutants for circadian genes *Clk*^{Jrk}, *per*^{01}, *cyc*^{01}, *tim*^{01} and *pdf*^{01} (n=32 for each group). Statistical significance (p≤0.017) indicated by: c: comparison of baseline activity to after first administration; d: comparison of activity after first and second exposures; e: comparison of baseline activity to after second administration. All tests were within the group, using ANOVA for repeated measurements with Bonferroni post-hoc test. **A)** Population data for vCOC exposure. Average locomotor activity (counts/min) during baseline, 5 minutes before exposures (bsl), 5 minutes after first exposure (1st) and 5 minutes after second (2nd) exposure to vCOC. B) **)** Population data for vMETH exposure. Average locomotor activity (counts/min) during baseline, 10 minutes before exposures (bsl), 10 minutes after first exposure (1st) and 10 minutes after second (2nd) exposure to vMETH.

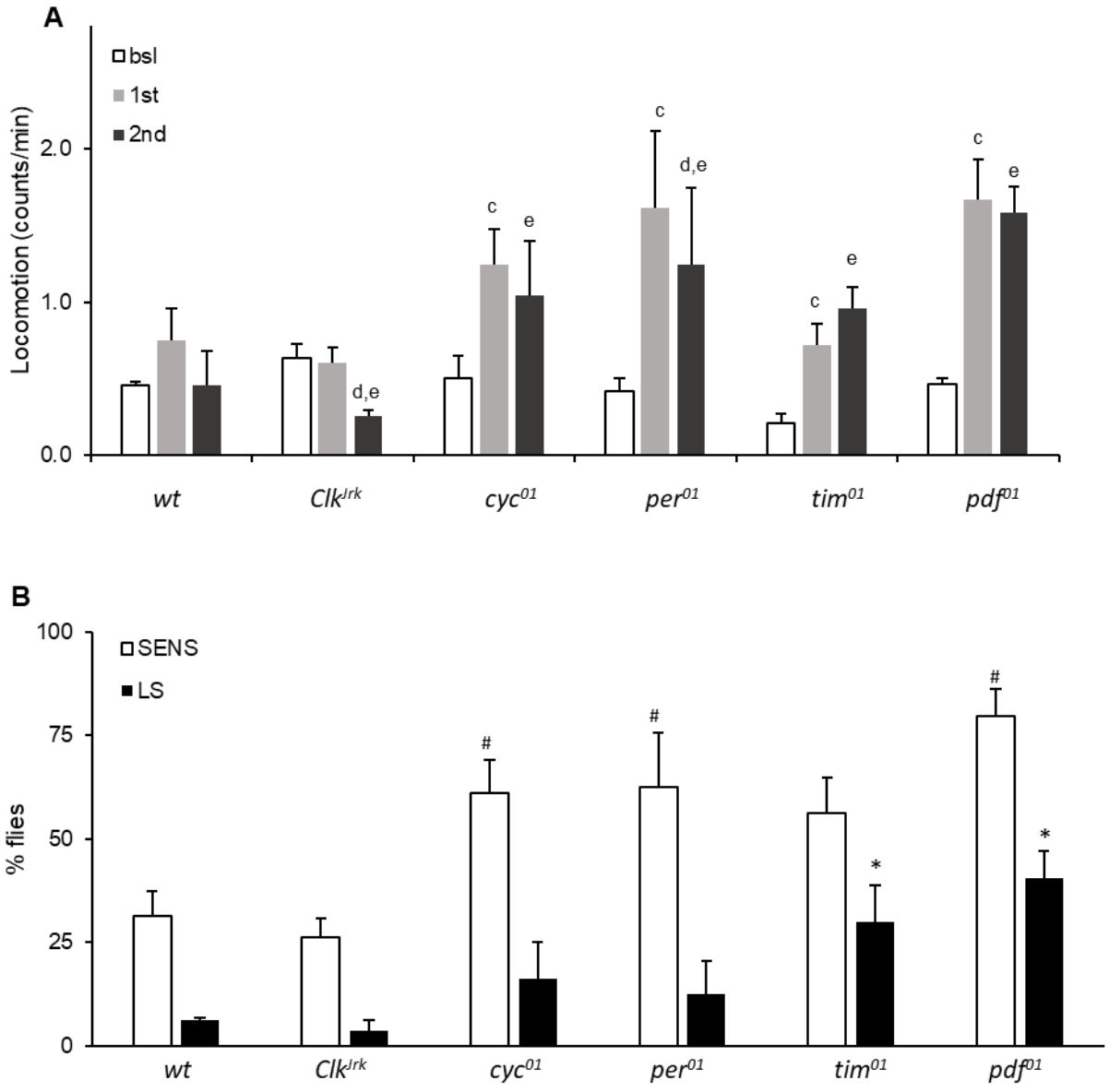


Figure 8. Influence of warm airflow on sensitivity and locomotor sensitization of circadian mutants controls for COC. A) Average locomotor activity (counts/min) during baseline, 5 minutes before exposures (bsl), 5 minutes after first exposure (1st) and 5 minutes after second (2nd) exposure to warm air flow (2.5 L/min, for 1 minute after 8 minutes of heating), given 6 hours apart. Fly populations were either wild type (*wt*) or mutants for circadian genes Clk^{Jrk}, per^{01}, cyc^{01}, tim^{01} and pdf^{01} (n=32 for each group). Statistical significance (p≤0.017) indicated by: c: comparison of baseline activity to after first administration; d: comparison of activity after first and second exposures; e: comparison of baseline activity to after second administration. All tests were within the group, using ANOVA for repeated measurements with Bonferroni post-hoc test. **B)** Percentage of individual flies showing sensitivity or increased locomotor activity to a first exposure to warm airflow (2.5 L/min, for 1 minute after 8 minutes of heating) and flies showing further increase in locomotor activity to a second exposure (LS). Kruskal-Wallis H-test for nonparametric analysis of independent multiple samples showed statistical significance (p≤0.05) for comparison of *wt* to mutants in SENS (#) and (*) LS with Dunn's post-hoc test.

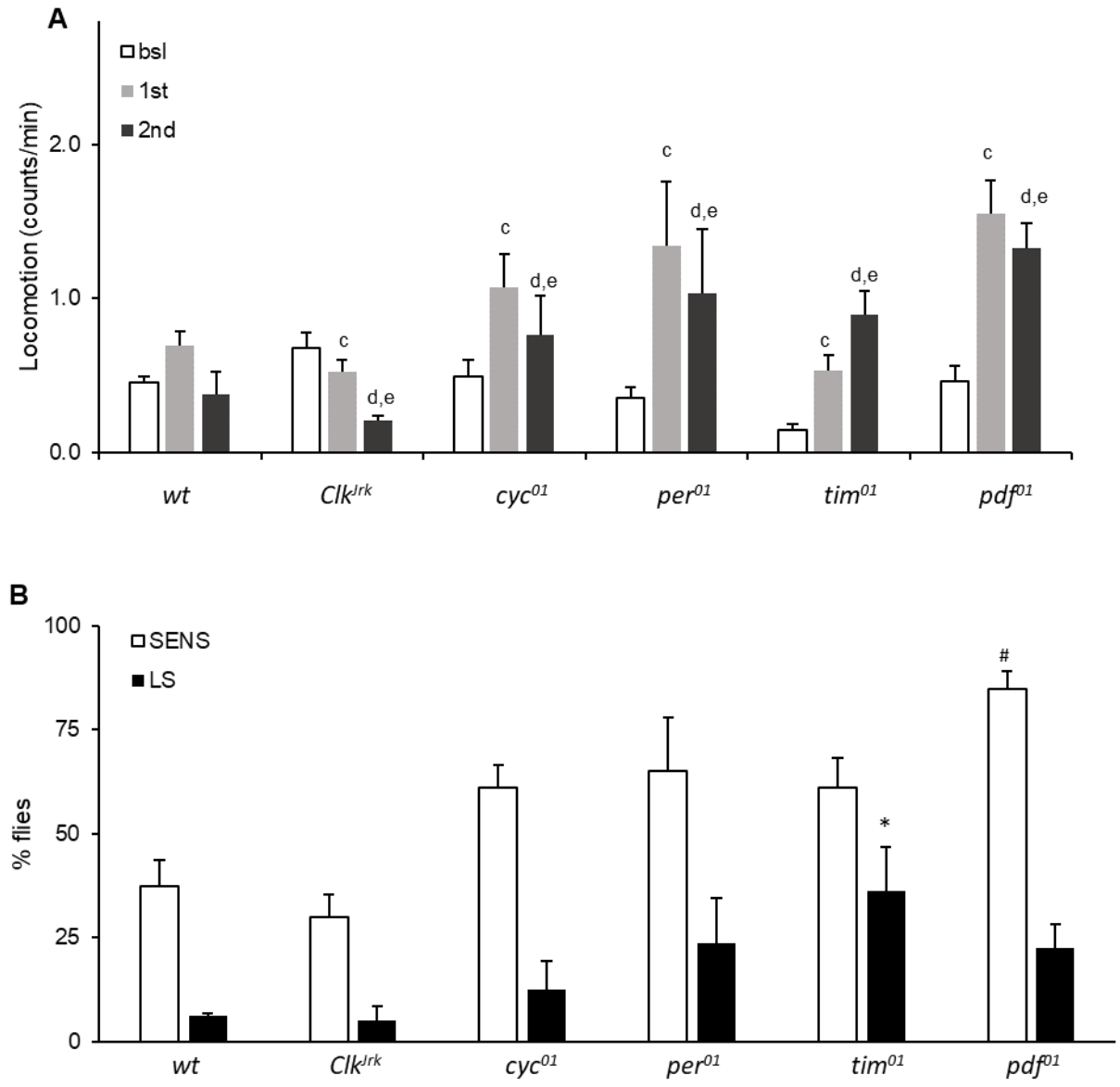


Figure 9. Influence of warm airflow on sensitivity and locomotor sensitization of circadian mutants controls for METH. A) Average locomotor activity (counts/min) during baseline, 10 minutes before exposures (bsl), 10 minutes after first exposure (1st) and 10 minutes after second (2nd) exposure to warm airflow (2,5 L/min, for 1 minute after 8 minutes of heating), given 10 hours apart. Fly populations were either wild type (*wt*) or mutants for circadian genes *Clk*^{Jrk}, *per*^{01}, *cyc*^{01}, *tim*^{01} and *pdf*^{01} (n=32 for each group). Statistical significance (p≤0.017) indicated by: c: comparison of baseline activity to after first administration; d: comparison of activity after first and second exposures; e: comparison of baseline activity to after second administration. All tests were within the group, using ANOVA for repeated measurements with Bonferroni post-hoc test. **B)** Percentage of individual flies showing sensitivity or increased locomotor activity to a first exposure to warm air flow (2.5 L/min, for 1 minute after 8 minutes of heating) and flies showing further increase in locomotor activity to a second exposure (LS). Kruskal-Wallis H-test for nonparametric analysis of independent multiple samples showed statistical significance (p≤0.05) for comparison of *wt* to mutants in SENS (#) and (*) LS with Dunn's post-hoc test.

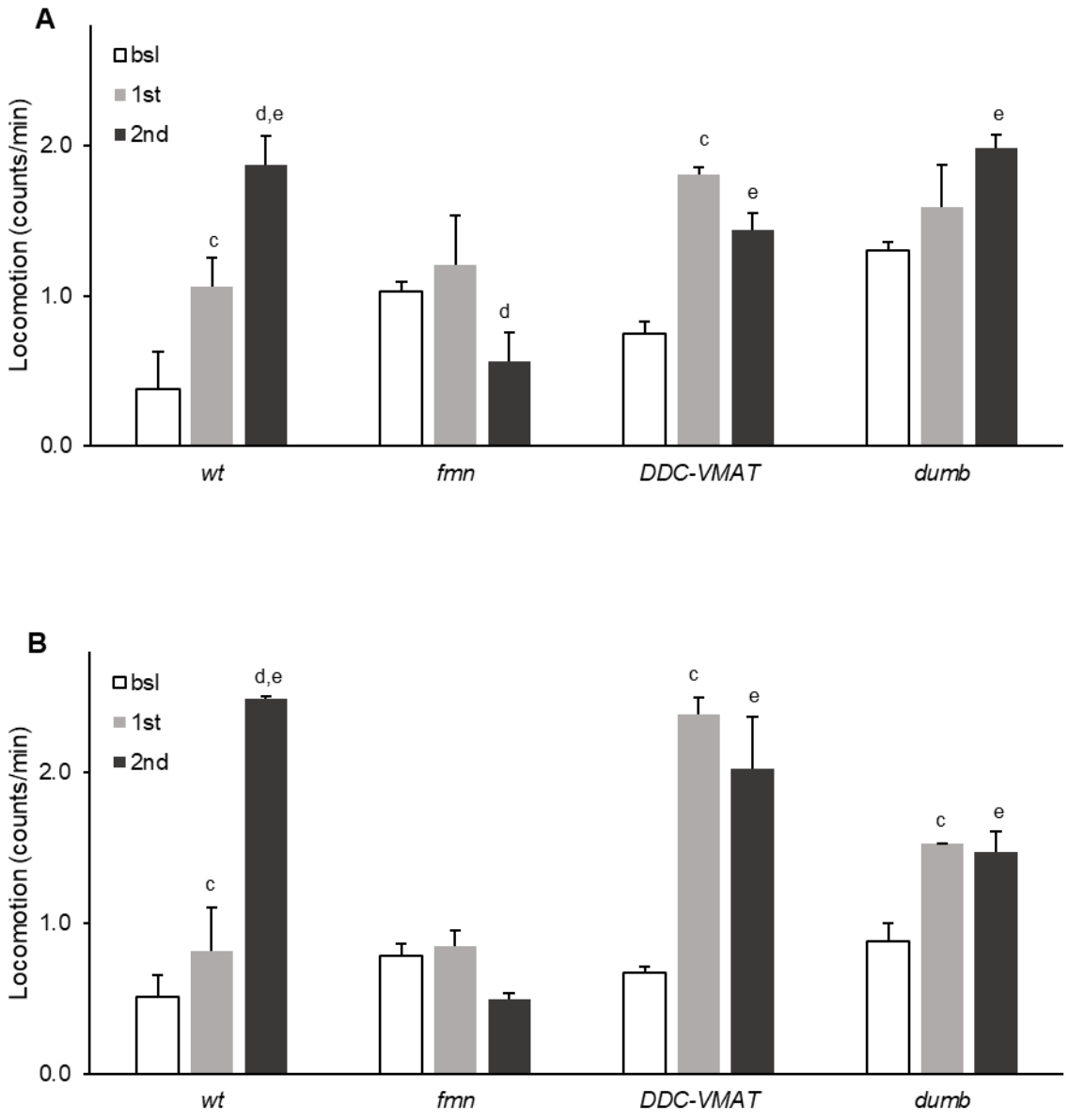


Figure 10. Population data on dopamine transporters and receptor mutants influence on locomotion before and after PS exposures in male flies. Fly populations were either wild type (*wt*) or mutants in dopamine transporter (*fmn*), dopamine receptor (*dumb*) and transgene flies in vesicular monoamine transporter (DDC-VMAT) (n=32 for each group). Statistical significance ($p \leq 0.017$) indicated by: c: comparison of baseline activity to after first administration; d: comparison of activity after first and second exposures; e: comparison of baseline activity to after second administration. All tests were within the group, using ANOVA for repeated measurements with Bonferroni post-hoc test. **A)** Population data for vCOC exposure. Average locomotor activity (counts/min) during baseline, 5 minutes before exposures (bsl), 5 minutes after first exposure (1^{st}) and 5 minutes after second (2^{nd}) exposure to vCOC. **B)** Population data for vMETH exposure. Average locomotor activity (counts/min) during baseline, 10 minutes before exposures (bsl), 10 minutes after first exposure (1^{st}) and 10 minutes after second (2^{nd}) exposure to vMETH.

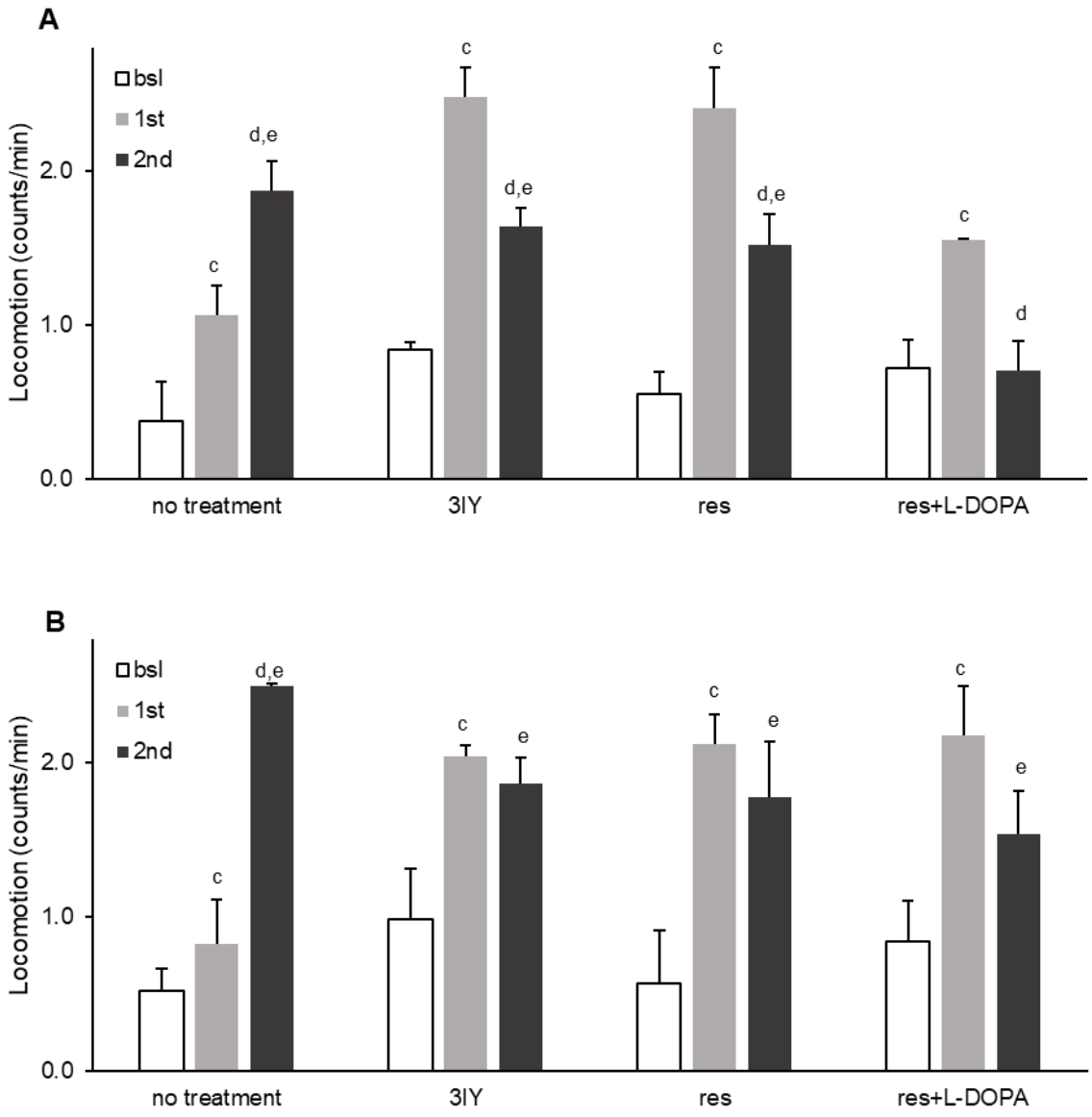


Figure 11. Population data on pharmacological reduction of monoamines influence on locomotion before and after PS exposures in male *wt* flies. Fly populations were either wild type (*wt*) or *wt* pre-treated with 3-iodo tyrosine (3IY), reserpine (res) and reserpine and L-DOPA (res+L-DOPA) (n=32 for each group). Statistical significance ($p \leq 0.017$) indicated by: c: comparison of baseline activity to after first administration; d: comparison of activity after first and second exposures; e: comparison of baseline activity to after second administration. All tests were within the group, using one-way ANOVA with Dunnett post-hoc test. **A)** Population data for vCOC exposure. Average locomotor activity (counts/min) during baseline, 5 minutes before exposures (bsl), 5 minutes after first exposure (1^{st}) and 5 minutes after second (2^{nd}) exposure to vCOC. **B)** Population data for vMETH exposure. Average locomotor activity (counts/min) during baseline, 10 minutes before exposures (bsl), 10 minutes after first exposure (1^{st}) and 10 minutes after second (2^{nd}) exposure to vMETH.

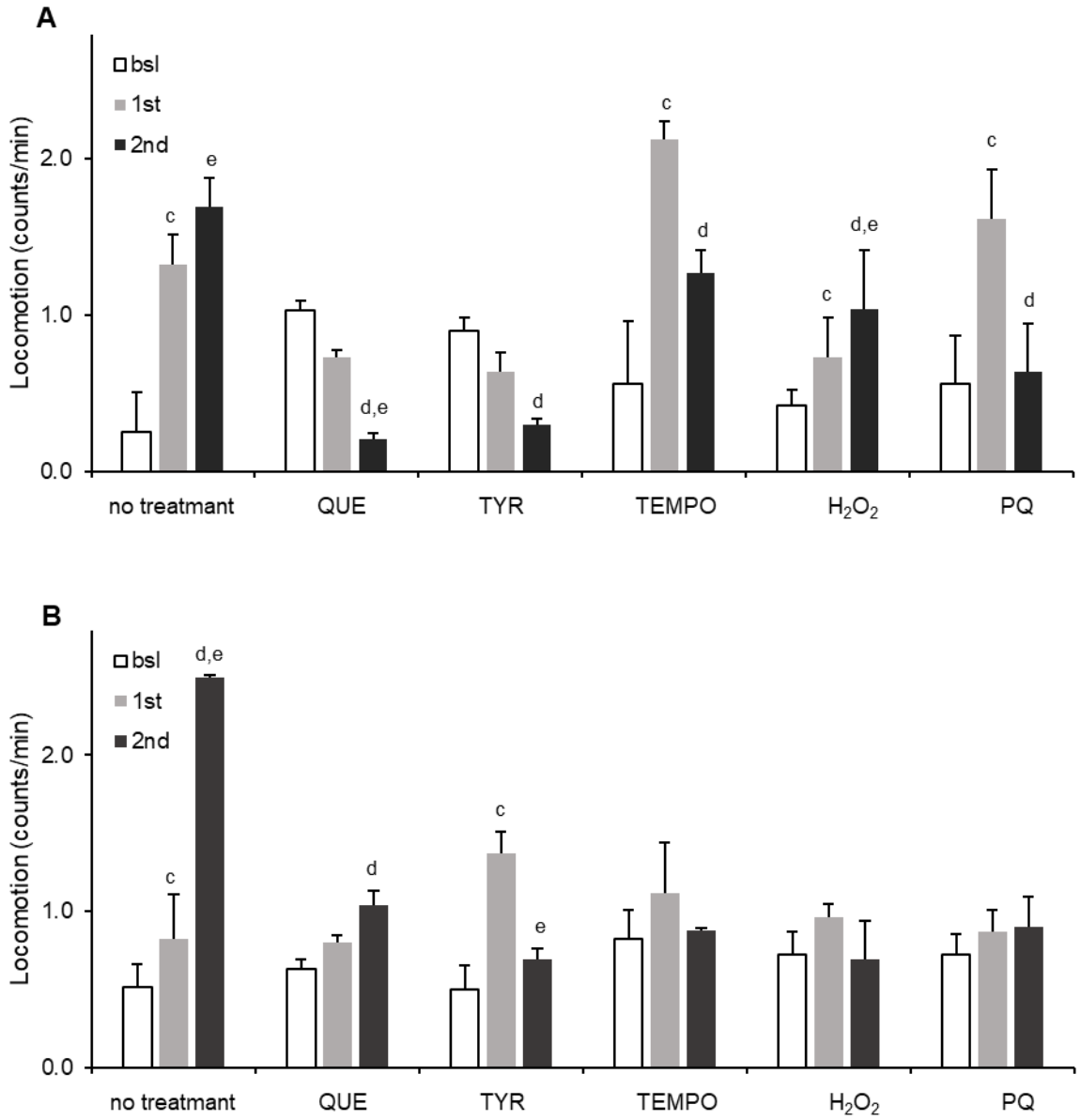


Figure 12. Population data on pro- and antioxidant influence on locomotion before and after PS exposures in male *wt* flies. Fly populations were either wild type (*wt*) or *wt* pre-treated with anti-oxidants quercetin (QUE), tyrosol (TYR), TEMPOL and prooxidants hydrogen peroxide (H_2O_2) and paraquat (PQ) (n=32 for each group). Statistical significance ($p \leq 0.017$) indicated by: c: comparison of baseline activity to after first administration; d: comparison of activity after first and second exposures; e: comparison of baseline activity to after second administration. All tests were within the group, using ANOVA for repeated measurements with Bonferroni post-hoc test. **A)** Population data for vCOC exposure. Average locomotor activity (counts/min) during baseline, 5 minutes before exposures (bsl), 5 minutes after first exposure (1^{st}) and 5 minutes after second (2^{nd}) exposure to vCOC. **B)** Population data for vMETH exposure. Average locomotor activity (counts/min) during baseline, 10 minutes before exposures (bsl), 10 minutes after first exposure (1^{st}) and 10 minutes after second (2^{nd}) exposure to vMETH.

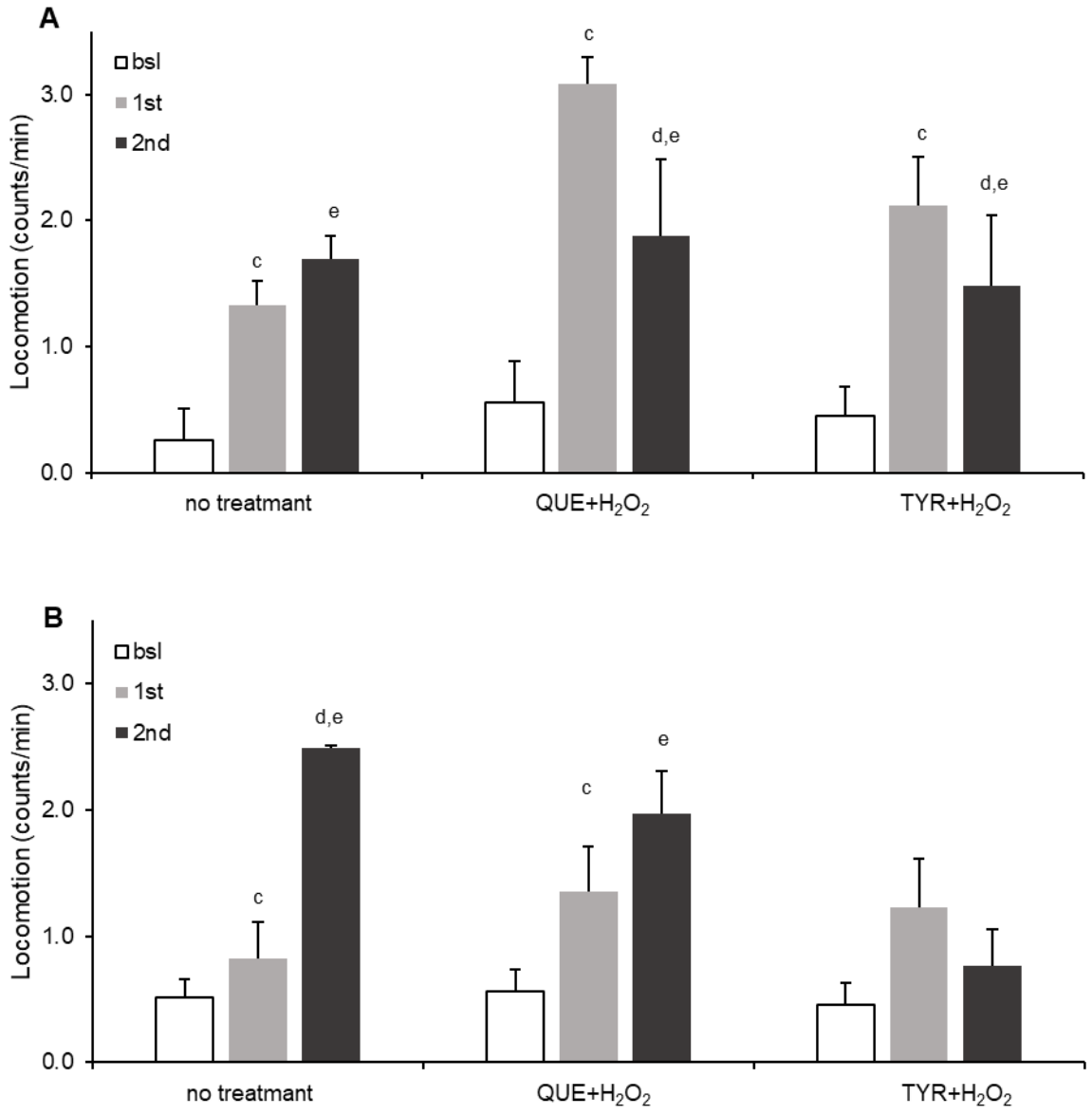


Figure 13. Population data on pro- and antioxidant combination influence on locomotion before and after PS exposures in male *wt* flies. Fly populations were either wild type (*wt*) or *wt* pre-treated with antioxidant quercetin (QUE) and prooxidant hydrogen peroxide (H_2O_2) (QUE+ H_2O_2) and antioxidant tyrosol (TYR) and prooxidant hydrogen peroxide (H_2O_2) (TYR+ H_2O_2) (n=32 for each group). Statistical significance ($p \leq 0.017$) indicated by: c: comparison of baseline activity to after first administration; d: comparison of activity after first and second exposures; e: comparison of baseline activity to after second administration. All tests were within the group, using ANOVA for repeated measurements with Bonferroni post-hoc test. **A)** Population data for vCOC exposure. Average locomotor activity (counts/min) during baseline, 5 minutes before exposures (bsl), 5 minutes after first exposure (1^{st}) and 5 minutes after second (2^{nd}) exposure to vCOC. **B)** Population data for vMETH exposure. Average locomotor activity (counts/min) during baseline, 10 minutes before exposures (bsl), 10 minutes after first exposure (1^{st}) and 10 minutes after second (2^{nd}) exposure to vMETH.

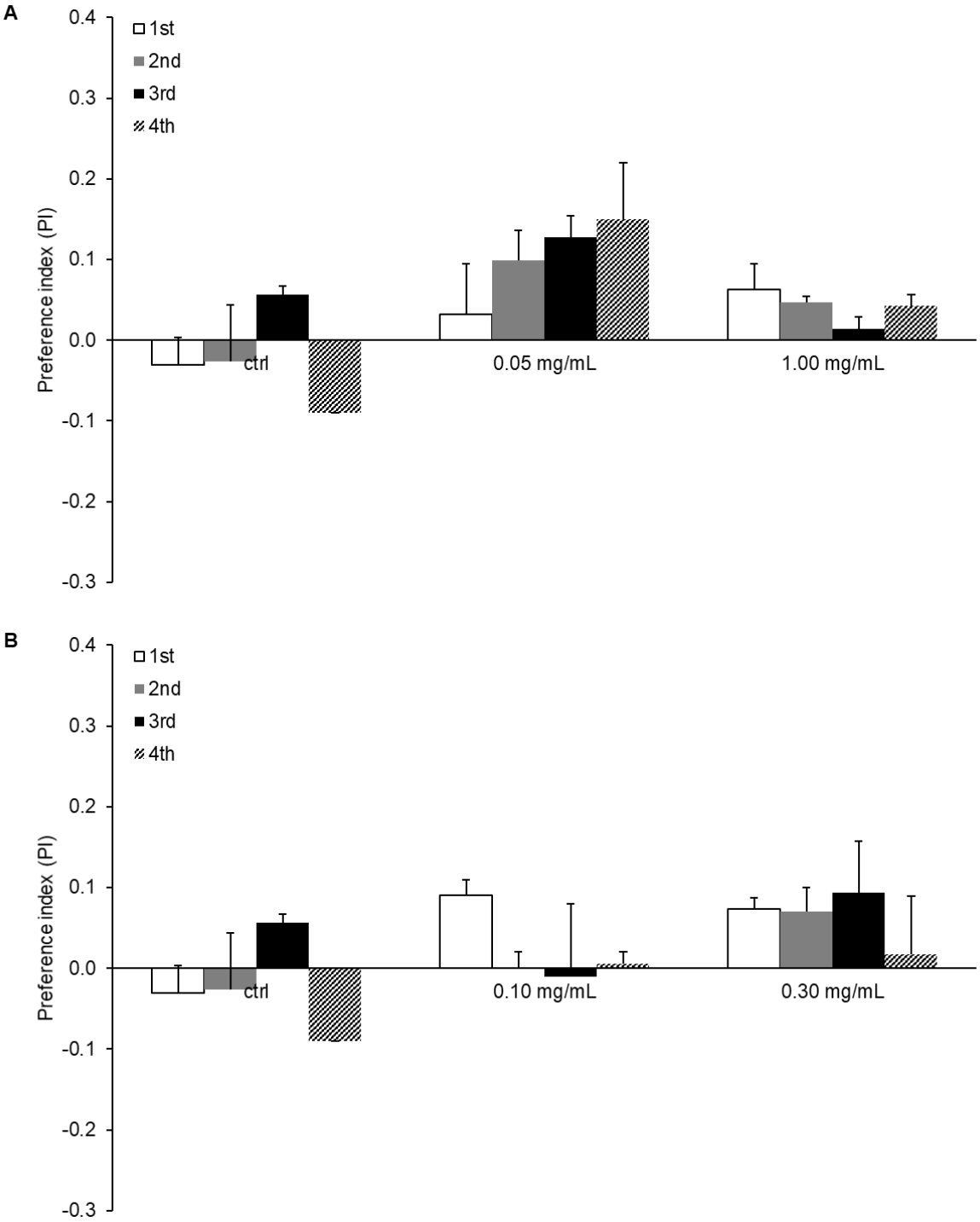


Figure 14. Preferential consumption over consecutive days depends on drug concentration. In the experiment, male flies aged 3-5 days were fed in the control group with aqueous solution of sucrose in all four capillaries, and in the test groups two capillaries contained a sucrose solution and in the other two aqueous solutions of drug. The volume of consumed food in each capillary was measured every 24 hours and was converted to the preference index. The experiment was repeated 2 times with 2 tubes each containing 6 flies (n=24). One way ANOVA followed by the Tukey's multiple comparison showed no differences within groups. **A)** Control group was exposed to only sucrose solutions during all 4 days, while test groups were exposed to COC solution and sucrose solution in concentrations 0.05 and 1.00

mg/mL. **B)** Control group was exposed to only sucrose solutions during all 4 days, while test groups were exposed to 0.10 and 0.30 mg/mL METH solution and sucrose solution.

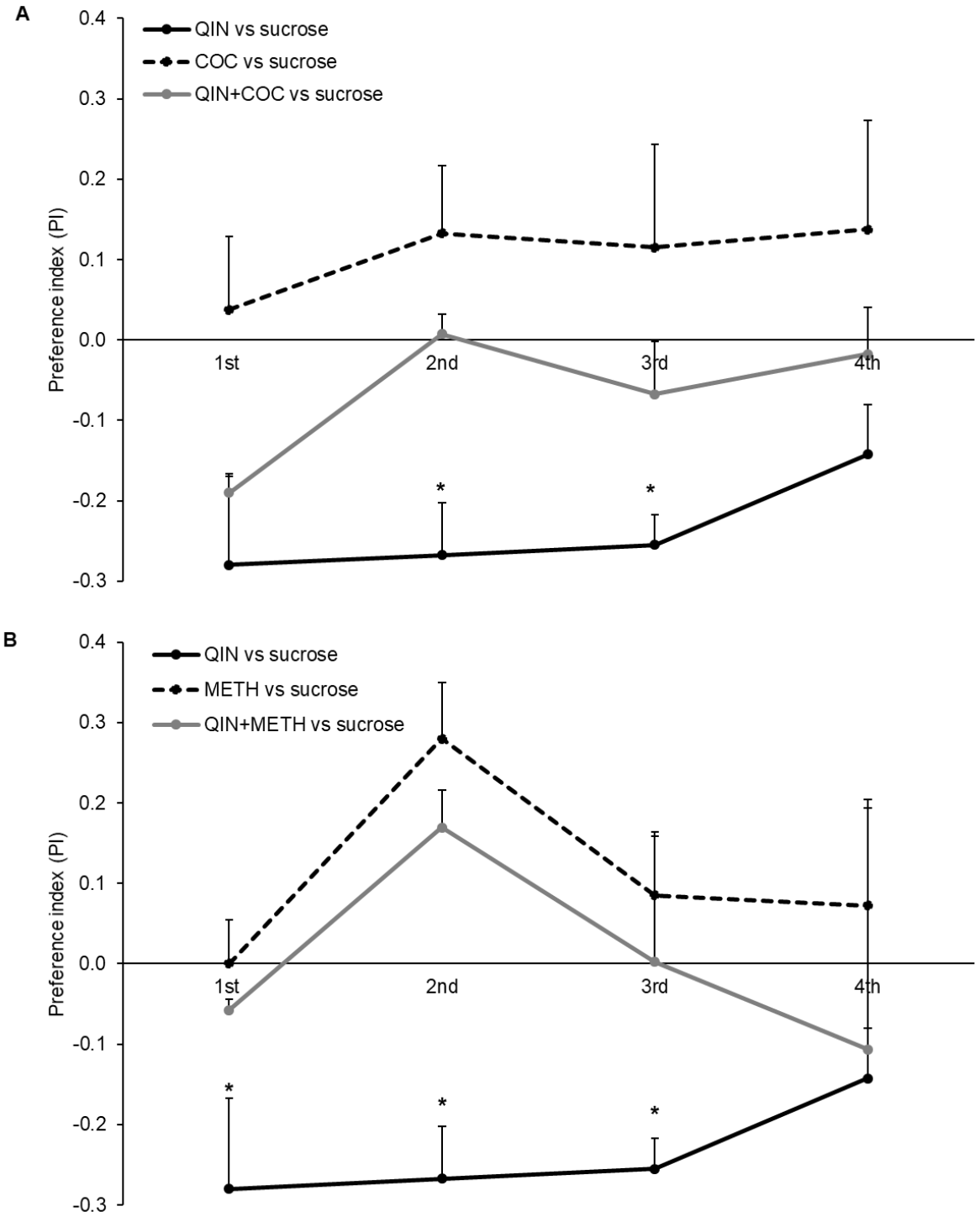


Figure 15. Flies self-administered COC and METH despite the bitter taste of quinine. Flies were divided into three groups: 300 μM QIN solution versus sucrose solution, drug solution (COC 0.15 mg/mL and METH 0.20 mg/mL) versus sucrose solution and 300 μM QIN and drug (COC 0.15 mg/mL and METH 0.20 mg/mL) solution versus sucrose solution. After each of 4 days, the amount of consumed

solutions was measured and PI was calculated for each of the solutions versus sucrose solution. Data are presented as mean PI ± SEM over 4 days in row. The experiment was repeated 2 times with 3 tubes each containing 6 flies (n=36). One way ANOVA followed by the Tukey's multiple comparison was used to determine differences between groups (*p<0,05).

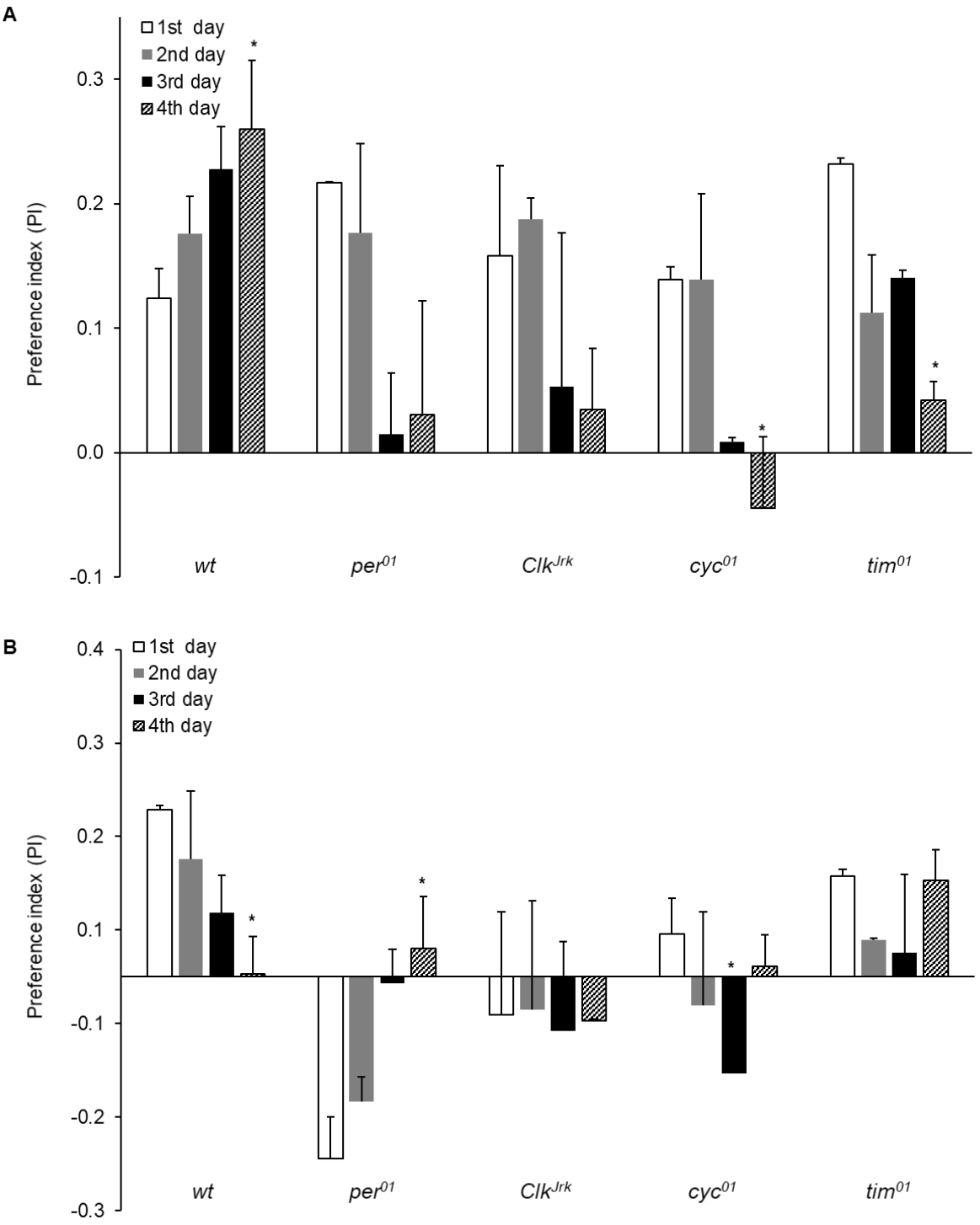


Figure 16. PI values for circadian mutants over four days in row for COC and METH. The flies were divided into groups according to the genotype: *wt*- wild type, *per*^{01}- mutant in period gene, *Clk*^{Jrk}

mutants for Clock gen, *cyc*^{01} mutants for cycle gen and *tim*^{01} mutants for timeless gen. During the four days flies had a choice between the dug solutions (COC 0.15 mg/mL and METH 0.20 mg/mL) and the sucrose solutions. Every 24 hours, the amount of solutions consumption was measured and converted to the preference index. The graph shows the average index of preferences during 4 administrations for each genotype. The experiment was repeated 2 times with 3 tubes each containing 6 flies (n=36). One-way ANOVA and Tukey's multiple comparison (*p<0.05) were used to determine the difference between the groups. **A)** Preferential consumption for COC represent as mean PI ±SEM for four days in row for all five genotypes. **B)** Preferential consumption for METH represent as mean PI ± SEM for four days in row for all five genotypes.

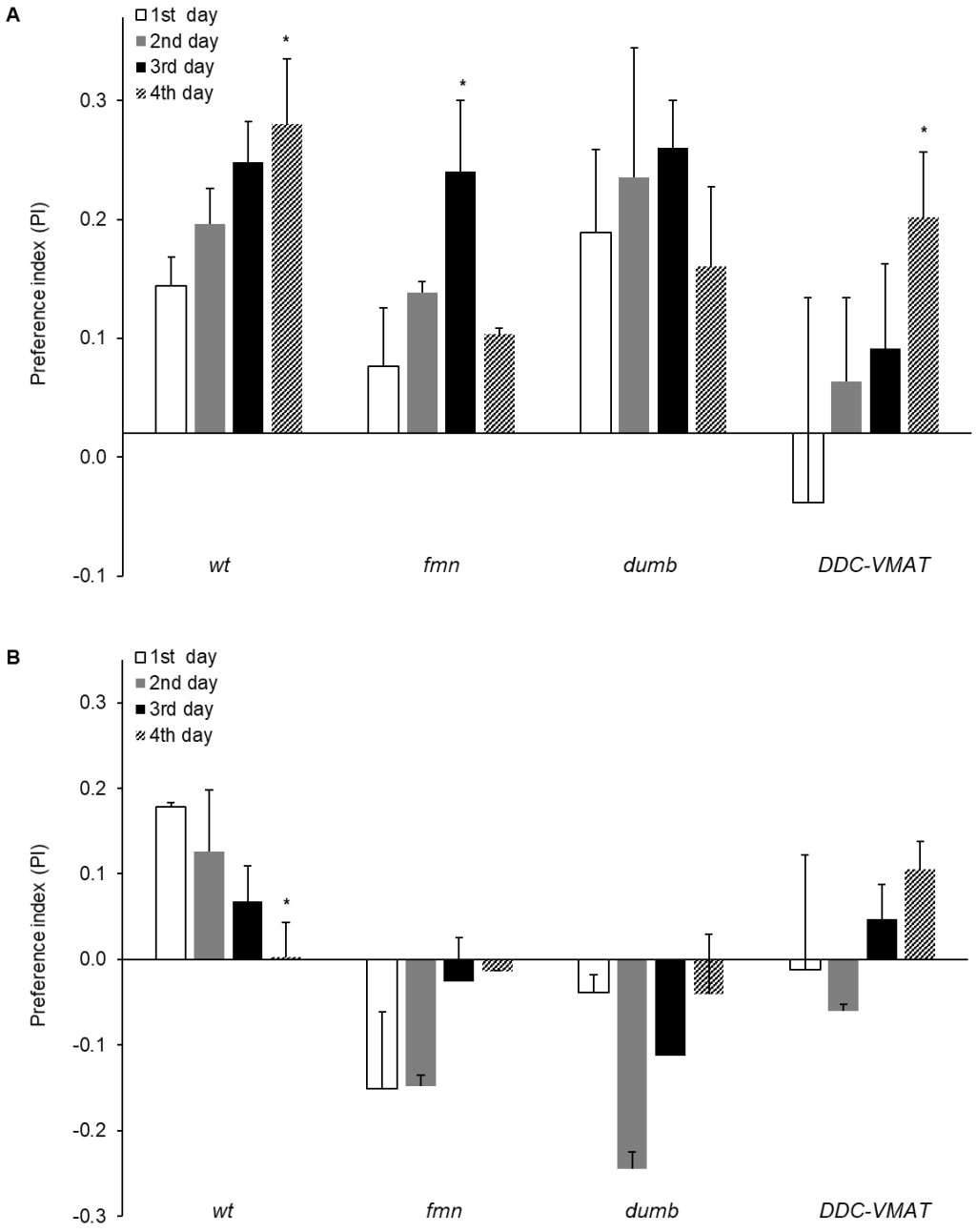


Figure 17. PI values for *fmn* and *dumb* mutants, and transgene flies DDC-VMAT over 4 days for COC and METH. The flies were divided into groups according to the genotype: *wt*-wild type, *fmn*-fumin, dopamine transporter mutant, *dumb* mutant in dopamine-like receptor 1 and DDC-VMAT flies caring RNAi in dopaminergic and serotonergic neurons. During the four days, flies had a choice between the COC 0.15 mg/mL or METH 0.20 mg/mL and the pure solution of sucrose. Every 24 hours, the amount of both liquids was measured and converted to the preference index. The experiment was repeated 2 times with 2 tubes each containing 6 flies (n=24). One-way ANOVA and Tukey's multiple comparison (*p<0.05) were used to determine the differences between the groups. **A)** Preferential consumption for COC represent as mean PI ± SEM for four days in row for all four genotypes. **B)** Preferential consumption for METH represent as mean PI ± SEM for four days in row for all four genotypes.

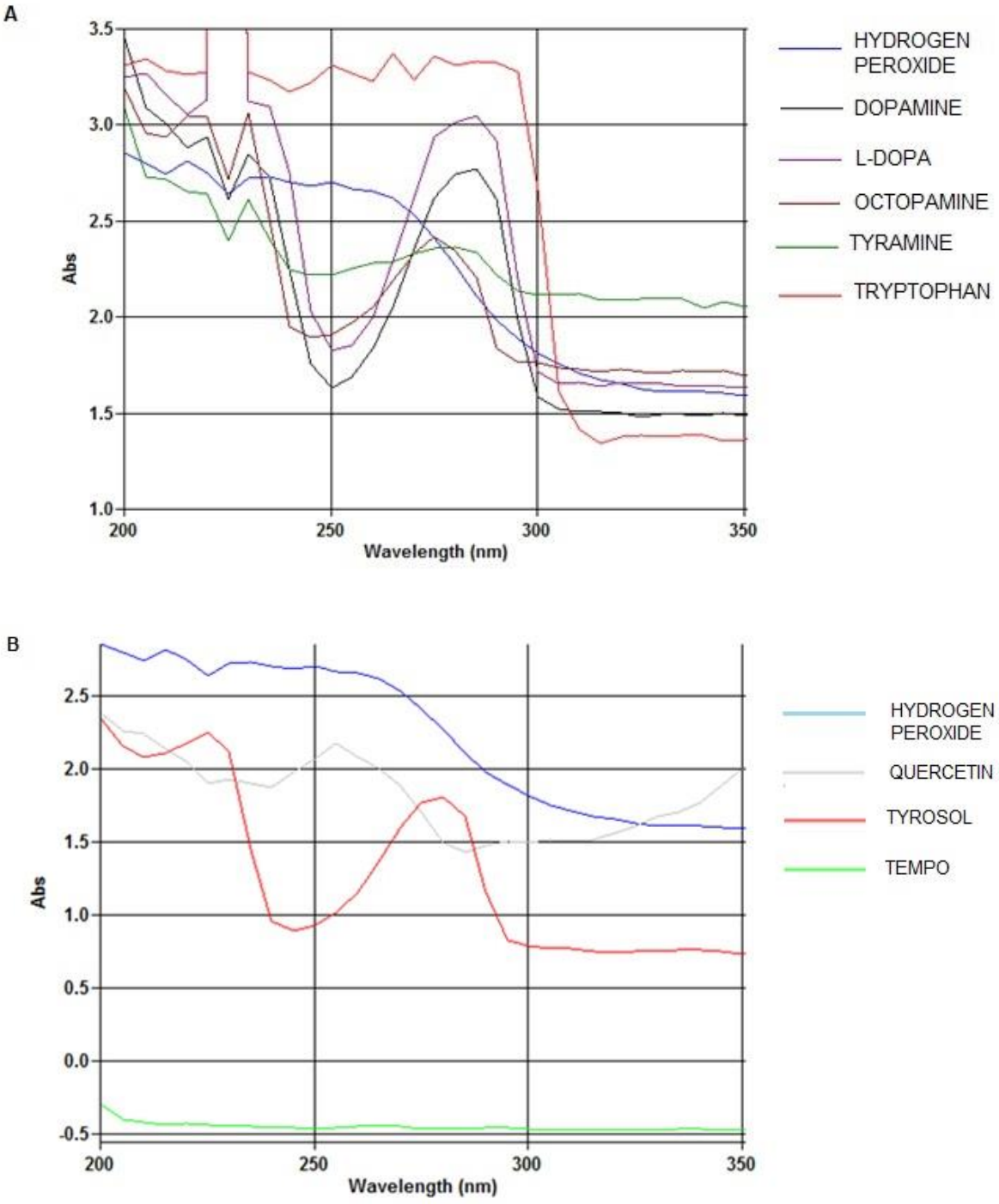


Figure 18. UV-VIS spectra of A) hydrogen peroxide, DA, L-DOPA, octopamine, TYRA, tryptophan and B) hydrogen peroxide, QUE, TYR , TEMPOL dissolved in methanol using quartz cuvettes in wavelength range 350-200 nm. From graph, it can be seen that all tested molecules have same absorption peak around 230 nm, which is also maximum of hydrogen peroxide absorbance in UV-VIS spectra. Based on overlapping of tested molecules and hydrogen peroxide it is not possible to follow hydrogen peroxide decomposition in presence of this molecules by using UV-VIS.

12. BIOGRAPHY

PERSONAL INFORMATION

Ana Filošević

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Sex: female | Date of birth: 30/06/1987 | Nationality: Croat

WORK EXPERIENCE

31.01.2017. -	**Assistant, Scientific Area: Natural Sciences, Field: Chemistry - behavioral neuroscience** University of Rijeka, Department of Biotechnology, Radmile Matejčić 2, 51 000 Rijeka, Croatia Laboratory for behavioral genetics
01. 01. 2015. -31. 12. 2016.	**Expert Associate in the natural sciences, field of chemistry - behavioral neuroscience** University of Rijeka, Department of Biotechnology, Radmile Matejčić 2, 51 000 Rijeka, Croatia Laboratory for behavioral genetics
01. 02. 2014. - 31. 12. 2014.	**Expert Associate for the preparation and implementation of EU projects** University of Rijeka, Department of Biotechnology, Radmile Matejčić 2, 51 000 Rijeka, Croatia "Ruđer Bošković" Institute, Bijenička cesta 54, 10 000 Zagreb, Croatia Thalassotherapia Opatija - Clinic for treatment, rehabilitation and prevention of heart and blood vessel diseases, Ul. Maršala Tita 188, 51410 Opatija, Croatia
01. 02. 2014. – 31. 12. 2014.	**Expert Associate in the natural sciences, field of chemistry** University of Rijeka, Department of Biotechnology, Radmile Matejčić 2, 51 000 Rijeka, Croatia Panaceo International Active Mineral Production GmbH, Finkensteiner Str. 5, 9585 Villach-Gödersdorf, Austria
25. 11. 2013. – 31. 12. 2014.	**Teaching Assistant** University of Rijeka, Department of Biotechnology, Radmile Matejčić 2, 51 000 Rijeka, Croatia
01. 02. 2013. –31. 01. 2014.	**Laboratory manager** University of Rijeka, Department of Biotechnology, Radmile Matejčić 2, 51 000 Rijeka, Croatia

EDUACATION

16. 09. 2013. –

Postgraduate studies (Ph.D.)
Course: „Medicinal Chemistry“
Orientation: biotechnology in biomedicine
Department of Biotechnology, University of Rijeka, Radmile Matejčić 2, 51 000 Rijeka, Croatia

06. 07. 2012.

Master of Chemistry (M.Sc.)
Course: research
Orientation: inorganic and structural chemistry and biochemistry
Faculty of Science, University of Zagreb, Department of Chemistry, Horvatovac 102a, 10 000 Zagreb, Croatia

24. 09. 2010.

Bachelor of Chemistry (B.Sc.)

Faculty of Science, University of Zagreb, Department of Chemistry, Horvatovac 102a, 10 000 Zagreb, Croatia

ADDITIONAL TRAINING

Scientific training

- 11.-20.11.2016. training in the field of rhythmicity and chronobiology , **Prof. Charlotte Förster, Ph.D.**, Lehrstuhl für Neurobiologie und Genetik, Universitaet Wuerzburg, Biozentrum Am Hubland, Wuerzbur, Germany
- 22.07.2016. sample preparation and *in vivo* imaging **Prof. Gero Miesenböck, Ph.D.**, Centre for Neural Circuits and Behaviour, University of Oxford, Oxford, UK
- 07.-13.06. 2016. video monitoring and quantification of *Drosophila* behavior using the echoscope method, **Prof. Giorgio F. Gilestro, Ph.D.**, Imperial College London, Faculty of Natural Sciences, Department of Life Sciences, London, UK
- 01.10.-01.11.2014. work on essays for the quantification of learning and memory (reward and punishment), the basis of work with flies, genetic tools and manipulations, **Prof. Scott Waddell, Ph.D.**,"British Scholarship Trust" scholarship for scientific research at UK universities, Center for Neural Circuits and Behaviour, University of Oxford, Oxford, UK
- 21.-25.07.2014. and 22.-26.09.2014. training in the field of colloidal and interfacial chemistry, **Prof. Tajana Peročanin**, **Ph.D**. and **academician Prof. Nikola Kallay, Ph.D.**, at Department for physical chemistry, Faculty of science, University of Zagreb, Department of chemistry, Horvatovac 102a, 10 000 Zagreb, Croatia
- 15.-16.05.2014. and 17.-19.09.2014. surface characterization using XPS **Prof. Mladen Petravić Ph.D**, Centre for Micro and Nano Science and Technology, University of Rijeka, Rijeka, Croatia

Summer schools

- 24.–29.07.2016., Oxford Sleep and Circadian Neuroscience Summer Schools, Sleep & Circadian Neuroscience Institute, University of Oxford, Oxford, UK
- 29. 05. – 4. 06. 2016., FENS-SfN Summer School, “Cellular Mechanisms and Networks in Addiction”, Bertinoro, Italy

- 26. 06. - 16. 07.2015., DROSOPHILA NEUROBIOLOGY: GENES, CIRCUITS & BEHAVIOR, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, SAD

- 01.-12. 09. 2014., 2014. Zürich Summer School on Biomedical Imaging, 8th Zürich Interdisciplinary Summer School on Biomedical Imaging, ETH Zürich, Switzerland

- 12.-13. 06. 2014., 15th International Chromatography School, Zagreb, Croatia

Training in the field of science popularization and education

- 03.-05.03.2017. Višnjan Scientific and Educational center/Astronomical Society Višnjan, education for new mentors in national and EU context, principles, organization and preparation of educational project, **Korado Korlević,** Višnjan, Croatia

- 12.07.2016. and 14.07.2016. Improvement of evaluation procedures at the University of Rijeka, UNAPRORI, Training for teacher competence improvement, Prof. Svjetlana Kolić-Vehovec,Ph.D., Ass.Prof. Sanja Smojver-Ažić , Ph.D.

- 14.-15.03.2016. The training program in science communication, working with the media, presentations live and work in front of the cameras, organized by British Council Croatia as FameLab finalist in Croatia, mentors **Karl Byrne** and **Krešimir Macan,** Terme Jezerčica, Donja Stubica, Croatia

Advanced education and training in the field of economics and management

- 06.-08.03.2017. Career Booster Rijeka, organizations Start-Up, Faculty of Economic, University of Rijeka, Rijeka, Croatia

- 13.09.2013. Mapa znanja, Tendering and EU funds for bidders and contracting authorities – Methodology of investment projects financed by EU funds in connection with public procurement, lector**: M.Sc. Zdravko Pandžić**, Zagreb, Croatia

Other training

- ECDL-European Computer Driving Licence, University of Zagreb, University Computing Center (SRCE) 08. 12. 2012.

PERSONALS SKILLS

Mother tongue: Croatian

Other languages	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	A2	B2	A2	B2	A2
German	A2	B1	A2	A2	B1

Levels: A1/2: Basic user - B1/2: Independent user - C1/2 Proficient user
Common European Framework of Reference for Languages

Communication skills

- good communication and organization skills developed at the position of laboratory and project manager

Organizational / managerial

- experience in managing projects and teams

skills

- accurate in respect of deadlines
- analytical in the organization of tasks

Job-related skills

- chemistry and biochemistry related techniques: FT-IR, TGA, DSC, UV-VIS, X-ray diffraction on single crystal and polycrystalline, cell cultures, isolation of DNA and RNA, PCR, methods of recombinant DNA, isolation and purification of proteins, ICP-MS, XPS-MS, UHPLC, GC-FID and determination of electro-kinetic parameters of colloidal systems
- behavior assays using model organism *Drosophila melanogaster*: reward and punishment learning and memory in controlled environment using a T-maze, sleep and activity using DAMS, negative geotaxis, PER assay for learning and memory, addiction
- methods of confocal microscopy and visualization using a fluorescent dye / protein
- good knowledge of methods in quality management of projects (in charge for finance and economic suitability and the implementation of the EU project)
- experience in preparation of SWOT analysis and risk assessment of project proposals as well as the economic and financial sustainability of the project
- knowledge of the new products introducing methodology to the market as well as the recruitment of new personnel (human resources management)
- knowledge of the legal framework relating to patents and intellectual property
- experience in tendering procedure (EU and Croatian low framework)
- keeping records and procurement at the department level
- coordination between employees, managers and head of the Laboratory of the Department

Computer skills

- great computer skills proficient in MS Office programs (MS Word, MS PowerPoint, MS Excel, MS Access, MS Outlook)
- knowledge of scientific database search (SCOPUS, Science Direct, PubMed, Protein Data Bank (PDB), Cambridge Structural Database (CSD),) as well as Web technologies
- knowledge of working with modern computational methods that are used to interpret and predict the structure and reactivity of molecules Marvin, Gaussian
- knowledge of work with Statistica and MedCalc to set up numerical models and methods
- other application software for the analysis and interpretation of experiment results

Other skills

- Molecular biology of addiction,
- Neurochemistry and molecular neurobiology

Driving license

- B

ADDITIONAL INFORMATION

Scientific and other projects

Project leader:

- "Fly-high – influence of oxidative stress" P940, scientific project financed by Student Council of University of Rijeka in 2017.
- "Biotechnologist for Rijeka" P870, community project financed by Student Council of University of Rijeka in 2017.

Associate on project:

- Expert associate on project "Defining the role of circadian genes in behavioral sensitization to psychostimulants in *Drosophila melanogaster*", financed trough Croatian Science Foundation (HRRZ), project leader: Ass. Prof. Rozi Andretić Waldowski, Ph.D.
- Expert associate on Projects of the University of Rijeka, Funds for scientific research at the University of Rijeka "Defining the role of circadian genes in behavioral sensitization to psychostimulants in *Drosophila melanogaster*", project leader: Ass. Prof. Rozi Andretić Waldowski, Ph.D.
- Expert associate on international development and research project with industry partners on development of new products "Medical agents for detoxification on artificial stomach and duodenum model", project leader: Prof. Sandra Kraljević Pavelić, Ph.D.
- Center for high throughput in biotechnology, European Union Infrastructural project: Research Infrastructure for Campus-based laboratories at University of Rijeka, 2013, RISK, project leader: Prof. Pero Lučin, Ph.D. and Prof. Nevenka Ožanić, Ph.D.

Teaching

- undergraduate program Biotechnology and Drug research at Department of Biotechnology:
- Physical chemistry - practical laboratory exercise - 2013./2014.
- Bioassay in drug discovery – teaching assistant - 2014./2015
- Organic chemistry - practical laboratory exercise - 2013.-up to now

Mentorship of master thesis

- 2017. S. Al-samarai, Differences in the development of behavioral sensitization to cocaine compared to methamphetamine in *Drosophila melanogaster*
- 2017. M. Brkljačić, Investigating of antioxidant activity of various clinoptilolite materials on *Drosophila melanogaster* model
- 2017. A. Selimović, Defining the preferential consumption of cocaine and methamphetamine in *Drosophila melanogaster*
- 2017. J. Kolobarić, Influence of oxidative stress on development of locomotor sensitization in *Drosophila melanogaster*
- 2016. F. Rigo, Influence of tyrosol and quercetin on indicators of aging and redox status in *period* mutants of *Drosophila melanogaster*

- 2016. T. Žic, Influence of tyrosol and quercetin on indicators of aging and redox status in *Drosophila timeless* circadian mutants

- 2016. I. Ćoso, Investigating characteristics of behavioral sensitization in *Drosophila melanogaster*

- 2015. A. Bolonja, Investigating the effect of polyphenols and ethanol on aging, behavior and longevity in *Drosophila melanogaster*

Honors and awards

- IBRO Travel grant for attending "1st Instituto de Neurociencias PhD Student & Postdoc Meeting, Building Neuroscience: The future of a multidisciplinary field", Alicante, Spain, 18.-19. 02.2016.

- Scholarship Foundation of the University of Rijeka for the co-financing student activities - Student training on summer school Drosophila Neurobiology Genes, Circuits & BEHAVIOR, CSHL, New York 26.06.–16. 07.2015.

- Best poser presentation award 5th Student Congress of Neuroscience, NeuRi 2015. NEURI 2015. 24.-26. April 2015. Rijeka/Rab, Croatia with poster presentation of work. „Development of a new high-throughput assay for behavioral sensitization to psychostimulants in Drosophila melanogaster", Maja Badurina, **Ana Filošević**, Ivan Odak, Rozi Andretić Wladowski

- "British Scholarship Trust" scholarship for scientific research at UK universities 2014. University of Oxford

- Two consecutive years 2011th and 2012th with a 4.84 grade point average out of 5.00, in the top 10 students at Department of Chemistry, Faculty of Science, University of Zagreb, Croatia

Membership in science organizations

- from September 2012. member of the "Croatian Chemical Society" in sections of Organic and Medicinal Chemistry

- from September 2015. member of "Croatian Society for Neuroscience"

- from February 2016. member of "Federation of European Neuroscience Societies" - FENS

- from June 2016. Postgraduate study representative at Student Council of the Department of biotechnology

Scientific papers

- **A. Filošević,** S. Al-samarai, R. Andretić Waldowski, High throughput measurement of locomotor sensitization to volatilized cocaine in Drosophila melanogaster, Methods, Front. Mol. Neurosci. – in the review

- S. Kraljević Pavelić, V. Micek, **A. Filošević**, D. Gumbarević, P. Žurga, A. Bulog, Y. Yamamoto, T. Preočanin, J. Plavec, R. Peter, M. Petravić, D. Vikić-Topić, K. Pavelić, Novel, oxygenated clinoptilolite material efficiently removes aluminium from aluminium chloride-intoxicated rats in vivo, Micropor Mesopor Mat, DOI: 10.1016/j.micromeso.2017.04.062 (Q1)

Active participation in conferences and scientific meetings

<u>Oral presentations:</u>

- 7th Student Congress of Neuroscience, NeuRi 2017., Rijeka/Rab, Croatia, 21.-23.04.2017., "Drosophila melanogaster a model organism in the addiction research", **A. Filošević**, A. Selimović, R. Andretić Waldowski

- Neurofly 2016., 16th European Neurobiology of Drosophila Conference, Platanias, Chania, Crete, Greece, 02.-06.09.2016., „Psychostimulants induce behavioral sensitization and tolerance in Drosophila melanogaster“, **A. Filošević**, I. Ćoso, R. Andretić Waldowski

- 20th Young Neuroscientist Meeting, Rijeka, Croatia, 30.06.2016., "Complex behavior in simple organism: challenges in the quantification of behavior", **A. Filošević**, R. Andretić Waldowski

Poster presentations:

- Lisabon addictions, 2nd European conference on addictive behaviours and dependencies, Lisabon, Portugal, 24.-26.10.2017., „Self-administration of cocaine and metamphetamine in Drosophila melanogaster“, **A. Filošević,** A. Selimović, R. Andretić Waldowski

- 2017. IEBMC, Biological Clocks: Mechanisms and Applications, Rijeka, Croatia, 6.-8.10.2017., “Circadian genes have phenotype-specific roles in psychostimulant-induced neuronal plasticity in *Drosophila*“, **A. Filošević**, J. Kolobarić, A. Selimović, S. Al-samarai, R. Andretić Waldowski

- 6th Croatian Neuroscience Congress, Osijek, Croatia, 16.-18.09.2017., „Interaction between redox status and psychostimulants-induced neural plasticity in *Drosophila*“, **A. Filošević**, J. Kolobarić, S. Al-samarai and R. Andretić Waldowski; „Preferential consumption of psychostimulants in *Drosophila melanogaster*: introduction of self-administration paradigm“, A. Selimović, **A. Filošević**, R. Andretić Waldowski

- Advances in Biomedical Research, MedILS, Split, Croatia, 03.-07.07. 2017., „Circadian Genes and Redox Regulate Neuroplasticity to Psychostimulants in *Drosophila*“, **A. Filošević**, J. Kolobarić, S. Al Samarai, R. Andretić Waldowski

- 19th Annual Genes, Brain and Behavior Meeting of IBANGS, Madrid, Spain, 15.-18.05.2017., „*Drosophila melanogaster* as model for studying drug addiction – introduction of self-administration paradigm: preferential consumption of cocaine in *Drosophila melanogaste*“ **A. Filošević**, A. Selimović, R. Andretić Waldowski

- 7th Student Congress of Neuroscience, NeuRi 2017., Rijeka/Rab, Croatia, 21.-23.04.2017., „Role of oxidative stress in behavioral sensitization to psychostimulants in *Drosophila*“, S. Al-Samarai, J. Kolobarić, **A. Filošević**, R. Andretić Waldowski

- 10th FENS Forum of Neuroscience, Copenhagen, Denmark, 02.-06.07. 2016., „Defining the behavioral sensitization to psychostimulants in *Drosophila*“, **A. Filošević**, I. Ćoso, R. Andretić Waldowski

- 6th Student Congress of Neuroscience, NeuRi 2016., Rijeka/Rab, Croatia, 22.-24. 04. 2016., „Characterizing short and long-term behavioral sensitization in *Drosophila*”, **A. Filošević**, I. Ćoso, R. Andretić Waldowski

- “1st Instituto de Neurociencias PhD Student & Postdoc Meeting, Building Neuroscience: The future of a multidisciplinary field”,., Alicante, Spain, 18.-19. 02. 2016., “Influence of diet enriched with polyphenols on indicators of aging in *Drosophila melanogaster*”, **A. Filošević**, R. Andretić Waldowski
- 5th Croatian Congress of Neuroscience, Split, Croatia, 17.–19.09.2015., “Effect of polyphenol compounds on ageing in *Drosophila melanogaster*”, A. Bolonja, **A. Filošević**, R. Andretić Waldowski

- CroArtScia 2015. Symposium Technological innovations: atr & science,

Zagreb, Croatia, 27.-30.05.2015., „*Drosophila melanogaster* as model organism in neurobiology of addiction - behavioral genetics approach“, **A. Filošević**, R. Andretić Wladowski

▪ 5th Student Congress of Neuroscience, NeuRi 2015., Rijeka/Rab, Croatia, 24.-26. 04.2015. „Development of a new high-throughput assay for behavioral sensitization to psychostimulants in Drosophila melanogaster“, M. Badurina, **A. Filošević**, I. Odak, R. Andretić Wladowski

Student script

▪ N. Malatesti**, A. Filošević**, Organic chemistry practical for students of 2nd year in undergraduate program Biotechnology and Drug research at Department of Biotechnology

Science popularization projects

▪ 20.-27.07.2017. Višnjan Scientific and Educational center/Astronomical Society Višnjan, mentor at Youth Science Camp 3 for 7th and 8th grade Primary school talented students on project “Coffee addiction in *Drosophila melanogaster*”

▪ 2014.-2017. *Tetragon* and “Department Open Day”, as a part of “Festival znanosti” project, manager of Department budget, organization and preparation of students to perform chemical experiments and coordination of administrative duties at Department of Biotechnology

▪ 21.03.2017. FameLab finalist in Croatia organized by British Council Croatia, presentation of scientific theme in terms of science popularizing

▪ 27.09.2013. Researchers' Night 2013. In Rijeka, Croatia, Project financed through FP7 EU funds Coordinator of Department activities and active participant