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SVEUČILIŠTE U RIJECI

FAKULTET BIOTEHNOLOGIJE I RAZVOJA LIJEKOVA

Preddiplomski sveučilišni studij

"Biotehnologija i istraživanje lijekova"

Petra Cikač

Utjecaj suplementacije glutaminom i kolinom kloridom tijekom izolacije na fenotip preferencijalne konzumacije metamfetamina kod D. melanogaster

Završni rad

Rijeka, rujan 2024.

Mentor rada: *izv. prof. dr. sc. Rozi Andretić Waldowski*

Ko-mentor rada: *dr. sc. Ana Filošević Vujnović*

Undergraduate thesis was defended in September 2024.

In front of Committee:

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3. dr. sc. Ana Filošević Vujnović

Undergraduate thesis has 28 pages, 8 figures, 0 tables and 24 references.

Abstract:

Addiction represents a global challenge to the world, affecting many individuals and communities, making it necessary to examine the mechanisms of addiction. One major environmental stressor, social isolation, disrupts the balance of neurochemicals in the brain that subsequently influence the behaviors related to addiction. This study examines the effects of neurotransmitter precursor supplementation and social isolation on methamphetamine (METH) preference in *D. melanogaster*.

Wild-type three to five day-old male flies were isolated for five days and supplemented with either 10 mg/mL L-Glutamine (GLU) precursor of glutamate, or 3 mg/mL choline chloride (CH.CL) precursor of acetylcholine. After isolation and pretreatment, we used the FlyCAFÉ assay where flies were offered a choice between regular food and METH-supplemented food for three consecutive days. METH preference was calculated as difference between METH-food and regular food for induvial fly, and data were presented as average for every day of the experiment. The results of this study have shown that supplementation with GLU or CH.CL during five days of isolation increased METH preference in FlyCAFÉ, what contrasts with previous studies where it was shown that isolation reduces METH preference relative to non-isolated flies.

This research reveals that GLU or CH.CL supplementation reduced effect induced by isolation, considering social and biochemical aspects in test for METH preference in flies. Future genetic tests are needed to confirm role of glutamine and acetylcholine in the regulation of this addiction endophenotype in flies.

Key words: *Drosophila melanogaster*, social isolation, FlyCAFÉ, methamphetamine, preference, glutamine and acetylcholine

Sažetak:

Ovisnost je globalni problem koji pogađa mnoge ljude i zajednice, zbog čega je važno istražiti molekularne mehanizme ovisnosti. Jedan od glavnih okolišnih stresora, socijalna izolacija, narušava ravnotežu neurokemikalija u mozgu, što posljedično utječe na ponašanja povezana s ovisnošću. Ova studija ispituje učinke dodavanja prekursora neurotransmitera i socijalne izolacije na sklonost metamfetaminu (METH) kod *D. melanogaster*.

U ovoj studiji, divlji tip mušica starih tri do pet dana izoliran je na pet dana uz dohranu s 10 mg/mL L-glutamina (GLU) prekursora glutamata ili 3 mg/mL kolin klorida (CH.CL) prekursora acetilkolina. Nakon izolacije i predtretmana mušice su prebačene u FlyCAFÉ esej, gdje mi je tri uzastopna ponuđen izbor između obične hrane i hrane s dodatkom METH-a. Preferencija za METH-a je izračunata kao razlika između volumena konzumirane METH-hrane i uobičajene hrane za individualnu mušicu, a podaci su prikazani kao prosjek za svaki dan eksperimenta. Rezultati ove studije su pokazali da suplementacija s GLU ili CH.CL tijekom pet dana izolacije povećava preferenciju METH-a u FlyCAFÉ-u, što je suprotno prethodnim studijama gdje je pokazano da izolacija smanjuje preferenciju za METH u odnosu na neizolirane mušice.

Ovo istraživanje otkriva da dohrana s GLU ili CH.CL smanjuje učinak izazvan izolacijom, uzimajući u obzir socijalne i biokemijske aspekte u testu preferencije METH kod mušica. Budući genetski testovi trebaju potvrditi ulogu glutamina i acetilkolina u regulaciji ovog endofenotipa ovisnosti kod mušica.

Ključne riječi: *Drosophila melanogaster, socijalna izolacija, FlyCAFÉ, metamfetamin, preferencija, glutamat i acetilkolin*

1 INTRODUCTION

1.1. Methamphetamine molecular mechanism of action

Methamphetamine (METH) is a psychostimulant that influences the central nervous system (CNS) through various molecular pathways [1]. The main targets of METH are the dopamine transporter (DAT) and vesicular monoamine transporter (VMAT), which regulate dopamine (DA) levels in parts of the brain associated with rewarding effects [2]. METH binds to DAT and reverse its function, causing release of DA into the synaptic cleft. VMAT function is also disrupted by METH, leading to DA being released from synaptic vesicles into the cytosol, which then results in an increased release of DA from neurons through the reversed function of DAT [1]. Among other monoamines, besides DA, METH affects serotonin and norepinephrine, increasing their extracellular levels and prolonging receptor stimulation [3]. As a result of this elevation of free monoamine concentrations, METH significantly enhances stimulant effects (Figure 1.).

METH primarily affects dopaminergic neurons in the striatum and induces neurotoxicity. Neurotoxicity leads to cell degradation and impaired signaling, largely due to increased oxidative stress resulting from elevated levels of DA. An excess of dopamine in the synaptic clefts or cytoplasm undergoes oxidation, resulting in the production of reactive oxygen species (ROS). These ROS damage lipids, proteins, and DNA, leading to extensive cell damage. Exposure to METH results in mitochondrial malfunction, leading to increased production of ROS and subsequent Adenosine triphosphate (ATP) depletion [2]. This oxidative stress leads to neuroinflammation and ultimately damages neurons, which contributes to neuronal loss similar to that observed in Parkinson’s and other neurodegenerative diseases [1]. At higher concentrations, along with other neurotransmitters, DA may promote excitotoxicity through excessive activation of glutamatergic receptors, thus causing further injury to neurons. All these mechanisms eventually result in chronic brain damage, which occurs because of long-term abuse of METH [3].

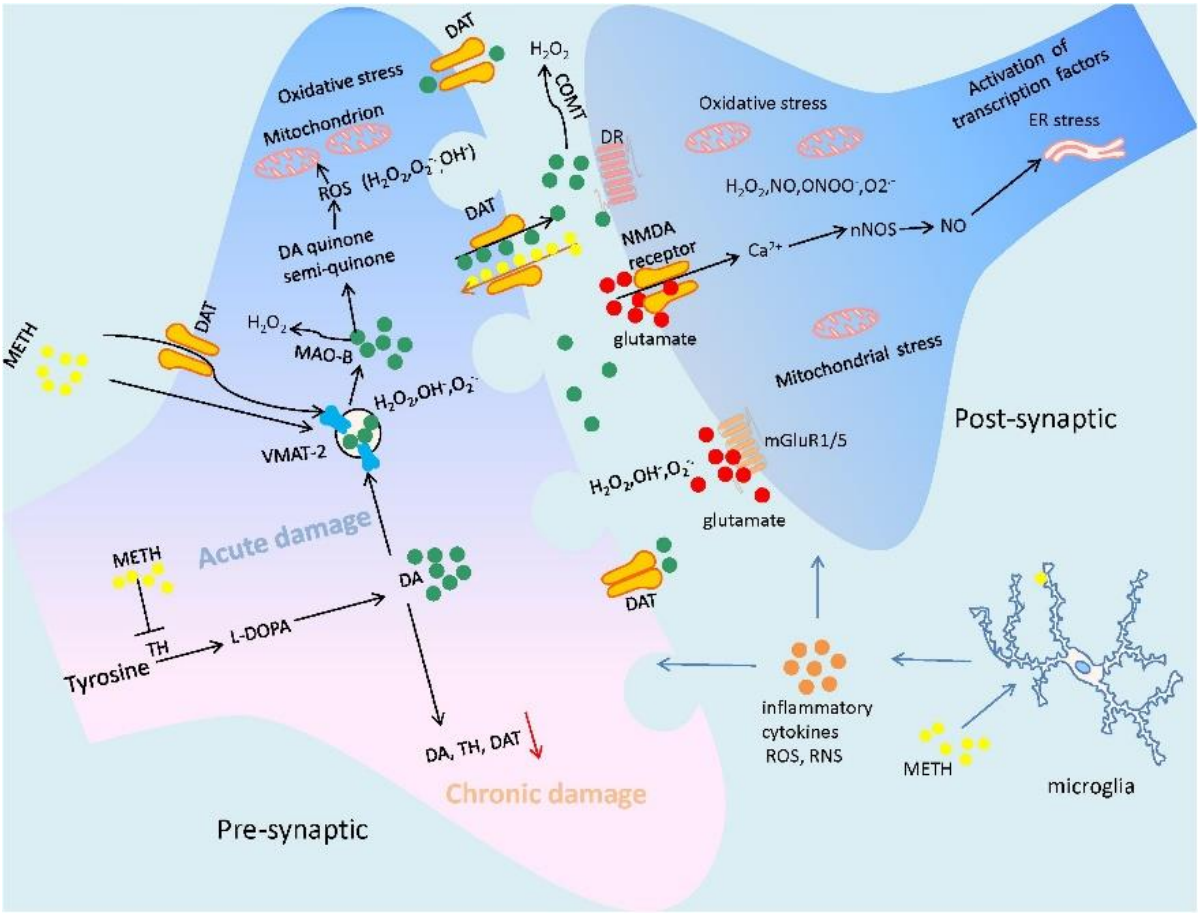

FIGURE 1. Illustration of methamphetamine (METH) neurotoxicity. Key mechanisms include dopamine (DA) oxidation, excessive glutamate production, and increased reactive oxygen (ROS) or nitrogen species (RNS), which in turn lead to mitochondrial dysfunction as well as endoplasmic reticulum stress. Microglial-mediated neuroinflammation and cytokine release are also implicated in neuronal damage. With increased METH consumption, the levels of dopamine indicators, including dopamine and tyrosine hydroxylase, reduce [1].

1.2. Glutamate and neurotransmission

1.2.1. Effects of glutamate on behavior and addiction

Glutamate (GLUT) plays an important role through ionotropic and metabotropic receptors in excitatory synapse. GLUT is involved in various neurological processes and behaviors as the main brain's stimulating neurotransmitter (Figure 2.). GLUT contributes to neurodevelopment as well as neuroplasticity that leads to the neuronal growth and maturation [4]. Additionally, N-Methyl-D-Aspartate (NMDA) and Alpha-Amino-3-Hydroxy-5-

Methyl-4-Isoxazoleproprionic Acid (AMPA) ionotropic glutamate receptors found in mammals directly influence synaptic strength and neural pathways [5]. Cocaine or alcohol addiction, influence the normal cell-to-cell communication that utilizes glutamate as a mediator. Modifications in the release and receptor activity of GLUT induce changes in excitatory synaptic plasticity associated with addictive behavior. Prolonged drug use can lead to a long-lasting change in the functioning of glutamatergic receptors, thus facilitating susceptibility to relapse during withdrawal.

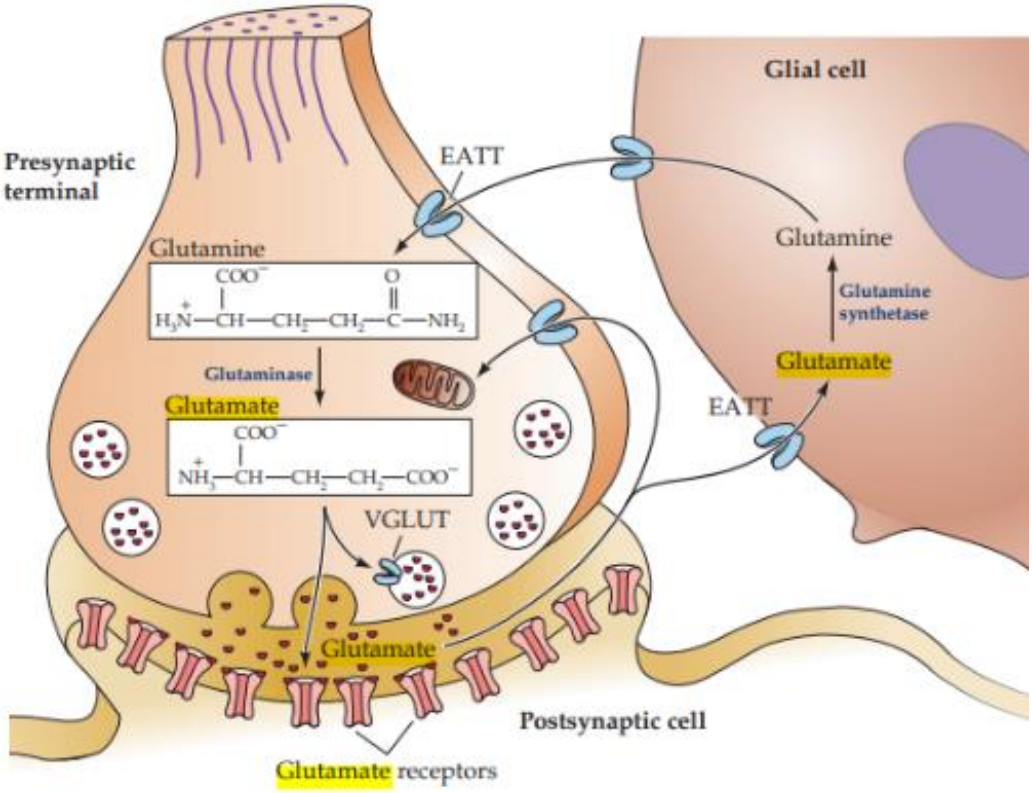

FIGURE 2. Between neurons and glial cells, glutamate is synthesized and recycled by a variety of mechanisms. Once it is released into the synapse, its actions are short-lived because of specialized transporters that retrieve it from both neurons and surrounding glial cells. Inside nerve endings, glial cells release glutamine that is taken up by neurons and converted back to glutamate. Excitatory amino acid transporters (EAT) move glutamate into the cell, whereas it enters presynaptic terminals through glutamate transporter proteins (VGLUT) [6].

D. melanogaster used in addiction studies shows that glutamate plays a significant role [4]. In this model, the molecular mechanisms that influence the change in synaptic plasticity resemble those that occur when glutamatergic receptor activity is altered [5]. In studies with fruit flies, changes in drug attractiveness are due to adaptations of specific signaling pathways that influence movement and similar functions [5]. These models use specific genetic variants or signaling pathways involved in the glutamatergic signaling [6], thereby providing insights into the cellular basis of addiction. Alterations in AMPA and NMDA functions in flies are seen to be the same as with mammals, indicating that these mechanisms have remained conserved throughout evolution. Therefore, behavioral consequences of addiction, like those associated with glutamatergic signaling are similar in both fruit flies and mammals.

1.3. Choline chloride and acetylcholine

1.3.1. Impact of acetylcholine on behavior and addiction

The principal excitatory neurotransmitter in the brain is acetylcholine (ACh). as the several brain functions and neuro-muscular activities depend on it. It can be synthesized from choline by the action of choline acetyltransferase across synapses through muscarinic and nicotinic receptors [7]. The presence of different forms of choline, such as choline chloride (CH.CL), is essential for maintaining the stability of the acetylcholine structure.

At the molecular level, ACh interacts with either ionotropic nicotinic acetylcholine receptors (nAChRs) or metabotropic muscarinic acetylcholine receptors (mAChRs). The activation of these two receptors results from change in the conductivity of ion channels, cAMP modulation, and phosphorylation of proteins [8], leading to low levels of ACh. As a key neurotransmitter that is widely distributed in the brain, can be greatly influenced by CH.CL, which significantly affects cognitive function and behavior (Figure 3.). In mammals, ACh represents one of the most important transmitters involved in learning, memory and synaptic plasticity.

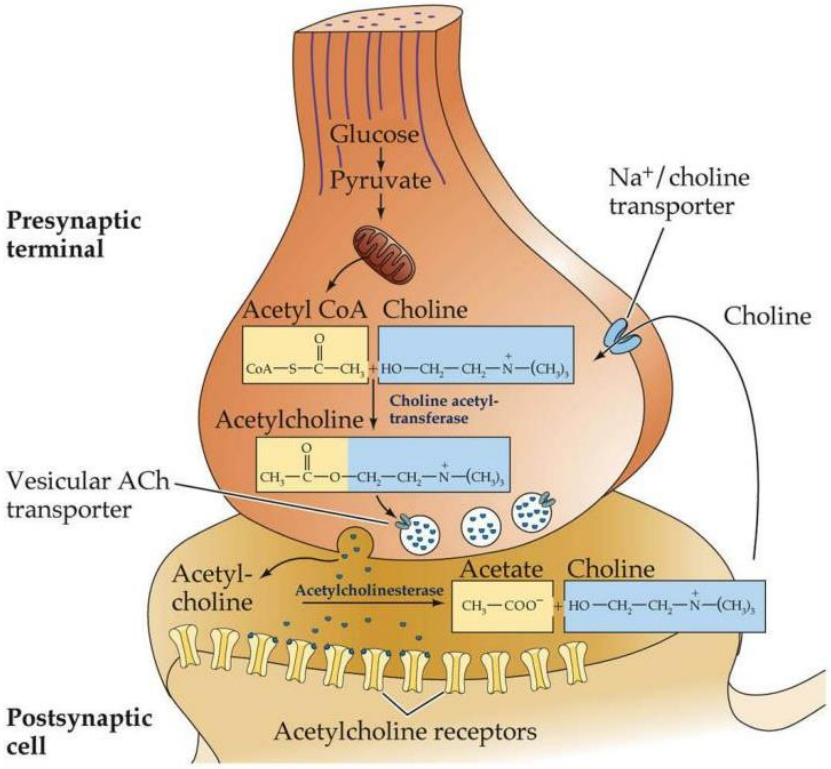

FIGURE 3. Synthesis of acetylcholine (ACh) in nerve endings receptive to cholinergic stimuli. Choline acetyltransferase (ChAT) is the enzyme needed for synthesis of ACh from choline and acetyl CoA. Acetyl CoA comes from pyruvate, which is product of glycolysis, while choline gets into terminals through a Na^+-dependent transporter. There are synaptic vesicles where the loading of ACh occurs trough vesicular transporter. As soon as released, ACh rapidly breaks down because of ChAT, while choline is transported back into the terminal [6].

Choline ions are released into synapses between nerve cells, where they serve as precursors for the synthesis of ACh, increasing brain activity and stimulation [7]. There is a significant decrease in stress and anxiety when choline chloride supplementation is applied [8], contributing to long-term brain health by participating in the creation of chemicals necessary for communication between cells and the preservation of their connections [9]. In studies with *D. melanogaster*, choline chloride improved performance in maze navigation tests and increased ACh levels, suggesting broader neural effects [9, 10]. Such observations show that brain energy can be enhanced in different organisms by supplements containing CH.CL.

1.4. *Drosophila* as a model for substance addiction

The study of addiction relies on the use of animal models in order to gain an understanding of the biological and neurochemical mechanisms underlying addictive behaviors. These models allow for controlled addiction studies and its potential treatments. Various methods, such as *in vivo* electrophysiology, optogenetics, or neuroimaging, are used to investigate this drug addiction on flies. Drug self-administration and conditioned place preference are among the several paradigms that mimic human drug addiction in animals by explaining the rewarding properties of addictive substances [11] [12].

D. melanogaster has become an important organism for studying addiction due to simple genome and nerve system, followed by short lifespan. Although it may have a simple brain, this species shares several fundamental biological processes with higher organisms, such as major neurotransmitters and cell types [13]. Genetic tools develop for genetic, molecular and behavioral studies are RNAi (RNA interference), CRISPR-Cas9, or transgenic expression systems [13]. These genetic tools are used preferentially to assess the impact of particular gene on addiction-related behavior. For instance, *D. melanogaster* models mammalian responses to addictive substances like alcohol, cocaine, and nicotine [14]. Locomotion, sedation, and preference may be affected by exposure to ethanol. Additionally, it has been shown that feeding patterns and locomotor activities of flies are changed by cocaine or nicotine [15]. These behaviors associated with addiction are measured reproducibly, allowing for the studies of the properties of drugs that are rewarding or the identification of genetic factors that make individuals vulnerable to addiction. Automated assays for drug-induced behaviors in conjunction with genetic interactions enable the identification of addiction phenotypes through neurotransmitter systems.

1.4.1. Modeling methamphetamine addiction in *Drosophila melanogaster*

The *D. melanogaster* organism serves as a model for studying the effects of METH on neurobiology and behavior. It has been shown that exposure to METH causes many types of behavior like those found in mice and rats, such as increased movement and preference towards food containing METH [16]. METH leads to hyperactivity and alterations in the daily sleep-wake cycle indicative of its stimulant nature. Moreover, when voluntary or non-voluntary exposed to METH, flies show hyperactivity. Exposure to one dose of volatile METH leads to sensitivity, shown as increase in locomotor activity. Furthermore, when flies are given a second dose in the same concentration, a gradual increase in locomotion occurs, known in the literature as locomotor sensitization [15]. When flies are offered to selects between a capillary containing drug versus regular sugar food in the FlyCAFÉ assay they chose more of drug containing food, which suggests that METH is rewarding to the flies [15]. In genetic research using this organism, some genes and signaling pathways stimulated through METH have been identified [16], such as those connected with dopamine signaling and neural plasticity.

1.4.1.1. CAFÉ and FlyCAFÉ assay

A method known as CAFÉ (Capillary Feeder) assay is used to assess the intake of liquid food in a population of fruit flies, by monitoring their feeding behavior using a capillary containing fluid sustenance (Figure 4.). This technique allows to measure how much group consumes over a certain period. It has proved very valuable in investigations on drugs such as cocaine and ethanol, which cause dependence [17]. The research has shown that flies prefer food consumption supplemented with METH or cocaine relative to non-supplemented food.

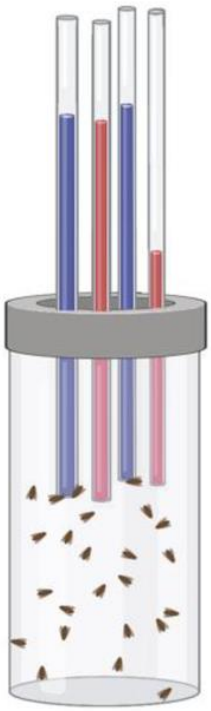

FIGURE 4. Schematic of the CAFÉ (Capillary Feeder) assay. The CAFÉ chambers are sealed at the bottom with non-woven fabric to allow for air circulation. Four trimmed pipette tips were inserted into the lid to hold glass microcapillaries containing liquid food. These capillaries were replaced daily at designated times. Identical chambers without flies were used as controls to account for evaporation, and these measurements were subtracted from the consumption data [15].

The FlyCAFÉ assay [15] is modification of the existing CAFÉ assay, which incorporates features for both feeding behavior and preference, but on the level of individual fly. The FlyCAFÉ assay uses a two-choice setup where individual fly can choose between a drug-containing food and regular food [15]. This setup allows for measuring not only the amount of substance consumed and the preference for the drug over a consecutive day, but also locomotor activity and time spent by capillary containing regular food or food with METH. The FlyCAFÉ has made it possible to understand how much food each fly eats individually. Additionally, with FlyCAFÉ, it can be determined whether the preference for food choices is due to a group effect or if it is truly present in individual flies. The assay has been applied to study MPC administration in flies [15].

1.5. Effects of social isolation on behavior and neurochemistry

Isolation, or social deprivation, represent minimal contact with other individuals, and has been reported to have significant impact on mental health. Prolonged isolation can lead to anxiety, depression, and cognitive decline, linked to changes in brain chemistry and structure. Using the *D. melanogaster*, it is possible to study neurological effects of isolation due to simple nervous system and well-mapped genetics. Isolation in *D. melanogaster* results in behavioral changes, such as increased aggression and altered sleep patterns [18]. Neurochemically, it affects neurotransmitter levels, impacting pathways related to mood regulation and social behavior [19].

1.5.1. Impact of social isolation on *Drosophila melanogaster* behavior

Social isolation has similar behavioral and neurological consensuses in *D. melanogaster* as in mammals. The absence of social interaction appears to elevate and increase aggressive behavior compared to flies that are housed in group [20]. Isolated flies show irregularities in their circadian patterns, suggesting that social isolation can affect physiological processes associated with internal time keeping mechanisms [18]. On a neurobiological level, isolated flies exhibit modifications in neuronal activity that correlate with heightened anxiety-like behaviors and diminished social engagement [20]. The relationship between social interactions and brain function underscores the importance of social environments in maintaining behavioral and mental health.

1.5.2. Studies on the neurochemical changes induced by social isolation

At the brain molecular level, social isolation affects many neurotransmitters responsible for sending messages from one neuron to another. Social isolation changes serotonin and dopamine concentrations, both involved in

regulating mood and different behaviors [20]. Correspondingly, reward processing and motivation are affected, leading to changes in behavior as consequence of modified social involvement [21]. The effects of social isolation can be seen at the level of neural circuits and gene expression [21]. Such transformations highlight how complexly intertwined social interaction is with neurobiological activities.

1.5.3. Influence of social isolation on vulnerability to substance addiction

Social isolation significantly impacts susceptibility to substance addiction, as shown in studies with both rodents and *D. melanogaster*. Research involving rats and mice has demonstrated that social isolation increases the vulnerability to addictive substances like ethanol and cocaine [13]. Isolated animals exhibit increased drug-seeking behaviors and consume higher amounts of addictive substances compared to their socially housed counterparts. For instance, rats living in small areas show increased locomotor activity and spend more time within drug-paired area than those living in larger spaces. In other words, drugs are more reinforcing to isolated animals [22]. Isolation disrupts levels of dopamine and serotonin, necessary for normal transmission in reward circuitry, and puts individuals on higher chances of being reinforced by drugs. Isolated mice shown different disfunctions of dopamine receptors, which leads to their greater susceptibility and consumption of drugs [22]. *D. melanogaster* studies also showed that isolation increases cocaine sensitivity as well as ethanol intake, indicative of disturbances in dopamine neural pathways [23]. Such findings emphasize how complicated is the relationship between social interaction and addiction. The changes in transmitters systems disrupted by isolation on rats or flies demonstrate that this condition alters neurobiological reactions in a way that enhances vulnerability towards substance abuse.

1.6. *Drosophila* model of social isolation and addiction

The *D. melanogaster* is a good model to study how social isolation affects addiction-related behaviors and neural mechanisms. In a study of ethanol preference, the ethanol consumption is changed significantly following isolation of fruit flies. Isolated flies demonstrate a higher preference towards the consumption of ethanol when matched against their counterparts who live in groups, what implies that isolation leads to more vulnerability to addiction. The rise in consumption is connected to alterations made by the brain on reward system especially in pathways of dopamine [23]. Not only does ethanol intake increase when a fly is isolated but also its locomotion and feeding behaviors are changed as shown in behavioral analysis. Neurochemical studies support this finding by showing that there is change in serotonin levels and dopamine signaling coinciding with more drug use [21].

Previous studies from our laboratory looked at influence of different isolation duration in three to five day-old male flies on METH preferential consumption (MPC) using FlyCAFÉ. It was shown that flies which have been isolated for one day have lower MPC compared to non-isolated flies. When isolation was prolonged to five days, reduction in MPC was even higher than with one day of isolation [24]. Based on the obtained results, an LC-MS/MS analysis of the concentration of monoamines in the heads of flies that were isolated for one and five days was performed, and it was found that there was a reduction of dopamine, octopamine, glutamate and acetylcholine. The reduction was more pronounced for five days of isolation compared to one day of isolation (unpublished results). Considering the above results and the fact that by feeding flies with precursors of glutamate, which is glutamine, and the precursor of acetylcholine, which is choline chloride, it is possible to pharmacologically increase their concentration, the impact of supplementation during five days of isolation on MPC was tested.

2. AIMS

The main goal of the research was to determine the effects of social isolation on METH preference in *D. melanogaster* can be changed by supplementation with L-glutamine (GLU) or choline chloride (CH.CL).

To achieve this, we isolated three to five day-old male flies for five days and supplemented them with either CH.CL or GLU. Over three successive days, we used the FlyCAFÉ method to measure quantitatively the preference for MPC relative to the regular food. This enabled us to track and compare food consumption between non-supplemented (control) flies and those receiving supplements.

We hypothesized that supplementing with GLU or CH.CL during isolation would significantly alter MPC when compared to the control group.

3. METHODS AND MATERIALS

3.1. Fly Strains and maintenance

For experiments we used adult three to five day-old male *Drosophila melanogaster*, of wild type (*wt*) *Canton S* background. The flies were raised in the vials containing standard cornmeal/agar medium under controlled conditions of 25°C and 70% humidity, with a 12-hour light/dark cycles.

3.2 Chemicals

L-Glutamine (GLU) (≥98%) was purchased from Alfa Aesar, choline chloride, (CH.CL) (≥ 99%) was purchased from Thermo Scientific, methamphetamine hydrochloride (METH) (≥)98%), and mineral oil were purchased from Sigma Aldrich.

3.3 Oral Administration of Glutamate and choline chloride

To investigate the effects of monoamines reduction after five-day isolation, we fed *wt* male flies with 10 mg/mL GLU or 3 mg/mL CH.CL. First, using CO_2 anesthesia we have collected three to five day-old male flies from cultivation bottles. Flies were then individually housed for five days on cornmeal/agar medium supplemented with GLU or CH.CL. Pretreatment was conducted in an incubator under controlled conditions (25°C; 70% humidity; 12-hour light/dark cycle) before measuring the preferential consumption using the FlyCAFÉ assay (Figure 5.).

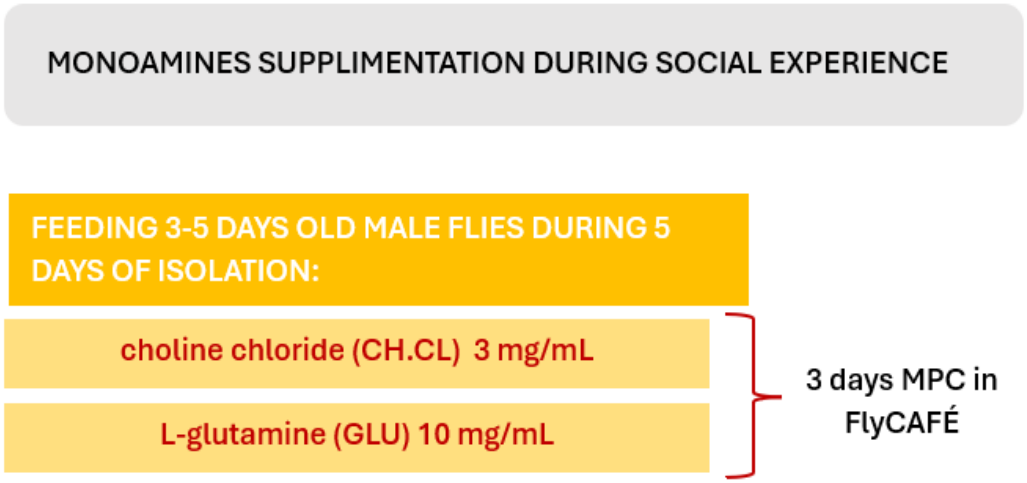

FIGURE 5. CantonS male flies, raised on cornmeal food, were separated at three to five day-old for the isolation process. For isolation, two groups of 32 flies received different supplemental food treatment. After isolation, both groups were placed in a glass tube for FlyCAFÉ assay. Preference was measured in constant darkness for three consecutive days (MPC), with food consumption measured every 24 hours.

3.4 FlyCAFÉ Assay

In our study, we utilized the FlyCAFÉ assay, which combines the standard two-choice CAFE assay with the Drosophila Activity Monitoring System (DAMS) (TriKinetics, Waltham, MASS). Individual flies were placed in standard DAM system glass tubes (65 × 5 mm) modified by attaching a 1.5-cm rubber tube to each end. The rubber cap was covered with nylon mesh and fixed with parafilm to allow water vapor entry and prevent dehydration. A hole was drilled in the upper middle of each rubber cap to fit a 200-µl pipette tip, which was modified to hold a 5-µl glass capillary (Hirschmann) (Figure 6A). Capillaries were filled via capillary action with mineral oil to minimize evaporation and then with liquid food. Liquid food was a 100-mM sucrose and 0,05 mg/mL yeast solution prepared in tap water, while METH food was a mixture of liquid food with 0,15 mg/mL METH. The height of the liquid food in the capillary was measured using a ruler, and the capillaries were then inserted into the pipette tip to be easily accessible to the flies. The amount of liquid consumed in the capillaries was measured daily at 10:00 AM and replaced with freshly prepared capillaries. Control flies had a choice of liquid food from both capillaries, whereas experimental flies were divided into two groups of 11 with METH and food offered on alternate sides of the glass tubes to eliminate potential side bias (Figure 6C). The DAM5M monitor was placed on a pedestal in a plastic tub with 1 L of tap water, covered with cling film to minimize humidity fluctuations and evaporation. The tub was placed inside an incubator set at 24°C in constant darkness to prevent side preference due to environmental cues (Figure 6B). To account for potential evaporation, we had tubes without flies containing liquid food in both capillaries. Each day, the amount of consumed food was corrected for average evaporation using these evaporation control tubes.

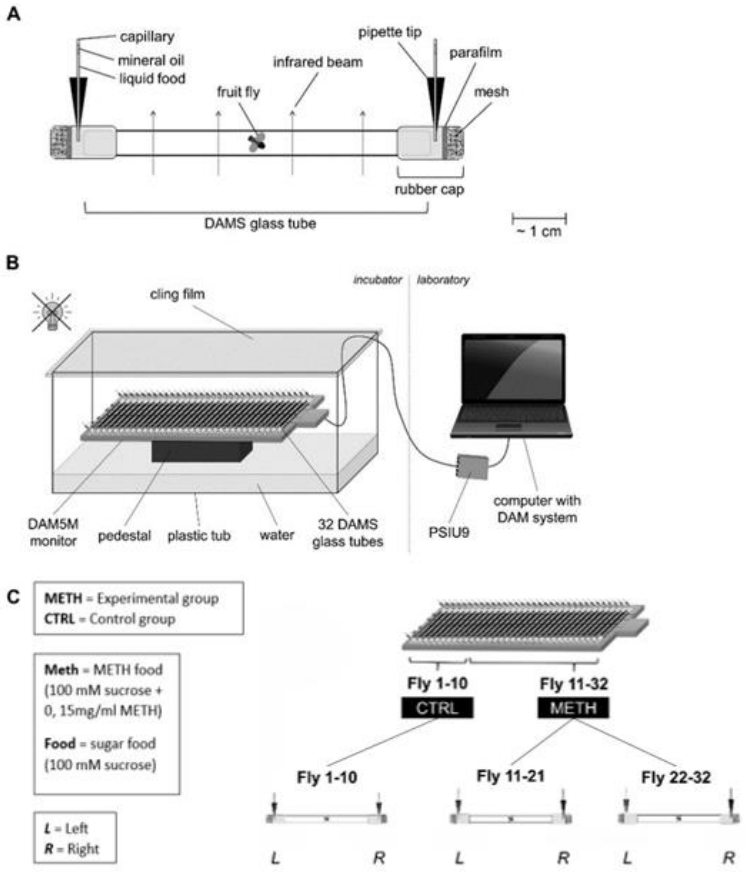

FIGURE 6. FlyCAFÉ: a method for measuring preferential consumption of food in individual flies. A Drosophila Activity Monitoring System (DAMS) glass tube with modified ends for insertion of glass capillaries with liquid food. B, FlyCAFÉ experimental setup consisting of DAM5M DAMS monitor, that holds 32 glass tubes with individual flies, placed in a sealed container with water, and connected to a computer that collects locomotor activity data every minute during a 24-h period. C, Standard procedure for positioning glass tubes in a DAM5M monitor. METH, methamphetamine.

This study included three groups of flies:

- ***CTRL (Control Group):***

This group represents the control and consists of male flies aged three to five days, kept under standard conditions (grouped on sugar based liquid food) [24].

- ***Experimental Group 5D (Isolated Without Supplementation):***

This group consists of three to five-day-old male flies that have been isolated and fed sugar based liquid food [24].

- ***Experimental Group CH.CL or GLU (Isolated With Supplementation):***

This group consists of three to five-day-old male flies that have been isolated and fed sugar based liquid food with supplementation with CH.CL or GLU

3.5 Data analysis and statistics

All data from the FlyCAFÉ assay was processed using MS Excel to calculate preference. Statistical analyses and visualizations were conducted using (GraphPad Prism 10.4.3). Differences between treatments across different days were analyzed using two-way ANOVA, followed by Tuky post hoc test. Difference between averages of three days were analyzed by One-way ANOVA with Bonferroni post hoc test. Differences were considered significant if $p < 0.05$

4. RESLUTS

Previous studies have shown that social isolation reduces monoamine concentrations and MPC in *D. melanogaster* (unpublished results). The study included three groups: control group (CTRL), and 5 days isolated experimental groups; flies without supplementation (5D) and supplemented (CH.CL or GLU). CTRL and 5D results are from V. Dukić master thesis. To investigate the role of monoamine reduction in MPC following five days of isolation, flies were supplemented with either 10 mg/mL L-Glutamine (GLU) or 3 mg/mL choline chloride (CH.CL) during the isolation period. The MPC was then assessed using the FlyCAFÉ assay over a period of three days.

4.1. Supplementation with choline chloride leads to positive preference for METH in isolated male flies

In male flies aged three to five days, we examined the effects of supplementing choline chloride (CH.CL) for five days during isolation on their MPC. Flies were offered a choice between two capillaries, one containing liquid sugar-based food and the other sugar food with METH. The difference in volume of food consumed by individual flies from each capillary over a period of twenty-four hours in three successive days determined preference in FlyCAFÉ assay.

Using these results, we can compare how flies preferred food containing METH (Figure 7.A). The CTRL group consistently exhibited a mild positive preference for METH. Similarly, on the first day, both 5D and CH.CL flies showed a positive preference for METH, but in the 5D, this preference dropped dramatically by the third day ($*p < 0.05$). CH.CL flies maintained a positive preference for METH across all three days, and on the second and third day, their preference was significantly higher than that of the 5D flies. We did not observe any significant difference between the CTRL and CH.CL flies.

When comparing the three-day averages, we found a notable difference (*p < 0.05) between the CTRL and 5D flies (Figure 7.B). The 5D flies showed a negative preference, while the CH.CL flies exhibited a preference similar to that of the CTRL.

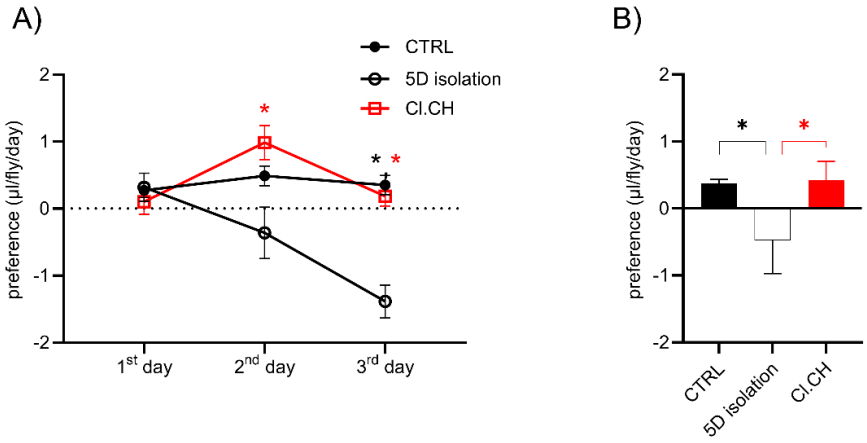

FIGURE 7. Supplementation with the choline chloride, precursor of acetylcholine, during five days of isolation leads to increased preference for METH in the FlyCAFÉ assay. **A)** Preference was measured on three consecutive days in the FlyCAFÉ assay as the difference in the ingested volume of food with METH and food without METH in CTRL (flies without isolation *n*=22), 5D isolated (flies that were in isolation for five days *n*=22) and Cl.CH (flies that were fed with choline chloride during five days of isolation *n*=22). Data are presented as mean +/- SEM. Two-way ANOVA with Tuky post hoc test, * for p< 0.05. **B)** Average value +/- SEM for three days of flies under A). One-way ANOVA with Bonferroni post hoc test * for p< 0.05.

4.2. Supplementation with L-Glutamate leads to positive preference for METH in isolated male flies

The experiment was performed similarly to the previous, but with the addition of GLU supplementation. We investigated the effect of GLU supplementation during five days of isolation on MPC in three to five-day-old male *D. melanogaster*. Results for the CTRL and 5D groups are based on V. Dukić's master's thesis.

GLU group had a significantly higher preference than the 5D group on the second and third day (Figure 8.A), while difference is similar as in the Figure 7.A between CTRL and 5D.

Flies supplemented with GLU had higher MPC compared to those who were only isolated for 5 days (Figure 8.B). There was no difference between CTRL and GLU group in MPC (Figure 8.B).

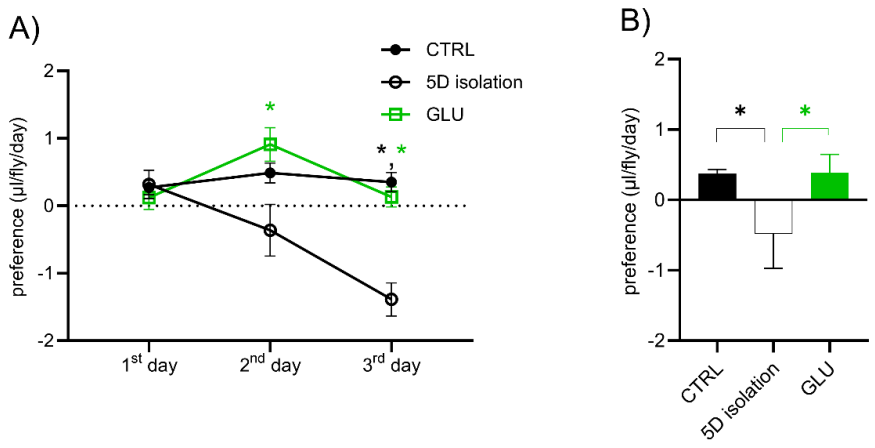

FIGURE 8. Supplementation with the glutamine, precursor of glutamate, during five days of isolation leads to increased preference for METH in the FlyCAFÉ assay. **A)** Preference was measured on three consecutive days in the FlyCAFÉ assay as the difference in the ingested volume of food with METH and food without METH in CTRL (flies without isolation *n*=22), 5D isolated (flies that were in isolation for five days *n*=22) and GLU (flies that were fed with glutamine during five days of isolation *n*=22). Data are presented as mean +/- SEM. Two-way ANOVA with Tuky post hoc test, * for p< 0.05. **B)** Average value +/- SEM for three days of flies under A). One-way ANOVA with Bonferroni post hoc test * for p< 0.05

5. DISCUSSION

The goal of our study was to examine the effects of neurotransmitter precursor supplementation on MPC in socially isolated in *D. melanogaster*. The previous observations showed that a five-day period of isolation significantly diminishes METH consumption in flies (V. Dukić, Master Thesis, 2022) and that social isolation leads to a significant decrease in several major brain neurotransmitters (unpublished results). To explore whether the decrease in neurotransmitters associated with isolation directly influences MPC, we supplemented the flies with GLU and CH.CL. These two neurotransmitter precursors are known to be reduced during isolation.

Both glutamate (GLUT) and acetylcholine (ACh) are essential neurotransmitters with important roles in brain function, making them particularly significant for neurobiologists. GLUT is the prevalent excitatory neurotransmitter in the brain that is involved in synaptic plasticity processes as well as reward pathways. An increase in GLUT levels is potentially capable of affecting the activity of glutamatergic receptors, which changes drug behavior [5]. In comparison, ACh is also well known for a number of functions such as memory and coordination of motor actions. Moreover, its involvement in reward mechanisms and addiction is also widely established; disruptions of cholinergic signaling can affect dopaminergic pathways linked to drug-seeking behavior [7] [9]. As a main result, our experiment revealed that adding the neurotransmitter precursor glutamine (GLU) and choline chloride (CH.CL) led to a significant change in MPC in isolated flies. More precisely, our results suggest that METH reward pathways are somehow altered and that supplementation with these neurotransmitter precursors may induce changes in the activation of reward pathways, which could diminish or even alter the reward response to METH, as reflected in changes in preference for this substance.

Our research reveals some key insights into how GLU and CH.CL supplementation affects MPC. The greatest difficulty, perhaps, is determining the level of recovery in terms of GLU and CH.CL. Without precise measurements, it is very hard to predict the effects of these changes on the other neurotransmitters in the brain. Since neurotransmitter systems are so interconnected, a shift in one can affect many others. Another significant issue is the lack of clarity on how GLU and CH.CL interact with other neurotransmitter systems, like those involving dopamine, GABA, and serotonin. For example, GLUT may regulate dopamine's function which has to do with motivation and reward, while ACh uses its receptors to affect different neurotransmitter pathways, such as cholinergic pathway [4] [7] [8].

These interconnected systems suggest that changes in GLU and CH.CL levels could lead to complex and unexpected changes in brain chemistry, making it difficult to predict their impact on behavior. Besides, we still do not have enough information on how GLU and CH.CL supplementation influences important metabolic pathways. We know that ACh plays crucial roles not only in the nervous system but also in peripheral tissues, where it helps regulate inflammation, immune responses, and energy metabolism [7] [8]. Because of that, changes in ACh levels could also have more far-reaching effects beyond even the central nervous system, and therefore only make it harder to understand the full impact of these supplements. Additionally, how GLU and CH.CL interact with the brain's reward systems might lead to unexpected outcomes. By supplementing with these neurotransmitter precursors, the reactions to METH or other addictive substances are also likely to be changed.

This research indicates that supplementing with GLU and CH.CL brings about a change in the molecular system, reducing MPC in isolated flies. The reason why these flies do not show MPC after isolation may be related to complex neurochemical changes in the brain's reward systems. Increasing concentrations of GLU and CH.CL could also potentially

redistribute neurotransmitter resources and alter how these reward pathways respond to METH. This redistribution may decrease the rewarding value of METH or change the outlook towards METH as a reward [1] [2] [16].

However, the results of this study contrast with the previous studies, which state that isolation is likely to increase drug consumption. For example, prolonged isolation has been shown to increase ethanol consumption in mice [3] [18]. From these studies, it can be concluded that social isolation might in all probability cause drug-associated behavior as a means of relieving the stress or anxiety brought about by isolation [19] [20].

It should be highlighted that the methods of isolation, particularly for mice and flies, are different, and this is important in understanding the findings since the two models are being used. It is significant to note that in mouse studies, the preference for the substance is dependent on the sex of the individual, whereas our study dealt only with the male gender; therefore, we cannot determine how females might respond to this [22] [23]. From our previous research [24], we've also seen that mating does not increase METH preference, whereas in mice, there is a significant preference for ethanol, especially in those that were not able to mate. Another important factor that could explain the decreased METH consumption in flies is age, since we compare isolated flies of different ages while considering both the control and experimental groups [13] [21].

Further analysis of GLU and CH.CL in the context of METH addiction points to a strong connection between them. Further evidence in support of this idea comes from experiments that involve the use of RNA interference (RNAi) and genetic knockouts, which are used to knock down the expression of a specific gene. This allows the researchers to actually see the effects of lowering the levels of GLU or CH.CL on the animals' behavior [5] [6] [12]. These techniques provide scientists with a direct perspective on the effects of reduced GLU or CH.CL levels on animals. Studies employing these

systems have demonstrated that it is possible to influence reward-related behaviors, such as the response to METH, through modulation of the levels of peripheral neuropathic surgery drugs. This implies that variations in these neurotransmitter levels do not only affect one pathway, but rather help transform the overall structure of the brain that is important for related behaviors and addictions [11] [14].

Our results provide insight into the relationship between the levels of specific neurotransmitters and METH preference, suggesting that more studies are needed in order to clarify the complex mechanisms involved in drug-related behavior. Knowledge of such mechanisms is likely to be useful in developing new ways of dealing with addiction and other disorders associated with the brain's reward system [13] [17] [20].

6. CONCLUSION

Our research investigated how METH preference in *D. melanogaster* is affected by social isolation as well as supplementation with neurotransmitter precursors. According to our findings, a five-day period of social isolation significantly decreased the consumption of METH; this finding contradicts the common assumption that addiction is intensified by loneliness. In addition, we found out that when GLU or CH.CL were given during isolation that increased METH preference showing that such chemical messengers may nullify some consequences brought about by isolation.

These findings are surprising because they contradict findings that addiction is made worse by being isolated. According to what we have found, being isolated may affect brain chemicals that control the extent of drug attraction or aversion. This supports studies which demonstrate alterations in sensitivity to pleasure caused by stress-related events and their implications for drug choice.

Their different biochemistry makes it surprising that GLU and CH.CL impacts were not appreciably distinct. It may be because of the conditions under which experiments were conducted or doses administered not adequately accounting for their differential effects on organisms; therefore, this might suggest that more attention should be paid to baseline levels of neurotransmitters in studies related to addiction.

Our discoveries help in describing the intricate relationship of social aspects with chemical transformations in addiction. They highlight that we should take up more investigations to explore into the ways through which drug choice is influenced by neurotransmitter precursors and also consider social withdrawal. These findings may be used in future treatments because they give an idea on what kind of impact does socialization have over biological processes that lead somebody becoming addicted to drugs of abuse.

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O MENI

Kao studentica, vrlo sam motivirana u pronalasku posla u kojem bih mogla steći novo iskustvo i nove vještine. Vještine koje već posjedujem su komunikativnost, snalažljivost, ustrajnost i volja za rad. Uz to vrlo sam ambiciozna i svestrana stoga me zanimaju različita područja u kojima bih voljela napredovati i steći životno iskustvo. Moj osobni razvoj kroz posao za koji se prijavljujem očituje se u timskom radu kao i odgovornosti te stručnosti što i jest cilj fakulteta kojeg studiram.

RADNO ISKUSTVO

[01/08/2024 – 01/09/2024]

Prodavačica sportske opreme *INTERSPORT H d.o.o.*

Mjesto: Rijeka | **Zemlja:** Hrvatska

[01/07/2024 – 01/08/2024]

Prodavačica *ITX d.o.o.*

Mjesto: Rijeka | **Zemlja:** Hrvatska

[15/07/2022 – 30/08/2023]

Konobarica, ***Ugostiteljski obrt "MG", Vl. Gordana Dugandžić***

Mjesto: Funtana | **Zemlja:** Hrvatska

[05/07/2021 – 16/08/2021]

Sobarica, ***Valamar d.o.o.***

Mjesto: Funtana | **Zemlja:** Hrvatska

OBRAZOVANJE I OSPOSOBLJAVANJE

[04/10/2021 – Trenutačno]

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Odjel za biotehnologiju i istraživanje lijekova

[2017 – 2021]

Srednjoškolska diploma

Gimnazija Matije Antuna Reljkovića, Vinkovci

[2009 – 2017]

Osnovno obrazovanje

Osnovna škola Zrinskih Nuštar

JEZIČNE VJEŠTINE

Materinski jezik/jezici:

hrvatski

Drugi jezici:

engleski

SLUŠANJE **C1** ČITANJE **C1** PISANJE **B2**

GOVORNA PRODUKCIJA **B2** GOVORNA INTERAKCIJA **B2**

njemački

SLUŠANJE **B1** ČITANJE **B2** PISANJE **B1**

GOVORNA PRODUKCIJA **B1** GOVORNA INTERAKCIJA **B1**

DIGITALNE VJEŠTINE

Moje digitalne vještine

Komunikacijski programi (Skype Zoom TeamViewer) | MS Office (Excel, Power Point, Word, Outlook, Teams) | MS Office (MS Word, MS Excel, MS PowerPoint, MS Outlook, MS Teams) | -rad na računalu | -društvene mreže | Internet

Organizacijske vještine

Timski rad | Prilagodljivost | Dobro organizirana | Komunikativna

HOBIJI I INTERESI

Putovanja

Planinarenje

Slikanje

Različite sportske aktivnosti (joga, poledance, tenis, plivanje, kickbox)

KOMUNIKACIJSKE I MEĐULJUDSKE VJEŠTINE

Aktivno slušanje

Komunikativnost

Strpljivost

Empatičnost

Odgovornost

Susretljivost

VOLONTIRANJE

[04/03/2021 – 04/03/2021]

Projekt "Svaki razred jedno stablo"

Vladimira Gortana 16, 32100, Vinkovci

Druženje s osobama tjelesnog i mentalnog oštećenja u sklopu humanitarnog projekta "Svaki razred jedno stablo" u Centru za rehabilitaciju "Mala Terezija".

[19/04/2023 – 19/04/2023]

Projekt "Putujući znanstvenici"

Osnovna škola Čavle

Upoznavanje učenika s praktičnim pokusima u području kemije.

[24/04/2023 – 24/04/2023]

Projekt "Otvoreni dani fakulteta"

Radmile Matejčića 2, 51000, Rijeka

Upoznavanje studenata sa sadržajem fakulteta, njegovim zanimljivostima i praktičnim pokusima iz laboratorija.

[01/12/2023 – 02/12/2023]

Konferencija "Darwin"

Radmile Matejčića 2, 51000, Rijeka

Obilazak JGL-a s naglaskom na održive prakse u industriji te predavanja s tematikom zelene kemije, klimatskih promjena, korištenju otpada kao goriva i održivog uzgoja hrane.