Utjecaj izloženosti metafetaminu na fenotipske promjene u potomaka

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UNIVERSITY OF RIJEKA
DEPARTMENT OF BIOTECHNOLOGY
Undergraduate program
Biotechnology and drug research

Eva Jakuš

Effect of methamphetamine exposure on phenotypic changes in offspring

Undergraduate thesis

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SAŽETAK

Metamfetamin, kao i ostali psihostimulansi, uzrokuju mnoge fiziološke promjene povezane sa misregulacijom dopaminskog signalizacijskog puta u ljudi. Produljeno korištenje dovodi do promjena u signalizaciji mozga kao posljedica svojstva neuroplastičnosti, te se određenim epigenetskim modifikacijama koje utječu na gensku ekspresiju prenosi na potomke te stvara novi fenotip, unatoč neizloženosti stimulansu.

Cilj ovog rada bio je ispitati kako metamfetamin u formi aerosola (vMETH) utječe na stvaranje fenotipa s povišenom lokomotornom aktivnosti u F1 generaciji *Drosophile melanogaster*. vMETH je administriran metodom FlyBonga grupi mužjaka, od kojih je 23% izdvojeno s obzirom na najveću lokomotornu aktivnost te spareno s djevicama koje nisu tretirane vMETH-om. Utjecaj izloženost mužjaka je zatim praćen u F1 generaciji mjerenjem lokomotorne aktivnosti.

Rezultati su pokazali statistički veću lokomotornu aktivnost između tretirane F0 i F1 generacije, no pokazalo se da nema statistički značajne razlike između kontrolne i tretirane grupe. Rezultati bi mogli ukazivati na postojanje novog fenotipa koji bi bilo potrebno ispitati kroz više generacija.

Ključne riječi: *Drosophila melanogaster*, metamfetamin, fenotip, epigenetičke modifikacije

SUMMARY

Methamphetamine and other psychostimulants can instigate many physiological changes that lead to the misregulation of the dopamine signaling pathway in humans. Extended use can cause permanent changes in this signaling pathway due to neuroplasticity, and those changes are carried into the next generation by certain epigenetic modifications that affect gene expression. As a result, a phenotype with an increased locomotor activity is created and persists even without the initial stimuli.

The purpose of this thesis was to investigate how volazilized methamphetamine (vMETH) affects the creation of the new phenotype in the F1 generation of *Drosophile melanogaster*. vMETH is administered using the FlyBong method to a group of male flies and 23% of them were selected based on locomotor activity. They were then bred with untreated virgin flies. The effects of the methamphetamine dose wer observed in the F1 generation by measuring locomotor activity.

Results have shown a statistically higher activity in the F1 treated group, but there was no statistically significant difference between the control and the treated group. This could point to the existance of the mentioned phenotype, which should be investigated through several generations

Keywords: *Drosophila melanogaster*, methamphetamine, phenotype, epigenetic modifications

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1. INTRODUCTION

1.1. *Drosophila melanogaster* and its life cycle

Drosophila melanogaster has been recognized as an excellent model organism in various research, due to the length of its lifespan, quick generation time, cost-effective cultivation and many similarities in its physiology to mammals. *D. melanogaster* is colloquially known as a fruit fly, yet in contrast to the name, the microbes are what draws it to the fruit, not the fruit itself.

Being a holometabolous insect, it undergoes a complete metamorphosis from the egg stage to the imago (adult) stage. This cycle consists of several stages and the length varies due to many environmental factors. Specifically, it was shown to take longer in lower temperatures and in situations of overcrowding, while taking shorter in higher temperatures [1]. Flies cultivated on a nutritious sugar, yeast, agar, or cornmeal-based medium in 25°C and 60-70% humidity have a generation time of approximately 10 days.

The stages of *Drosophila melanogaster*'s development cycle are (1) a female fly lays the egg on the nutritious medium. After 24hr, it transitions into the larval stage, which itself consists of 3 larval stages. In the first larval stage, larvae remain on the surface of the medium. The second larval stage involves larvae burrowing into the medium. The third larval stage lasts about 48hr, in which larvae are searching for a place to transition into the pupal stage. *D. melanogaster* is used in its larval stage for studies on the nervous system and provides an even simpler model due to a smaller number of neurons compared to an adult fly.

The pupal stage lasts about 4 days and is comprized of first the encapsulation of a third stage larvae (12hr) and its time spent in the cocoon. New structures are made up from the undifferentiated cells of a larva, e.g. legs and wings, while the nervous and reproductive systems are preserved. Finally, pupae transition into adult fruit flies, with a lifespan of 30-40 days.

After first emerging from the cocoon, both male and female flies are considered virgin flies and are lighter and slightly larger than an older fly. Males reach sexual maturity before females and are sexually unresponsive for 8-12hr. The female flies are documented to mate with several partners throughout their life and can lay up to 3000 eggs. The possible large number of flies makes it ideal research, from the molecular cellular level to its physiology and pharmacology of many substances and potential drugs [2-4].

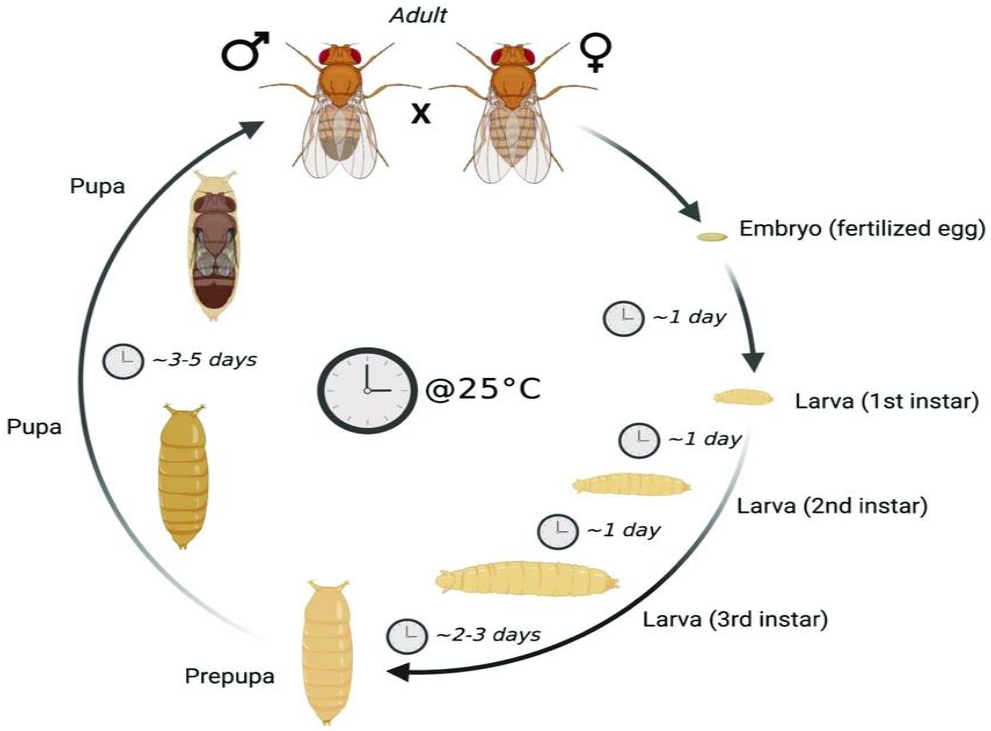


Figure 1. Life cycle of *D. melanogaster* under standard conditions. Consists of the embryonic stage, three larval stages, the pupal stage, and the adult stage. The temperature influencing the cycle is shown. [4]

The genome of a fruit fly represents only a fraction of the mammalian genome, but its homology makes it an excellent animal model in genetics research, as well as its impressive genetic repertoire, i.e. balancer chromosomes, palindromic repeats, RNAinterference, etc. [5]. Those

similarities are another advantage because the results of research on fruit flies can be translated and used in research on mammals. Furthermore, its nervous system, although rudimentary in comparison, is in many ways similar to a human's and evident by the fact that *Drosophila melanogaster* exhibits complex behaviors like learning, memory, social behaviors etc. [2]

1.2. **Addiction**

Addiction is defined as a chronic brain disease, whose onset is affected by genetic, environmental, and social factors [6]. Typical stages of addiction are (1) voluntary consumption of a drug, followed by a euphoric response, (2) repeated drug use that leads to compulsive use, and (3) physical and mental dependence on the drug [7].

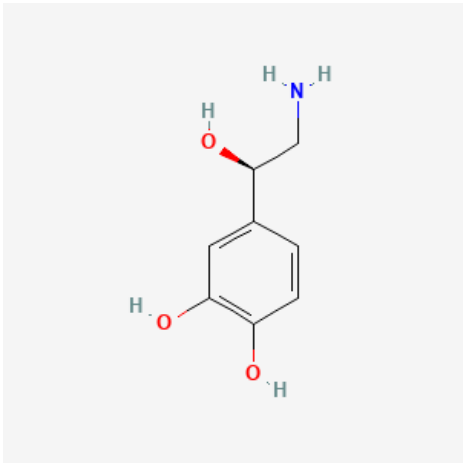
It has become a considerable medical, financial, and social issue [8]. Close to 300 million people have reported using psychoactive drugs, and approximately 39,5 million people are affected by drug addiction, with 580,000 deaths per year [8]. Most of the measures taken are essentially only preventative, which has somewhat reduced drug-related hospital admissions and minorly reduced the burden on the medical sector, but without any concrete pharmacological solution, the impact of those measures seems to be only temporary [9-10]. More research is needed to get to the root of the issue of a disorder as complex as addiction and its genetic factors and potentially one day find an effective treatment.

1.3. **Methamphetamine and dopamine signaling pathway**

Psychostimulants, such as amphetamines and cocaine, are drugs that lead to short-term physical and/or mental changes (increased alertness, loss of appetite, cardiovascular stimulation, improved mood, etc.) [12]. They target monoamine transporters, increasing monoaminergic transmission [13], and are a commonly used drug for a variety of mental

and physical disorders. They are also often abused, leading to addiction. At higher doses, or after prolonged use, psychostimulants can lead to many drug-induced disorders, e.g. schizophrenia. In animal models, increased locomotor activity decreased sleep, and changes in social behaviors have been documented. *D. melanogaster*'s dopaminergic neurons also use similar transporters and enzymes in the dopamine pathway, making the results applicable to humans [14-15].

Methamphetamine (METH) is a positively charged lipophilic molecule that alters the metabolism of dopamine, serotonin, and noradrenaline, by inhibiting the enzyme responsible for their removal from the synapses [16]. As an indirect monoamine agonist, it also prompts the release of monoamines, causing their release into the synaptic cleft. Without efficient removal, this process leads to a buildup of monoamines, i.e. dopamine, in the synapses, causing the euphoric effect of the drug [17]. The misregulation of the dopamine signaling pathway is shown to major factor in developing addiction [13].



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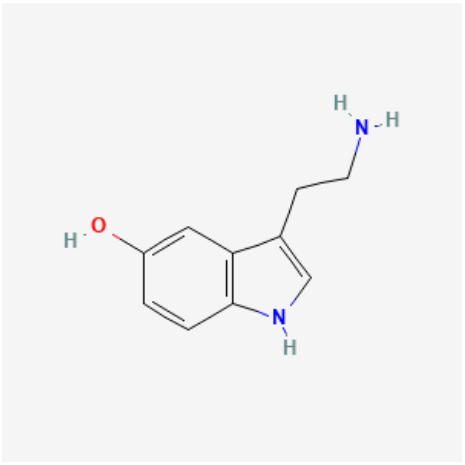

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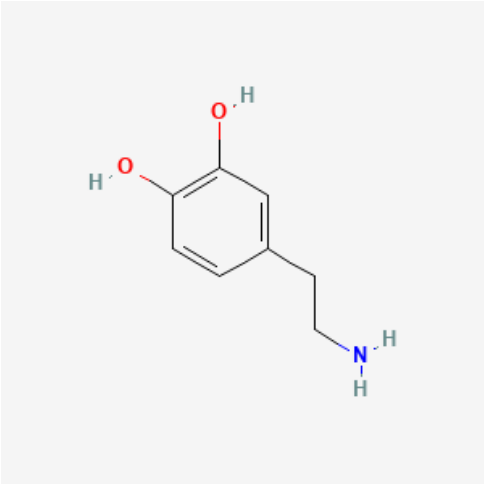
C 

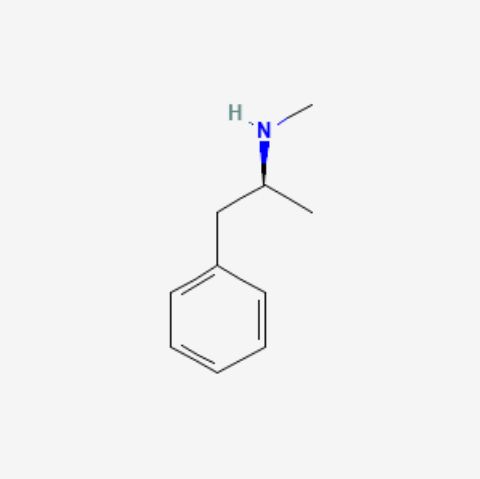
D 

Figure 2. Monoamine structures in comparison to the structure of N-methylamphetamine. Illustrations show chemical structures of (A) noradrenaline, (B) serotonin, (C) dopamine, (D) N-methylamphetamine. (Source: https://pubchem.ncbi.nlm.nih.gov/)

Dopamine (DA) is a highly conserved catecholamine neurotransmitter found in the central nervous system of animals. It has a crucial role in many important biological processes, such as motor coordination, motivation, learning, and memory. It is synthesized in a presynaptic dopaminergic neurons and is stored in vesicles before being released.

Disruptions in dopamine signaling are a major factor in many diseases, like Parkinson's disease, depression, and bipolar disorder [18]. DA has been closely connected with changes in neuroplasticity, i.e. the ability of the nervous system to be altered long-term due to external stimuli [19]. Therefore, drug use can be interpreted as a trigger to neuroplasticity, creating an new phenotype with the most changes documented in the *nucleus accumbens* (NAc) [20].

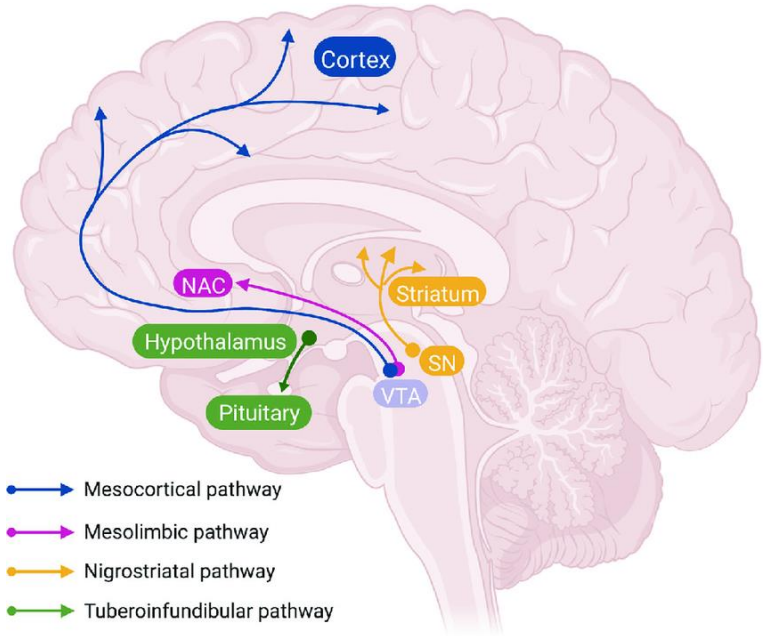


Figure 3. Dopamine pathways in the brain. Dopamine pathways are associated with physiological balance, memory, learning, emotion, and reward. [11]

1.4. Genetics and epigenetics of addiction

Epigenetics is a relatively new science that details changes in regulation mechanisms of transcription without DNA modifications, like DNA methylation, non-codingRNAs (ncRNA), and chromatin remodeling. Histones, the building blocks of chromatin, have an N-terminal tail, which is often a target of post-translational modifications such as methylation, which then trigger an activation or suppression of certain genes. These changes are often a consequence of an environmental stimulus and can create a new phenotype in the filial generation as a form of adaptation. These changes in gene expression can permanently influence the reward pathway, causing drug craving and relapse, hence addiction being interpreted as a harmful instance of drug abuse [22].

Epigenetic modifications mostly target somatic cells, which for some diseases increases the risk of onset in later years, e.g. in certain cancers and neurologic conditions like Alzheimer's disease or Parkinson's disease.

These changes can affect gametes, which then carry the specific phenotype into the next generation.

Transgenerational epigenetic effects refer to the phenotypes across multiple generations and do not follow classic genetics [21]. Some studies have shown compelling results in support of the existence of genetic components making individuals more susceptible to addiction, with various contributions of environmental factors [22]. The heritability of those components spans from 45% to 79% and can affect the next few generations without a repeat of stimuli. i.e. methamphetamine. Even with a vast number of studies suggesting a strong genetic contribution to drug abuse, the number of genes identified has been limited [25-26]. With more advancements in epigenetics, researchers hope to gain more insight into the specific changes in gene expression throughout the generations.

Another key component in the development of addiction is the brain's neuroplasticity, which is evident in two forms in the context of addiction: (1) tolerance, which is characterized by reduced effects due to drug dependency and withdrawal symptoms if the use is discontinued and (2) uncontrolled use, when drugs become similar to the brain as a physiological need. Changes to the brain made in the second form of neuroplasticity are permanent, causing relapses in individuals potentially years after discontinuing use [27]. In such individuals, there is evidence of changes in blood flow to certain parts of the brain when being shown 33 msec cues associated with the drug after years of sobriety. Similar evidence was shown through more simple behavior in animal models, with one such experiment having rats showing craving-associated behaviors up to 6 months after the last dose of the drug [27].

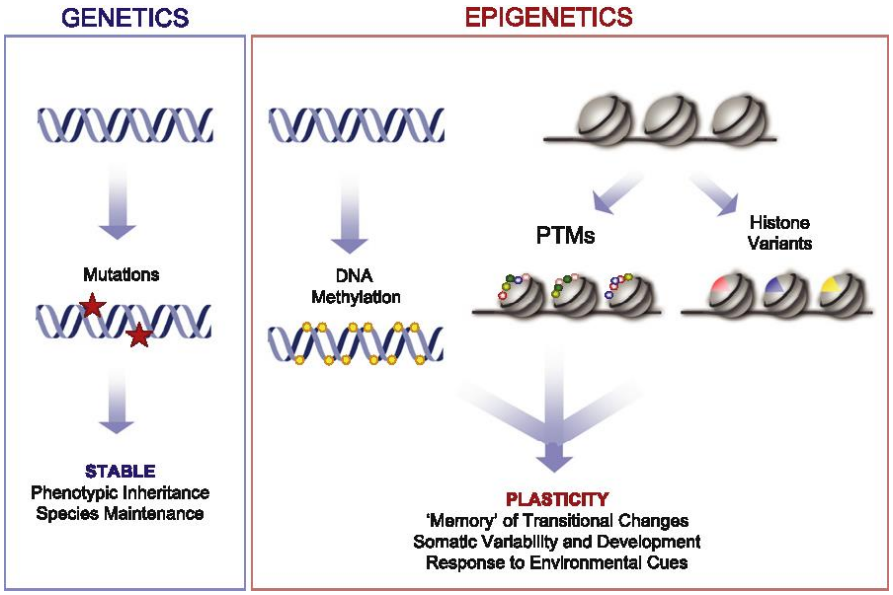


Figure 4. Comparison of epigenetic and genetic control. Genetic variation is mediated by mutations on the DNA. Epigenetic changes can occur after mutagenesis, or processes like DNA methylation or modifying chromatin. [20]

2. PURPOSE OF THE THESIS

The purpose of this experimental thesis is to examine the transgenerational effect of methamphetamine exposure on adult *Drosophila melanogaster* males and the resulting phenotypes in the filial generation. Due to vast research showing a strong genetic component in addiction, it can be hypothesized that the F1 generation of flies could exhibit similar behaviors (increased locomotor activity) as the F0 generation.

3. MATERIALS AND METHODS

3.1. Administration of methamphetamine (FlyBong method)

The FlyBong method measures locomotor activity after being exposed to stimuli. Approximately 4-6hr before administration, 0,1 μL of METH dissolved in alcohol is injected into the flask for the alcohol to evaporate. On the day of the experiment, the heating caps heat the flasks for close to 7 minutes (up to 185°C) to vaporize the previously injected METH, after which the aerosol is distributed into the tubes with individual flies. The control group was exposed to hot air. After one minute of exposure, the results are measured for 10 minutes to collect the data. Results are interpreted as a number of times a fly crossed the middle of the tube in an one-minute interval [26].

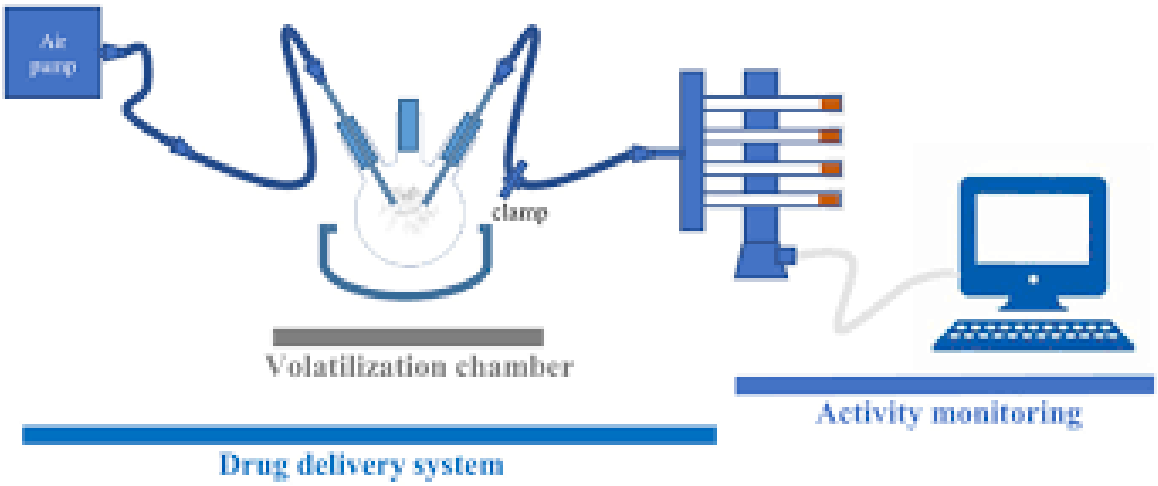


Figure 5. Illustration of the fly bong. The methamphetamine dissolved in alcohol is put into the three-neck flask and connected to the DAMS. The locomotor activity is monitored on a computer connected to DAMS. [26]

3.2. Preparation of the sample

D. melanogaster strain used in this experiment is the Canton S wild-type males. The flies were cultivated on a standard cornmeal-based medium

and were kept in the incubator in standard laboratory conditions (25°C, 70% humidity, 12hr:12hr light/dark cycle).

The day before the administration of the methamphetamine dose, 128 males were collected using CO_2 as an anesthetic. Tubes for the DAMS (*Drosophila* activity monitoring system) were prepared with the same food medium and parafilm was used to prevent drying up of the medium. The pre-prepared food medium was cut into 5 mm slices, after which it was inserted into each tube. The males were individually transferred into tubes approximately 24hr before the administration to adapt to the environment. There were 4 monitors prepared with 128 adult male flies. 64 males belonged to the control group and 64 males were in the treatment group. 75 µL of 0,1 mM METH was injected into the flask and left for about 12h for the alcohol to evaporate.

On the day of the experiment, the dose was administered in the morning in the way described previously.

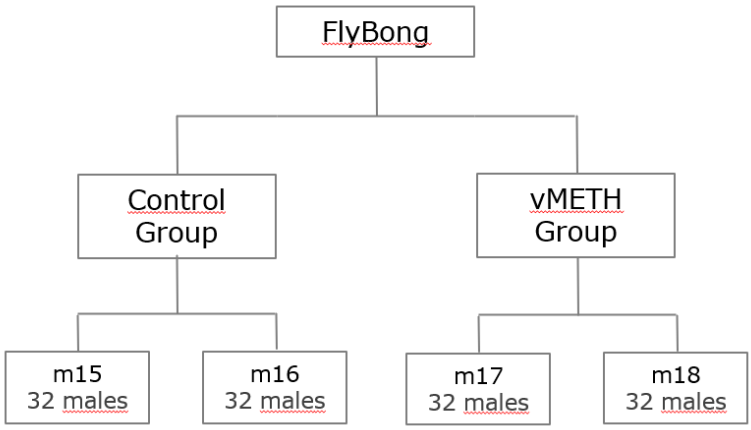


Figure 6. Design of the experiment. 128 male flies were transferred into four DAMS monitors, two of which were control monitors and two vMETH monitors.

3.3. Fly selection and quantification of life cycle stages

After 10 minutes of data collection, 30 control group males (23% of the control group) and 30 treatment group males (23% of the vMETH group) with the highest locomotor activity were selected and placed into larger test tubes with untreated virgin female fruit flies, creating a parental generation (F0). 15 untreated males were also individually placed into larger test tubes with untreated females. The adult male flies were taken out of the tubes after 48hr and the adult female flies 72hr after being placed into the tube. The first pupae appeared on the 7th day and were counted daily for 4 days. The first flies appeared on the 12th day and were counted for 4 days, after which they were collected for administration. The preparation of the sample followed the same steps as the first administration.

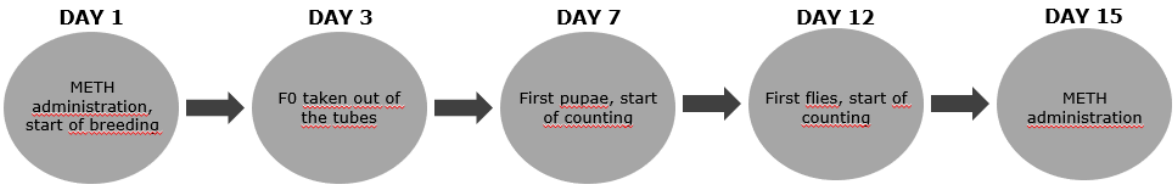


Figure 7. Chronological order of the experiment.

3.4. Data processing, statistical analysis, depiction of results

For the initial data analysis and the analysis of the locomotor activity of the control group and vMETH group after both administrations, MS Excel was used. The results were visualized in the form of linear and column graphs. Statistical analysis was carried out with the software GraphPad Prism 10.3.1. One-way ANOVA and the unpaired t-test were used.

4. RESULTS

This experiment was conducted to investigate if the exposure to methamphetamine would produce a phenotype with an increased locomotor activity compared to the control group in the F1 generation of *Drosophila melanogaster*. Methamphetamine was documented to increase locomotor activity in fruit flies and was administered to 128 males using the FlyBong method. Locomotor activity was initially measured to select the most active males and pair them with untreated virgin female flies. After breeding, pupae and hatched flies were counted in all three groups (CTRL, vMETH, CTRL no bong). In 72hr after breeding, 9 females bred with control males, 4 females bred with methamphetamine males, and 2 females bred with untreated males died without laying eggs.

4.1. Difference in the number of pupae among the groups

After breeding, females lay eggs in the nutritious medium. After 24hr, larvae were visible in the tubes. On the fifth day, larvae started climbing the walls of the test tubes, starting puparation. The first pupa was visable and the number was documented for the next four days.

There was no statistically significant difference between the averages of the control and vMETH group or between the vMETH and the control group that wasn't administered neither air or the drug. There was a statistically significant difference in the average number of the treated control group and the untreated control group. There was also no statistically significant difference between cumulative numbers of flies. (Picture 8). There is a trend of a higher number of offspring in the untreated group.

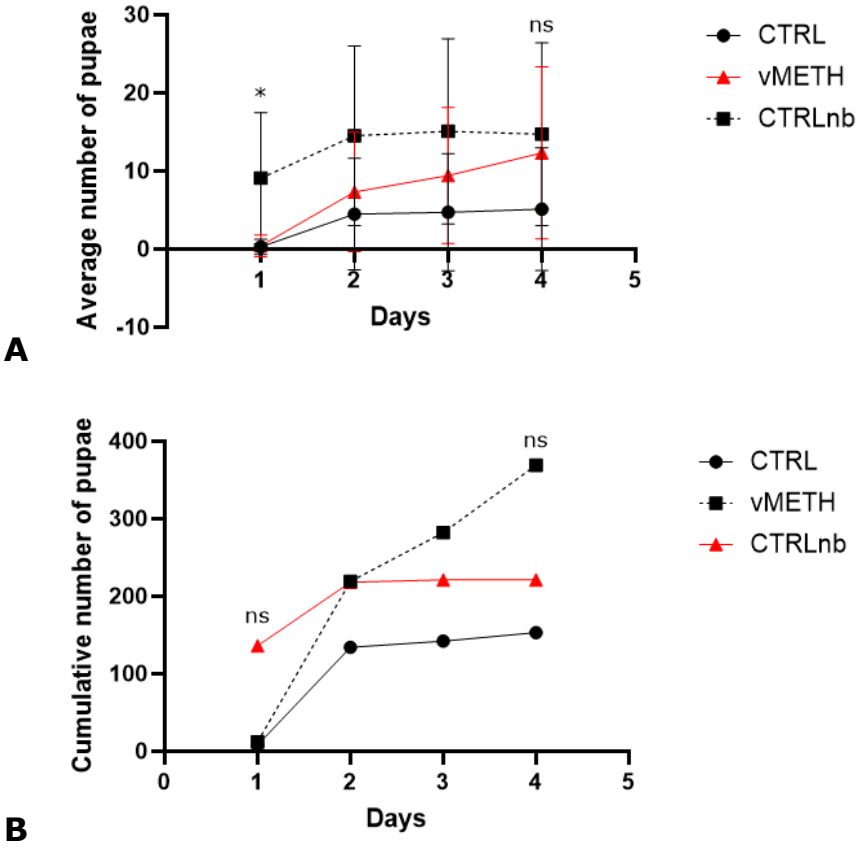


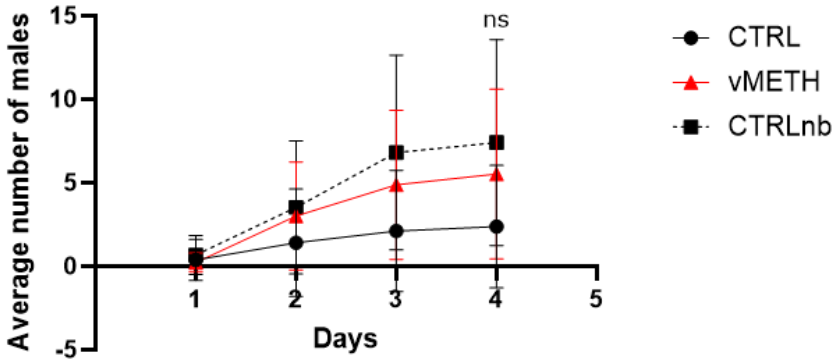
Figure 8. Methamphetamine did not lead to a change in the number of pupae. Pupae were counted daily for four days. **(A)** Average number of pupae. Results are presented as the mean value ± the standard deviation. * p=0,0126. **(B)** Cumulative number of pupae. The results are shown as absolute values. The One-way ANOVA test has shown a statistically significant difference between the initial group comparison. There is no statistically significant difference in the cumulative number of pupae. ns – no statistical significance.

4.2. Difference in the number of adult F1 flies among the groups

On average, the flies start emerging out of their pupilar stage four days days after enclosion. In this experiment, the first flies were visible on the twelfth day and the number was documented for the next four days.

There was no statistically significant difference between the control group and the treated group of F1 male flies and no statistically significant difference among the F1 female flies. There is a trend of a higher number

of both male and female adult flies in the last half of counting, in contrast to the first days of the experiment, where the values of all three groups are similar.



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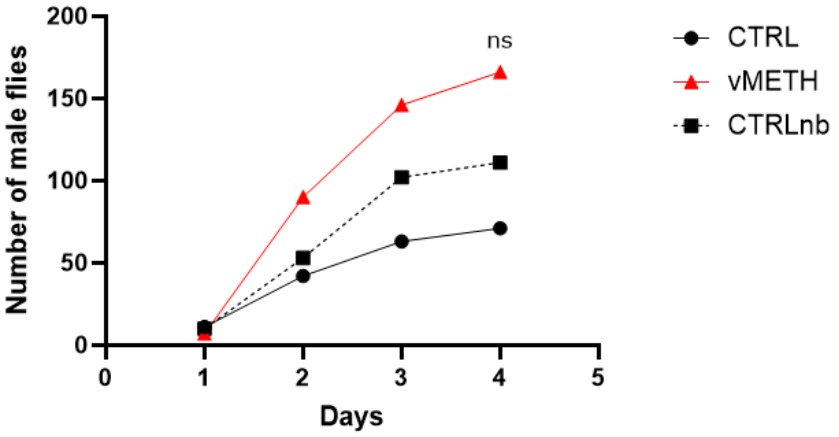

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Figure 9. Methamphetamine exposure did not significantlly affect the number of male flies in the F1 generation. Male flies were counted daily for four days. **(A)** Average number of male flies. Results are presented as the mean value ± the standard deviation. **(B)** Cumulative number of male flies. The results are shown as absolute values. The One-way ANOVA test did not show any statistically significant difference between the groups tested. ns – no statistical significance.

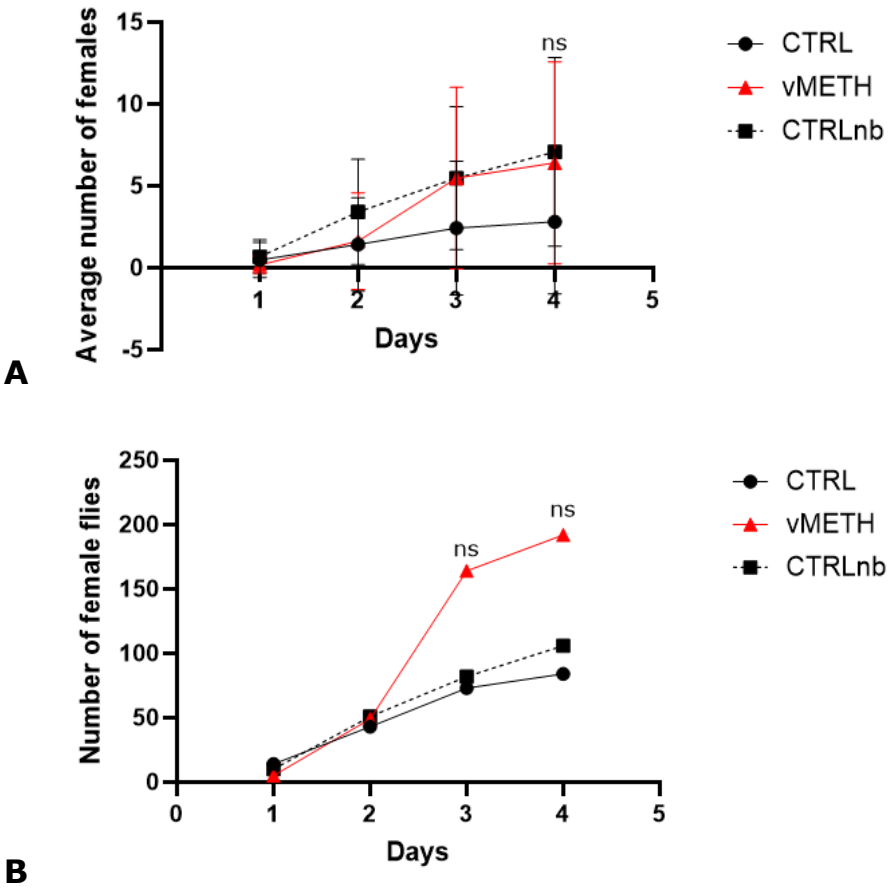


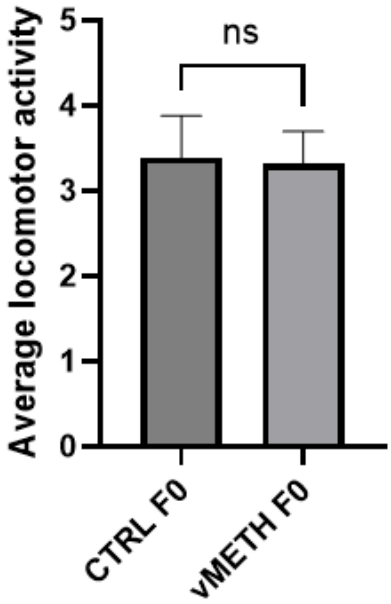
Figure 10. Methamphetamine exposure did not significantlly affect the number of female flies in the F1 generation. Female flies were counted daily for four days. **(A)** Average number of female flies. Results are presented as the mean value ± the standard deviation. **(B)** Cumulative number of female flies. The One-way ANOVA test did not show any statistically significant difference between the groups tested. ns – no statistical significance.

4.3. **Difference in locomotor activity**

Methamphetamine was shown to increase locomotor activity in flies, by inducing dopamine buildup in the synapses. After the administration of methamphetamine on F1 flies, their locomotor activity was measured in one-minute intervals for 10 minutes total and the average value of each flie's activity was noted.

The average locomotor activity in the F0 control group was similar to the average locomotor activity in the F0 vMETH group and there is no

statistically significant difference between them. The average locomotor activity in the F1 control group was higher than the average in the F1 vMETH group. When comparing different generations, the results have shown a statistically significant difference while comparing the F0 and F1 of both the control and vMETH groups, with a trend of an increase in average locomotor activity in the F1 generation.



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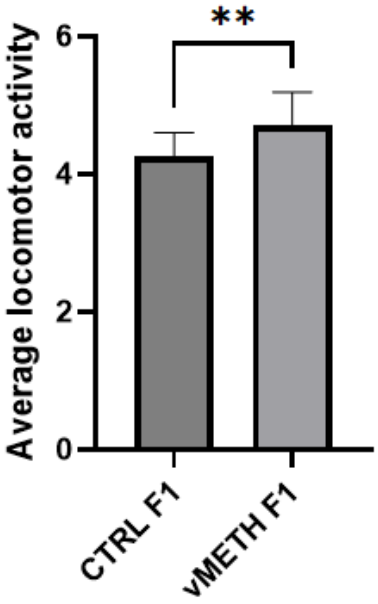

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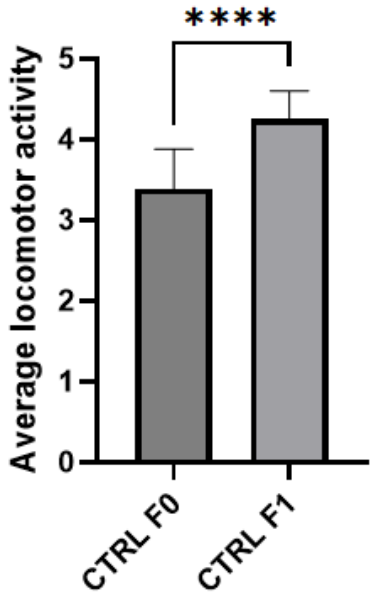

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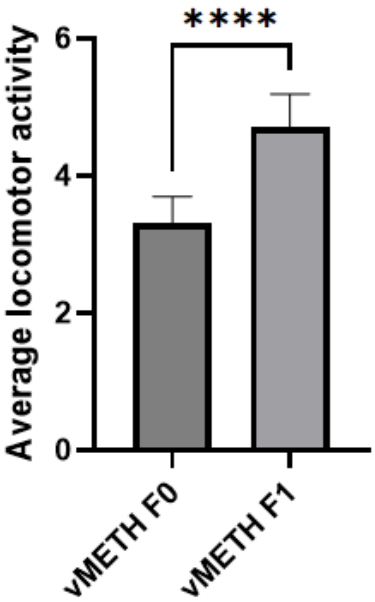

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Figure 11. Effect of methamphetamine on the locomotor activity of *Drosophila melanogaster*. The results were measured 10 minutes after administration. **(A)** The F0 group. **(B)** The F1 group. **(C)** The treated control group. **(D)** The treated vMETH group. The results were presented as the mean value. The unpaired t-test showed no statistically significant difference between in the F0 generation. Unpaired t-test has shown a statistically significant difference in the F1 generation. ns – no statistical significance. **p=0,0042; ****p<0,0001.

5. DISCUSSION

This experiment was meant to inspect the impact of methamphetamine on the phenotypes created in the F1 generation. It could be assumed that the next generation of flies exposed to vMETH would be sensitized to the drug more than the control group. It wasn't expected that there would be significant differences in the life cycle of the F1 generation.

The results have shown no statistically significant differences in the number of pupae and adult flies. There was no significant increase in locomotor activity comparing the F0 control and vMETH groups, but the difference inreased in the F1 generation. There was a statistically significant difference in locomotor activity when comparing the two generations in both the control and vMETH group, which could be a result of stress on the flies, overshadowing the effect of the drug itself. In previous research, methamphetamine was shown to significantly increase the locomotor activity compared to the F0 control.

This results could also be affected by the varied number of surviving parental flies and possible variables that could occur during administration. There is a trend of a higher average in the F1 generation of the vMETH group, which could implicate the higher sensitivity to drug exposure in the next generation. If the result is repeated through more generations, it could point to the transgenerational mechanism of creating a phenotype associated with drug exposure. Methamphetamine does indirectly affect gene transcription, strengthening that argument. Due to the early stages of

epigenetics and the exact genetic factors responsible for addiction being unknown, the exact mechanism of addiction vulnerability remains a topic of research.

This experiment has shown some trends, but due to a small number of generations tested, a relatively small number of samples, and a small number of repeats, the groups seeminly reacted similarly to being treated with air and methamphetamine. Due to those errors, there should be some modifications made to the methodology in the future, mainly more samples and more repeats. The nutritious medium could be modified to prevent flies from sticking to it. The breeding lasted for 48hr and prolonged breeding could ensure an adequate number of the F1 flies for analysis. Repeating this experiment through more generations could further uncover the phenotype in treated flies, after which the persistence of the gene without exposure could be explored.

6. CONCLUSION

In the experiment, Canton S males of *Drosophila melanogaster* were used in this experiment, with METH administrated with the FlyBong method. They were breed with virgin female flies and the number of flies and their locomotor activity were observed.

The results, even with a small number of modifications to the methodology needed, could point to a more concrete quantization of the methamphetamine effects and could offer a more detailed view of the consequential phenotypes created by drug exposure.

There is a large number of the effects of parental drug use on the filial generation, mainly being researched in pregnancy on rodent models and in human medical studies. Further epigenetic research into a specific genetic makeup that makes individuals more susceptible to addiction is needed to get closer to a concrete solution to this complex issue.

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