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3,4-METHYLENEDIOXYMETHAMPHETAMINE – INDUCED NEUROTOXICITY IN EXPERIMENTAL MICE

S 3,4-METILENDIOKSIMETAMFETAMINOM POVZROČENA NEVROTOKSIČNOST NA EKSPERIMENTALNIH MIŠIH

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I have performed and written my Master Thesis in University of Cagliari, in department for Biomedical Science, with collaboration of the Faculty of Pharmacy, University of Ljubljana. I have worked under the mentorship of Prof. Dr. Irena Mlinarič Raščan, PhD. and under the co-mentorship of Prof. Dr. Micaela Morelli, BScD.

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Statement

I declare that I have performed and written this Graduation Thesis solely by myself under the mentorship of Prof. Dr. Irena Mlinarič Raščan, PhD and co-mentorship of Prof. Dr. Micaela Morelli, BScD.

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ABSTRACT

Several preclinical reports suggest that amphetamine-related drugs may disrupt dopaminergic transmission and impair memory functions. In line with this, clinical observations report a higher propensity to develop Parkinson's disease in amphetamine users. Ecstasy, 3,4-methylenedioxymethamphetamine (MDMA) is an amphetamine-related drug largely consumed by adolescents and young adults, which raises concern about its acute and long-term effects. Our goal was to evaluate the effect of MDMA on memory functions and to evaluate if MDMA might modulate the effects of toxins known to induce dopamine neuron degeneration. Therefore we evaluated the vulnerability to neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) after a chronic administration of MDMA during late adolescence in mice.

We performed MDMA (10 mg/kg, i.p.) or vehicle (saline) for 9 weeks to simulate a chronic treatment in mice aged 8 weeks. Two weeks after treatment discontinuation, mice were treated with MPTP (20 mg/kg, i.p.) or vehicle for 4 days. To highlight any cognitive deficits in short-term/working memory, mice were evaluated in two cognitive tests: Novel object recognition and Spontaneous alternation behavior in a Y-maze at the following time points: after 24 hours, at four weeks, and at nine weeks of MDMA administration, after two weeks of MDMA wash-out, and after 24 hours from the last injection of MPTP.

Mice chronically treated with MDMA did not show any significant differences in the percentage of time spent in exploring the novel object till the last time point examined, when they spent a significant shorter time in exploring the novel object, comparing with vehicle-treated mice. After MPTP treatment, both MDMA-vehicle and vehicle-MPTP treated mice spent a significant less time in exploring the novel object comparing with vehicle-treated mice, and MDMA-MPTP treated mice showed a further reduction.

On the other hand, MDMA-treated mice did not show any significant modifications in spontaneous alternation behavior in a Y-maze at any time point.

In addition, mice were sacrificed and their brain sections were isolated for immunohistochemistry for detecting neuroinflammation and neurotoxicity of MDMA and MPTP, where in MDMA-pretreated mice MPTP treatment induced an increased activation of astroglia and microglia, and reduced numbers of dopaminergic neurons in SNc and striatum.

RAZŠIRJEN POVZETEK

Številne predklinične študije kažejo, da lahko droge amfetaminskih derivatov povzročijo selektivno nevrotoksičnost na dopaminergični prenos ter okvarijo tudi funkcije spomina. V skladu s tem poročajo o večji nagnjenosti k razvoju Parkinsonove bolezni pri uporabnikih amfetamina. 3,4-metilendioksimetamfetamin (MDMA, 'ekstazi') je derivat amfetamina, široko uporabljena droga pri mladostnikih in mladih, ki vzbuja zaskrbljenost zaradi svojih akutnih in kroničnih učinkov. Zlasti kritično je jemanje te substance v obdobju adolescence, ko so možgani toliko bolj občutljivi in dovzetni za poškodbe in je njihovo normalno delovanje ključnega pomena za nastajanje ali brisanje mednevronskih vezi. Znane so vzročne povezave med jemanjem drog v času adolescence in povečanim tveganjem za razvoj odvisnosti v obdobju odraslosti. Namen diplomskega dela je bil oceniti, ali uporaba MDMA med adolescenco okvari funkcije spomina in ali vpliva na učinke nevrotoksinov, ki povzročajo degeneracijo dopaminskih nevronov. Tako smo vrednotili škodljivost nevrotoksina 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) po kronični uporabi MDMA v pozni adolescenci pri miših.

Raziskave na miših so zelo specifične, saj lahko njihovi spol, starost in telesna masa različno vplivajo na obseg toksičnosti MPTP, kot tudi na možnost pojava lezij. Samice so manj občutljive na MPTP in kažejo večjo variabilnost v obsegu poškodbe kot samci, enako velja za miši, mlajše od 8 tednov in lažje od 25g. Optimalno vzbujanje toksičnosti MPTP dosežemo z izborom mišjih samcev, starih 8-10 tednov in težkih 25-30g.

V ta namen smo izvedli 9-tedenski poizkus, kjer smo 8 tednov stare eksperimentalne mišje samce kronično izpostavili substanci MDMA (10 mg/kg, i.p.), skupino kontrolnih miši pa smo obravnavali samo s topilom oziroma medijem. Vsaka miš je prejemala 2 odmerka dnevno, ločena s 4-6 urnim intervalom, dvakrat tedensko, torej je skupno prejela 36 odmerkov. Dva tedna po prekinitvi izpostavljenosti MDMA smo 19 tednov starim mišim dodajali MPTP (20 mg/kg, i.p.), kontrolnim mišim pa samo medij. Eksperimentalne skupine so bile torej štiri: MDMA-medij, MDMA-MPTP, medij-MPTP in medij-medij. Miši so prejemale MPTP enkrat dnevno 4 zaporedne dni.

Kognitivne okvare v kratkotrajnem oziroma delovnem spominu lahko dokazujemo z različnimi kognitivnimi testi. Za ocenjevanje kognitivnih sposobnosti testiranih miši smo uporabili dva kognitivna testa: prepoznavanje novega predmeta in spontano menjavanje

položaja v Y labirintu. Testi so bili opravljeni v naslednjih časovnih obdobjih: po 24 urah, pri 4 tednih in pri 9 tednih dajanja MDMA, po dveh tednih očiščenja MDMA ter 24 ur po zadnji injekciji MPTP.

Test prepoznavanja novega predmeta (NOR) je široko uporabljen kognitivni test, ki ga izvajajo na glodavcih za ocenjevanje kratkotrajnega oziroma delovnega spomina. Miši je potrebno privaditi na novo kletko, v kateri izvedemo test. Nepoznavanje in strah novega prostora bi namreč lahko povzročila nezanesljivost rezultatov. Tako smo vsako posamezno miš 24 ur pred začetkom testa dali za 5 minut v prazno kletko, da jo je raziskala. Na dan testa smo vsako miš dali v kletko za dva 3 minutna intervala (S1 in S2) in pustili, da je prosto raziskovala predmete. V prvem delu (S1) sta bila prisotna dva enaka predmeta, v drugem (S2) pa je bil prisoten en enak iz prejšnjega dela ter en nov predmet. S1 in S2 sta bila ločena s 60 minutnim natančno kontroliranim intervalom. Raziskovanje novega predmeta smo opredelili kot vohljanje, glodanje in dotikanje nosu miši s predmetom, medtem ko sedenje ali premikanje okoli predmeta nista bila definirana kot raziskovanje. Predmete smo po vsakem intervalu očistili vonja, saj bi ta lahko vplival na nepravilno raziskovanje novega predmeta. Med testom smo miši posneli z videokamero, raziskovalci pa smo zapustili prostor, da ni bilo nikakršnega vpliva na obnašanje miši pri raziskovanju novega predmeta. Videoposnetke smo nato analizirali s pomočjo računalnika, kjer smo določali naslednja parametra: a) čas, ki ga je vsaka miš porabila za raziskovanje predmetov v intervalih S1 in S2, ter b) prepoznavanje novega predmeta, ki je bilo izračunano kot razmerje med časom raziskovanja novega predmeta in celotnim časom raziskovanja obeh predmetov.

Miši, ki so bile kronično tretirane z MDMA, v primerjavi z ostalimi eksperimentalnimi skupinami niso pokazale nobenih signifikantnih razlik v odstotkih časa raziskovanja novega predmeta vse do zadnje časovne točke. Po dveh tednih očiščenja MDMA so MDMA-tretirane miši porabile signifikantno manjšo količino časa za raziskovanje novega predmeta v primerjavi z medij-medij tretiranimi mišmi. 24 ur po zadnji injekciji MPTP so tako MDMA-medij, kot tudi medij-MPTP tretirane miši porabile signifikantno manjšo količino časa v raziskovanju novega predmeta v primerjavi z medij-tretiranimi mišmi. MDMA-MPTP tretirane miši so pokazale še večjo okvaro spomina, saj so za raziskovanje novega predmeta porabile najmanj časa, v primerjavi z MDMA-medij, medij-MPTP in medij-medij tretiranimi mišmi.

Test vrednotenja spontanega menjavanja prostora v Y labirintu je prav tako pogosto uporabljen za preučevanje kratkoročnega spomina in splošnih kognitivnih funkcij pri glodavcih. V črnem labirintu v obliki črke Y smo miši testirali z 8 minutnimi intervali. Test smo izvedli vedno en dan po NOR, saj mišim povzroča večji stres in bi v obratnem vrstnem redu lahko vplival na spremenjeno raziskovanje novega predmeta v NOR. Spontano menjavanje prostora v Y labirintu smo prav tako posneli z videokamero in posnetke analizirali s pomočjo računalnika. V nadaljevanju smo izračunali odstotek spontanega menjavanja vej Y labirinta, ki je bil opredeljen kot razmerje med pravimi tripleti in vsemi tripleti.

MDMA-tretirane miši niso pokazale nobenih signifikantnih sprememb v spontanem menjavanju mest v labirintu Y v nobeni časovni točki raziskave v primerjavi z ostalimi eksperimentalnimi skupinami. Po 48 urah od zadnje injekcije MPTP so bile ponovno testirane v Y labirintu in prav tako nismo opazili nobenih signifikantnih razlik med skupinami miši.

Nadalje so bile miši žrtvovane, sekcije njihovih možganov pa izolirane za imunohistokemijo. Za odkrivanje nevrovnetja ter nevrotoksičnosti MDMA in MPTP so bili uporabljeni označevalci GFAP za aktivacijo astroglije, CD11b za aktivacijo mikroglije in TH za dopaminergično degeneracijo. Pri miših, kronično tretiranih z MDMA med pozno adolescenco, je administracija MPTP povzročila povečano aktivacijo astroglije in mikroglije ter zmanjšano število dopaminergičnih nevronov v črni substanci in striatumu.

Epidemiološke študije kažejo, da izpostavljenost vplivom okolja, na primer različnim kemikalijam, pesticidom ali drogam v mladosti, lahko pripomore k razvoju kognitivnih okvar in nevrodegenerativnih bolezni kasneje v življenju. Rezultati naše raziskave so pokazali, da kronična uporaba MDMA med pozno adolescenco poveča občutljivost miši za okvaro funkcij delovnega spomina, povzroči dodatno okvaro spomina zaradi dopaminergičnega toksina MPTP in nevrovnetje. Študija dokazuje, da uporaba drog amfetaminskih derivatov med adolescenco kritično vpliva na razvoj odvisnosti v obdobju odraslosti, povzroči pa tudi večjo občutljivost centralnega živčnega sistema na nevrotoksine, ki pozneje v življenju povzročijo okvare dopaminskih nevronov.

LIST OF ABBREVIATIONS

5-HT	serotonin
CNS	central nervous system
CSF	cerebrospinal fluid
DA	dopamine
DAT	dopamine transporter
MDMA	3,4-methylenedioxymethamphetamine
mPFC	medial prefrontal cortex
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA	noradrenaline
NOR	novel object recognition task
PD	Parkinson's disease
PFC	prefrontal cortex
SERT	serotonin transporter
SNc	substantia nigra pars-compacta

1 INTRODUCTION

1.1. 3,4-Methylendioxymethamphetamine (MDMA)

3,4-Methylenedioxymethamphetamine (MDMA), also famous as “Ecstasy” (Figure 1), is an amphetamine-related drug with psychostimulant and hallucinogenic properties. As the chemical name implies, it contains a basic amphetamine structure which bears a substitution with an N-methyl group and a methylenedioxy-ring on the third and fourth carbon of the phenyl ring.

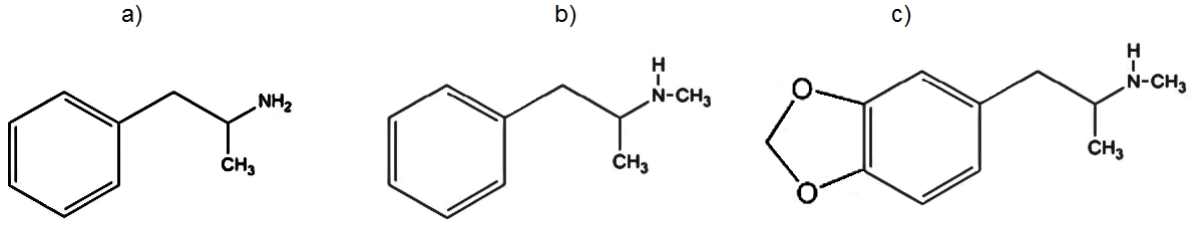

Figure 1 Chemical structures of amphetamine (a), methamphetamine (b), and MDMA (c)

MDMA was discovered in 1912 in the laboratory of Merck, when it was used to form other pharmacologically effective molecules. The first formal animal study on MDMA was done by Hardman and coworkers in 1953-54 (US army studies) and consisted of a number of LD50 determinations on five laboratory animal species, including the mouse. People tried MDMA at the end of 1960s, when it showed its ability to induce the atmosphere of happiness and increase in communication. Moreover, at the end of 1970s Leo Zeff tried to use drug in psychotherapy to facilitate the communication with patients during the therapy. In the same period, on the West Coast of the United Stated, Shulgin was synthetizing and testing MDMA and also described the psychoactive effects of MDMA in humans. First news of the harmful effects came out when in United States and Europe few people died because of a high MDMA ingestion. In 1980s, MDMA was usually taken by individuals in private parties, while at the end of the 1980s it has expanded to the big parties, where it had different names (“X”, “E”, “Molly”) (1).

In Slovenia, the first damaging consequences of illegal MDMA use were seen in 1990s, when eight people died of an acute drug-use and number of toxic deaths has risen in the next few years. The first MDMA-induced death in Slovenia happened in 2001. Even if many researches have shown possible adverse effects and fatal intoxication with this

substance, it has become widespread among young people, and therefore became very alarming (2).

MDMA is usually taken as tablets with different colors and printed symbols (seen on Figure 2) in order to capture the consumers' attention.

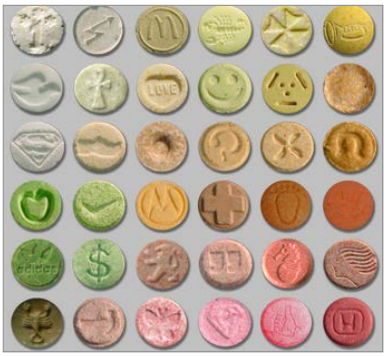

Figure 2 Examples of ecstasy tablets

"Ecstasy" tablets differ in amount of MDMA and can also include many other toxic substances (3). Moreover, Europe studies in 2005 demonstrated that "ecstasy" tablet contain around 100 mg of MDMA. In Slovenia, average values around 108 mg of MDMA per tablet were found, and the typical retail cost of each MDMA tablet varied between 3 and 10 euro (4).

1.1.1. Pharmacokinetics

MDMA, similar to other amphetamine-related drugs, is a weak base (pKa = 9.9) with low molecular weight that displays a high volume of distribution and binds poorly to plasma proteins (5). These properties allow MDMA to easily cross the lipid bilayer of biological membranes.

Numerous studies have indicated that in humans MDMA is better absorbed by the oral route, and reaches its maximum concentration after 1.5-3 hours from its intake, with a plasma half-life around 6-7 hours. In both humans and rodents, the majority of MDMA is metabolized by the liver (6).

In humans, the major metabolic pathway is O-demethylation catalyzed by CYP2D6, which leads to the generation of 3,4-dihydroxymethamphetamine, followed by O-methylation catalyzed by Catechol-O-methyltransferase, which results in 4-hydroxy-3-metoxymethamphetamine (6).

In rodents, the most important enzyme for metabolism is CYP2D1, which is comparable to CYP2D6 in people. Moreover, we have to be aware of variations among mice and rats when choosing an experimental model for a particular research (7).

With regard to renal excretion ability in people, almost all amount of the substance is metabolized by the liver, and a small percentage is eliminated unchanged by urine, in a dose-independent fashion (5, 8).

1.1.2. Pharmacology and toxicology of MDMA

The actions of MDMA on various areas of the CNS, as the striatum, the hippocampus and the cerebral cortex, involve complex mechanisms and multiple sites of action. MDMA binds all three presynaptic monoamine transporters, and exhibits the highest affinity for the serotonin transporter (SERT) (6).

Once translocated to the cytoplasm by SERT, MDMA inhibits the vesicular monoamine transporter type 2 (VMAT2) and produces rapid and acute release of serotonin (5-HT) from synaptic vesicles (9). As a consequence, an increase of extracellular 5-HT occurs, which is boosted by the block of reuptake on presynaptic terminals, and by inhibition of the monoamine oxidase type B (MAO-B), degradative enzymes. MDMA also blocks tryptophan hydroxylase (TPH), an effect that occurs as soon as 15 minutes after intake, and persists for up to two weeks (6).

It is necessary to distinguish between the neurotoxic effects observed after administration of MDMA in the rat and those observed in the mouse, since the neurotoxic profiles displayed by these two species are different (10). Additionally, it may not be ignored that (+)-MDMA is about 10 fold more potent than (+)-methamphetamine in inducing the release of 5-HT, whereas it is 6 fold less potent than (+)-methamphetamine(+) in promoting dopamine (DA) outflow (11).

MDMA is able to induce 5-HT degeneration in two phases. Rapid phase begins after a few minutes of drug intake, when MDMA causes acute increase of 5-HT level by blocking of its reuptake and forcing a release of 5-HT from presynaptic neurons. Moreover, mice treated with MDMA also display a decrease of tyrosine hydroxylase (TH)-positive neurons, as well as TH-positive and DAT-positive fibers in the striatum. After 24 hours, 5-HT content in brain tissues returns to proper levels, while TH activity stays reduced (12).

Long-term phase initiates during first few days and persists for months. It is characterized by drastic reduction of 5-HT, permanent deactivation of TPH and decrease of SERT binding and function. Furthermore, MDMA administration has been reported to produce an extensive reduction of DA and its metabolites in the striatum (13).

In addition to this evidence, it has been shown that co-administration of MDMA and caffeine in mice, induces astroglial and microglial activation, as assessed by analysis of the glial fibrillary acidic protein (GFAP), marker for astroglia, and by analysis of the complement receptor type 3 (CD11b), marker for microglia, in the substantia nigra pars-compacta (SNc) and striatum. This neurotoxic effect is probably due, on one hand, to the presence of pro-inflammatory mediators, such as cytokines and chemokines produced by the activated microglia, and on the other, to reactive oxygen species (ROS), which cause oxidative stress in the dopaminergic neurons of the SNc (14).

1.1.3. Effects of MDMA in humans

MDMA is, as mentioned above, one of the most abused substances in "rave" parties, and its effects on mood can be summarized with the rule of the three "Es": energy, empathy and euphoria. Indeed, the effects sought by MDMA consumers are essentially of two types: a stimulating effect and an effect of empathy. These effects are manifested with a latency of approximately 30 min after oral intake of MDMA, and last up to 3-4 hours. The MDMA “high” period is characterized mainly by disinhibition in social relations, greater openness of spirit, better acceptance of others (empathy effects), increased esteem and self-confidence, feeling of euphoria, increased vigilance, improvement of mood and abolition of fatigue (stimulatory effects). Some MDMA users also report “mystical experiences”, time distortions, altered perception of colors or lights, increased sensory experience, in particular as for tactile stimuli (15).

Besides these effects, which could be considered “positive”, MDMA may produce also several adverse effects. A series of disturbances can appear during the first few hours from drug intake, which affect the psychological sphere, like depression, mania, psychosis, panic attacks, irritability, hallucinations, insomnia, tiredness, fatigue, and paranoid ideas (16). Hallucinations and paranoia can persist for days or even weeks after the intake of MDMA. In addition, cases of potentially fatal neurological effects, such as subarachnoid and intracranial hemorrhage and thrombosis, have been reported in MDMA consumers (17). Remarkably, adverse effects have also been described after chronic MDMA use in

neuropsychiatric settings, which included psychosis, major depression, flashbacks, and aggressive behavior (16). Some recreational MDMA users display particularly cognitive deficits that are more marked in heavy drug users. In addition, investigations showed that MDMA users had long-term impairments in memory and learning and also impaired verbal memory function (18).

Peripheral adverse effects of MDMA in humans may occur at doses commonly used for recreational purposes (50-150 mg), and include increase in body temperature (hyperthermia) and blood pressure, sweating, tachycardia, palpitations, hyperreflexia, nausea, and headache. These effects may be also accompanied by vomiting, trismus and bruxism, hyperglycemia, muscle tension, dilated pupils, decreased libido, arrhythmias, cardiovascular arrests, and renal dysfunction. Importantly, all these adverse effects of MDMA can lead to severe intoxication and even death of the user (17).

Hyperthermia is a very relevant clinical problem in MDMA users, since body temperature elevation produced by the drug may reach up to 43°C. The problem of MDMA-caused increased body temperature is complicated, since the mechanisms included in producing fever by MDMA are not entirely understood. A potential mechanism could involve an interference of MDMA with the normal thermoregulatory mechanisms of the body. Irrespective of this uncertainty, several reports in humans and in laboratory animals confirm that rises in body temperature induced by MDMA are strictly influenced by the external environment. Thus, an important factor for the toxicity of the drug is the social gathering (called "aggregation toxicity"). Typical conditions of "raves" and clubs, where the music is deafening, room temperatures are high due to crowding, and people usually assume few water and lots of alcohol are crucial to amplify MDMA-induced hyperthermia. Moreover, these conditions may enhance all the acute toxic effects of MDMA described above (19).

Another important untoward effect observed after MDMA ingestion is the increase in the plasma concentrations of adrenocorticotropic hormone, cortisol, prolactin, oxytocin, arginine, and vasopressin, which are accompanied by a decrease in plasma sodium concentration (6, 20).

In people MDMA has shown adverse effect also on the immune system, causing immunosuppression, which may have a significant impact on the health of abusers. These effects may involve alterations of neutrophil phagocytosis, reduction of the production of inflammatory cytokines, suppression of the production of interferon γ (INT-γ), and

reduction of the expression of MCH-II molecules at the level of neuronal dendrites and macrophages. Furthermore MDMA reduces the number of circulating lymphocytes, especially of TCD4+, suppresses the proliferation of the T cell line, and distorts the production of cytokines favouring the specialization of Th0 into Th2. The immunosuppressive effects of MDMA are likely not a result of a direct action of the substance on immune cells, but rather stem from the release of immunomodulatory endogenous substances (21).

In contrast to the acute effects described so far, the evaluation of the long term effects of MDMA is complex, although these effects should not be underestimated. In this regards, the results acquired in experimental animals are essential to predict the MDMA cognitive deficits or neurotoxic effects in humans, even though only human studies may provide guidance on the schemes dose and frequency that expose the user to the risk of neurotoxicity.

Studies of long-term effects induced by MDMA on serotonergic transmission are based on the use of indirect methods, like measuring metabolites of 5-HT in the cerebrospinal fluid (CSF), or neuroimaging techniques with a ligand for SERT. In MDMA users, CSF analysis showed an important reduction in the levels of 5-HIAA versus controls. Positron emission tomography research using [11C] McN5652, a selective ligand for SERT, suggests that MDMA users have significant lower SERT binding abilities versus controls, and that reduction of SERT number is related with the quantity of MDMA used in the past (22). In addition to studies on brain serotonergic markers, other investigations have demonstrated the presence of long-term deficits in learning and memory in MDMA users (18).

1.1.4. Effects of MDMA on experimental animals

The most important acute effects of MDMA on different experimental models involve increased body temperature, excessive movement, and 5-HT behavioral syndrome.

MDMA causes a strong dose-dependent hyperthermia, one of the few effects of the drug that can be put in direct correlation with those observed in humans. In mice, MDMA-induced locomotion, body temperature and toxicity have been found enhanced by crowding, and this effect persisted even if each mouse was later allowed to occupy the cage alone. Moreover, mice treated with MDMA in a crowded cage, are more hyperactive and develop a stronger stereotypical behavior than singly-housed mice. This finding is in

agreement with previous evidence, showing the same effects also with other psychotropic drugs, such as methamphetamine, caffeine, and morphine (23).

Rats treated with acute MDMA display toxic effects at the level of the cardiovascular system, as a significant increase in blood pressure, tachycardia, and arrhythmia (24). Moreover, effects of MDMA on prolactin and corticosterone have been reported in rats as well. Thus, studies carried out by administering 1-3 mg MDMA per kilogram, have shown an increased secretion of prolactin and corticosterone, which can be reduced by pretreatment with the selective antagonists of 5-HT2 receptors ketanserin and mianserin (25).

Administration of MDMA has intense influence also on the basal metabolism of animals. MDMA is able to produce an increase the metabolic rate and evaporative water loss, which translates into increase of blood glucose, within 5 minutes after drug administration. This is attributable to the increased glycogenolysis, an effect exhibited by other drugs, such as cocaine (26).

Furthermore, investigations in rats have highlighted MDMA-induced cognitive deficits that were shown by difficulties in spatial learning and memory. Additionally, memory deficits induced by MDMA have been also observed in mice (27).

1.1.5. MDMA neurotoxicity

Regarding long-term outcome of MDMA on central nervous system (CNS), several studies have shown that the drug induces a neurotoxic insult to the serotonergic and dopaminergic terminals, as has been demonstrated by the decrease levels of DA and 5-HT and their metabolites (13).

As mentioned above, this neurotoxic effect is probably due to the presence of pro-inflammatory mediators and to ROS. Thus, MDMA can induce DA oxidation, causing an release of ROS and quinones (14). Interestingly, similar results have been reported in humans, as shown by a post-mortem study performed in a man who had used MDMA often for nine years. This report demonstrated a reduction of a half of 5-HT and its metabolite, 5-HIAA in the striatum (28).

Interestingly, a selective vulnerability towards striosomes was also found for the neurotoxicity induced by dopaminergic neurotoxins as MPTP (29), which suggests a similarity between MDMA and toxic agents acting on DA transmission.

1.2. Definition of adolescence and importance in the effects of psychoactive drugs

1.2.1. Adolescence in humans

In humans, adolescence is defined as the period of transition from childhood to adulthood, during which both psychological and physical changes takes place (30). Importantly, drug consumption during adolescence is probably related with a higher risk of developing substance abuse. In line with this view, epidemiological surveys indicate that the experience with different kinds of psychoactive agents is particularly prevalent during this stage of life.

A positive correlation between the age of the first drug approach and the probability of moving from recreational drug use to abuse and dependence has been suggested (31). This could possibly happen because the adolescent brain is particularly sensitive to the effects of psychoactive drugs, since it has not completed its development yet. In fact, adolescence is characterized by a change of the development strategy of the CNS, which passes from the production of a large number of neurons, to the generation of efficient neuronal pathways. Exogenous substances can disrupt this neuronal remodeling, and make the adolescent brain particularly vulnerable to their effects (32).

Neuronal remodeling also involves variations in the concentration of neurotransmitters, in their systems of reuptake, and in the number of receptors for different neurotransmitters. A very important role in the processes of synaptogenesis is carried out by glutamate, which plays a key role in synaptic plasticity, and is therefore important in cognitive processes like learning and memory. Since some drugs as cocaine, amphetamine, heroin, and alcohol, modify the action of different neurotransmitters, they can alter the normal conditions under which these neurological processes are implemented. Moreover, if drugs are used for a prolonged time, they may influence the neurobiological development of adolescent brain, together with its cognitive, emotional and behavioral functions (33).

1.2.2. Adolescence in experimental animals

Neurobehavioral characteristics of "adolescence" can be observed in rodents in the period ranging approximately from postnatal days 28 to 40, although this may slightly change according to the species considered. Different studies carried out in adolescent rats and mice have revealed a series of behavioral and physiological modifications similar to those featuring adolescence in humans (34).

Interestingly, a recent investigation in rats has shown that during adolescence marked fluctuations in the levels of endocannabinoids take place in the nucleus accumbens (NAc) and prefrontal cortex (PFC), which are brain areas involved in the mechanisms of reward, motivation and cognitive functions (30). This study suggests that consumption of psychoactive drugs of abuse during adolescence may lead to changes in the function of certain types of neurons, at the stage where their normal activity is crucial to create, or delete, interneuronal connections. This may produce alterations in brain development, which could have consequences on the psychological development of the adolescent individual (33).

1.3. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

MPTP is a neurotoxin, precursor of 1-methyl-4-phenylpyridinium (MPP+), which induces symptoms of Parkinson's disease (PD) by destroying dopaminergic neurons in the SNc. MPTP induces a parkinsonism characterized by a gradual onset and progression (35).

MPTP was discovered by a student of chemistry, Barry Kidston after inadvertent self-administration of MPTP as a side compound produced in the synthesis of an illicit meperidine,. Within three days he began exhibiting symptoms of Parkinson's disease (36). Later, administration of MPTP to non-human primates indicated that MPTP selectively induces lesions in the nigrostriatal system, and leads to behavioral changes remarkably similar to those featuring human PD (35).

As of today, several pieces of experimental evidence demonstrate that MPTP administration causes the reduction of DA in the striatum and nigrostriatal cell death in different experimental animals. The toxin is effective either after systemic or intracranial administration. When infused into the SNc, MPTP produces neuronal cell loss as well as a reduction of 40-70% of TH-immunostained cells in the SNc, associated with a significant reduction of DA in the dorsal striatum and PFC (37).

MPTP is highly lipophilic and rapidly crosses the blood-brain barrier and cell membranes, where it is converted to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) by MAO-B, localized in glial cells and serotonergic neurons. Drugs that block MAO-B, as selegiline, minimize the toxicity and prevent the neuronal damage induced by this metabolic conversion. This intermediate spontaneously oxidizes to MPP+, the toxic metabolite of MPTP (Figure 3).

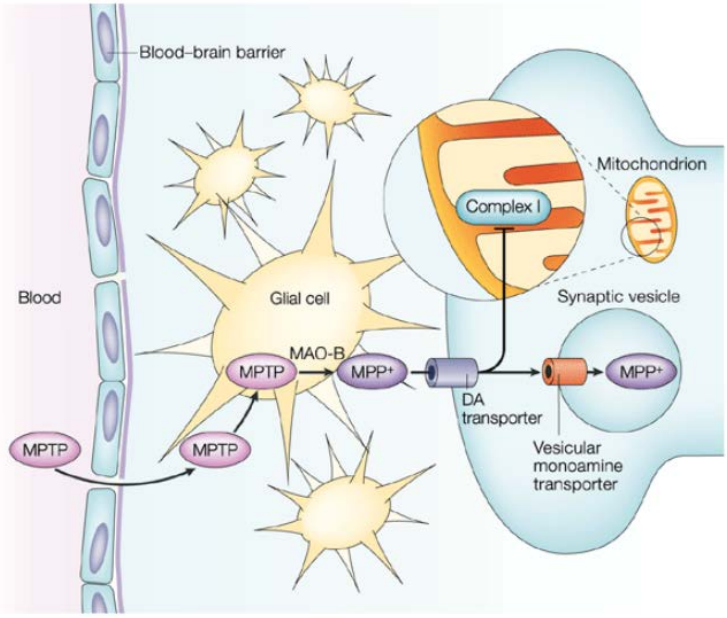

Figure 3 Shematic representation of mechanism of MPTP toxicity

MPP+ is selectively transported by the DAT into dopaminergic neurons, and drugs that block the reuptake of DA inhibit the action of MPP+. Moreover, MPP+ is a substrate also for VMAT2. MPTP has a different neurotoxic profile in laboratory animals, which depends on the different ability the synaptic vesicles have to uptake the toxin. Thus, in the rat the vesicles of striatal dopaminergic neurons have a high VMAT2 density compared with the same cells from the mouse. This might explain why the rat is less sensitive than the mouse to MPTP-induced neurotoxic damage, as a greater sequestration of MPP+ inside vesicles would not allow reaching striatal concentrations of MPTP, which are toxic for the dopaminergic neurons (38).

Gender, age, and body weight are also factors that modulate MPTP sensitivity, as well as lesion reproducibility. Female mice are less sensitive to the toxin and exhibit more variability in the extent of damage than males, as do mice that are younger than 8 weeks and weigh less than 25 g. Optimal reproducibility in MPTP neurotoxicity is obtained by using male C57BL/6 mice aged 8-10 weeks and weighing 25-30 g. These age-dependent differences may partly be due to modifications in MPTP metabolism, since the glial cells of older mice have a greater activity of MAO-B than those of younger mice (39).

The mechanisms by which MPTP induces cell death are still not completely understood, and this phenomenon likely stems from the combination of several factors. Thus, besides being accumulated in synaptic vesicles, MPP+ can also be stored in mitochondria, where it inhibits complex I of the respiratory chain (38), which is

responsible for electron transport and ATP synthesis. This causes a reduced synthesis of ATP, a decrease in the pool of cytoplasmic and mitochondrial NAD+, and a compensatory increase in glycolysis. The cytotoxic effect of MPTP is much more pronounced in cells particularly sensitive to deficiency of anaerobic metabolism, such as dopaminergic neurons of the SNc. The mitochondrial damage induced by MPP+ towards complex I seems to be also responsible for the production of ROS, and energy deficiency could reduce the ability of the neuron to curb the oxidative damage. ROS may also result from an alteration in the homeostasis of DA. Nitric oxide has been proposed as another molecule that may potentially contribute to MPTP-induced neurotoxic damage, as its interaction with the superoxide anion leads to the formation of peroxynitrite, a reactive species, capable of oxidizing cellular macromolecules and promoting neuronal death (40). Both nitric oxide and peroxynitrite are able to interfere with mitochondrial function, thus enhancing the toxic effect of MPP+ on ATP depletion (41).

1.4. Memory and cognitive process

Memory may be described as the procedure of encoding, storing and retrieving. Encoding is the process of transforming information from the environment to different stimuli for our senses (“sensory memory”). Storage is the second stage of memory, and allows information to be retained over time (short- and long-term memory). Finally, the third stage of memory involves the retrieval of the stored information. This model that envisions memory as a consequence of three stages is called Atkinson-Shiffrin model (42). Short-term memory may modify into long-term memory with the consolidation, which takes place in the hippocampus (43). For memory important parts of the brain are hippocampus, prefrontal cortex, and caudate nucleus.

1.4.1. MDMA and memory in humans

Chronic neuropsychiatric complications have been described in MDMA users, like psychosis, hysteria, aggressiveness, notable depression, and cognitive disturbance. Several reports indicate that occasional MDMA users show in particular cognitive deficits, which are more expressed in intensive drug users. These problems persist in the drug-free state, and may be also observed in abstinent MDMA users, who show working memory. Remarkably, all these studies highlighted also poorer verbal and visual memory, and observed that these deficits were in relation with reduction of 5-HIAA in CSF (44).

Furthermore, MDMA users had importantly increased time between external stimuli and behavioral response, and impaired abilities in working memory. Remarkably, numerous of the neuropsychological deficits shown by MDMA users seem not to be corrected even after many years of abstinence, probably due to particular neurotoxic injuries (45).

1.4.2. MDMA and memory in experimental animals

The effects of MDMA administration in experimental animals are crucial to predict the neurotoxic ability in occasional people users. Recurring injections of MDMA in just 24 hours induces permanent injury to DA in the striatum and 5-HT in prefrontal cortex (26, 46).

Many studies have formed rat with cognitive impairment produced by MDMA. Postnatal MDMA administration in rats created dose-related damage in sequential learning, spatial learning, and memory, while neonatal revealed almost no impact. These effects were associated with decreases in 5-HT in brain areas essential for learning and memory (47). These primal answers to MDMA may extend to the long-term learning and memory impairments. Taking into account that the neonatal period in rats is used as an example for the third trimester of fetal brain development in people (48), these findings indicate that children whose mothers use MDMA at the end of being pregnant have a higher possibility of developing numerous anomalies in brain development (49).

Other studies have shown that MDMA injuries in the hippocampus can produce reference memory impairment in rats, which might stem from alterations in 5-HT and DA. However, these effects could as well be related to MDMA-caused death of neurons in this area (50).

Memory deficits induced by MDMA have also been observed in mice. Trigo and colleagues treated mice with MDMA (1-30 mg/kg), and active avoidance acquisition and recall were evaluated. The study found that MDMA administered before the active avoidance sessions affected the retention and the recall of the task performance (51).

In a recent study of Ros-Simò in adult mice assessed declarative memory by means of novel object recognition task (NOR) and radial arm maze, 72 h after one MDMA injection. This acute neurotoxic dose impacted consolidation of declarative memory in both paradigms (52).

1.4.3. Cognitive deficits and MPTP-induced dopamine neuron degeneration

MPTP has widely been used by many researchers working on Parkinson's disease (PD) as a toxin to induce lesions in the SNc of experimental animals (mostly primates and rodents) in order to create preclinical model of PD. Within this context is widely used a PD model characterized by the bilateral intra-nigral administration of MPTP. Besides motor impairment, this model is characterized by a reduction of the animals' performance in an active avoidance task, impaired learning in both a cue version and a spatial working memory version of the water maze (53).

Moreover, rats bilaterally infused MPTP in the SNc display increased anxiety-like behaviors, and memory deficits manifested as a reduced exploration of the novel object in NOR. Furthermore, in this same model MPTP produced dopaminergic degeneration, increased microglial activity, and death of the hippocampus cells (54).

Scarce data are available on the psychological properties of MPTP in the mouse. It has induced a deficit in social recognition in the mouse, but this results probably out of noradrenaline (NA) levels in the olfactory bulb, rather than a reduction of DA in the striatum (55).

The modeling of cognitive deficits in rodents treated with MPTP may help to thoroughly understand cognitive deficits in humans.

2 RESEARCH AIM

Recent studies have shown that MDMA induces particular neurotoxic destruction of the dopaminergic transmission in mice. In addition, other investigations have highlighted the possibility that the adolescent brain may be particularly sensitive to the damage induced by amphetamine-related drugs. Moreover, it is now clear that various types of cognitive impairment occur at the early stages of PD, as well as in non-demented PD patients.

As of today, few clinical studies have examined the possibility that MDMA exposure during adolescence may impair memory functions or influence the effects of toxins known to induce dopamine neuron degeneration. Moreover, no preclinical studies have ever ascertained the influence of repeated treatment with MDMA during late adolescence on the cognitive deficits induced by MPTP administered at adult age. Therefore, there is a need for determining whether cognitive function may be affected by the exposition to both drugs.

To address this issue in the thesis we will perform a prolonged MDMA treatment (10 mg/kg, two times a day/twice a week/9 weeks) in late adolescent mice, which will continue till the adulthood. Throughout all pharmacological treatment, more precisely at the beginning (first week), in the middle (fourth week), at the end (ninth week) and after two weeks of MDMA washout, mice will be tested in NOR and spontaneous alternation behavior in a Y-maze, two cognitive tests widely used to investigate general cognitive function and short-term/working memory. Moreover, the same mice will be re-evaluated in these behavioral tasks after a subchronic MPTP treatment (20 mg/kg ×4) that will take place after two weeks of MDMA washout.

Further, after mice will be sacrificed, we will evaluate whether exposure to MDMA during adolescence is able to influence the neurotoxic damage produced by MPTP. To study the neuroinflammatory and neurotoxic potential we will assess microglial and astroglial activation in SNc and in striatum by CD11b and GFAP markers. Moreover, TH will be performed to evaluate the dopaminergic neuron destruction.

3 MATERIALS AND METHODS

3.1. Experimental mice

Male C57BL/6J mice (Charles River, Italy) weighing 20-23g at the beginning of experiments, were used. Mice were maintained at constant temperature (21±1°C) in a 12-hour light/dark cycle with food and water ad libitum. Experimental procedures were approved by the Ethical Committee of the University of Cagliari, in compliance with Italian guidelines for animal care (DL 116/92) and European Communities Council Directive (2010/63/EEC). Efforts were made to minimize the number of animals used and maximize humane treatment.

However, in our experiment we used the group of animal models, called induced animal model, because we created neurodegeneration by administrating certain substances to induce symptoms similar to those observed in humans.

3.2. Tested drugs MDMA and MPTP

MDMA was synthesized by Prof. A. Plumitallo, Department of Environmental and Life Sciences, University of Cagliari. MPTP was purchased from Santa Cruz, USA. MDMA was dissolved in saline, MPTP in distilled water. Both drugs were administered intraperitoneally (i.p.) in amount of 10 mg/kg.

3.3. Mice treatment protocol

Mice (8 weeks old) were treated with MDMA (10 mg/kg, i.p.) or vehicle according to a 9-week administration schedule. Each mouse received 2 administrations a day, separated by a 4-6 hour interval, twice a week, on Tuesday and Friday, for a total of 36 MDMA or vehicle administrations. Two weeks after treatment discontinuation, mice (19 weeks old) were divided into four groups and treated with MPTP (20 mg/kg, i.p.) or vehicle. Experimental groups were composed as follows: vehicle-MPTP, n=9; MDMA-MPTP, n=8-9; vehicle-vehicle, n=8-9; MDMA-vehicle, n=10. Mice received vehicle or MPTP once a day for four consecutive days. Three days after the last administration, mice were anesthetized with chloral hydrate, transcardially perfused with 4% paraformaldehyde

in phosphate buffer (0.1M, pH 7,4), and their brains were removed and used for immunohistochemistry.

Mice were tested in Novel object recognition task and spontaneous alternation behavior in a Y-maze in the following time points: at 48 hours from the beginning (first week), in the middle (fourth week), at the end (ninth week) of MDMA injections, after two weeks of MDMA washout, and after then after 24 hours from the last injection of MPTP in NOR or after 48 hours from the last injection of MPTP in Y-maze.

The doses of drugs used were chosen on the basis of previous studies (14) and may be regarded as medium doses according to use of MDMA in humans, considering that experienced users may take doses as high as 4 g over 24 hours. Similarly, the schedule of administration (twice a week) was chosen on the basis of the utilization of MDMA, which is occasional.

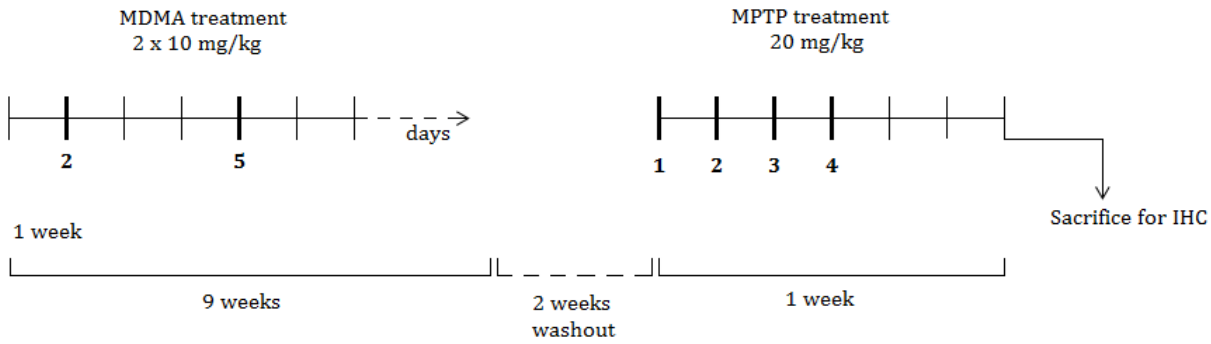

Figure 4 Treatment design

Mice received 2 administrations of MDMA (10 mg/kg, i.p.) a day, separated by a 4-6 h interval, twice a week, on 2nd and 5th day of the week, according to a 9-weeks administration schedule. 2 weeks after the last administration of MDMA, mice (19-week old) were treated with MPTP (20 mg/kg, i.p.) once a day for 4 consecutive days. Three days after the last administration, the mice were sacrificed for immunohistochemistry (IHC) studies. Tick marks labeled on each timeline indicate the day of the week on which the mice received the drug.

3.4. Neurotoxic substances MDMA and MPTP

Protection against toxic substances

Importantly, when working with substances with potential risk for toxicity, we have to handle them correctly and safely.

Both MDMA and MPTP are toxic substances, for that reason we protected our bodies with disposable materials every time entering in the place where mice were held. Hands were protected by double pair of gloves and body with special defend laboratory coat. For not breathing potential toxic substances we used special mask that covered both mouth and nose.

Moreover, even performing cognitive tests on days when the application of these substances was not made, we preferred to be protected, because mice's urine vapors could have been respired and toxic for our bodies (Figure 5).

Figure 5 Examples of protective gear

Additionally, for protecting examiners the following work regime was adopted: the examiner, who was administrating substance, touching caves, mice and all equipment, was allowed to handle all items except the video camera. Second examiner was in charge of opening the doors while entering and handling just the camera. Upper pair of gloves were removed and changed with clean pair if unintentional mistake happened.

3.5. Brain areas involved in cognitive processes

Hippocampus

The hippocampus is a main component of the human brain. It belongs to the limbic system, and plays important roles in the consolidation of information from short-term memory to long-term memory, and in spatial navigation. Moreover, psychologists and neuroscientists generally agree that the hippocampus plays an important role in the formation of new memory of experienced events called episodic or autobiographical memory. Part of this function involves the detection of novel events, places and stimuli. Some researchers regard the hippocampus as part of a larger medial temporal lobe memory system, which would be responsible for general declarative memory (including, for example, memory for facts in addition to episodic memory) (56).

The critical role of the hippocampus in spatial learning was demonstrated in rats by means of memory tasks that require discrimination between sets of cues that contain common elements like extra-maze cues visible from different points of a maze (57). There are a variety of tasks that allow identifying cognitive irregularities and enable measuring the consequent effects of drugs and/or lesions on spatial memory. Spatial learning and memory in rats can be tested utilizing different tests like Morris water maze task, the radial maze task or T-maze forced alternation task. Interestingly, neurologically intact rats can perform all these tasks correctly, whereas rats with lesions of the hippocampus can perform these tasks only when the right response can be associated with a single and not ambiguous cue (58).

Medial prefrontal cortex

The medial prefrontal cortex (mPFC) is implicated in planning complex cognitive behavior, personality expression, decision making, and moderating social behavior. The basic activity of this brain region is considered to be the orchestration of thoughts and actions in accordance with internal goals. The mPFC also plays a critical role in short-term memory maintenance (59).

The neural activation of mPFC is highly correlated with activity in the hippocampal cells, and these areas compensate each other for processing of spatial memory (60).

The critical role of mPFC in memory is demonstrated by studies showing that rats bearing a lesion of this area display a reduction in spontaneous alternation in a T-maze test (61).

Aging modifies the functioning of the PFC, leading to changes in cognition, motivation, and motor performance. The frequent comorbidity of these changes has led to a growing interest in determining the biological mechanisms that may account for their association (62).

Caudate nucleus (caudatus-putamen or striatum in rodents)

The nucleus caudatus is involved in procedural memory and in executive functions, which is an umbrella term encompassing cognitive processes that regulate, control, and manage other cognitive functions, such as planning, working memory, attention, problem solving, verbal reasoning, inhibition, mental flexibility, task switching, and initiation and monitoring of actions (63).

More recent studies have demonstrated that the caudate nucleus is deeply involved in learning and memory, particularly in habit learning that has been defined as an association between a stimulus and a response that develops slowly and automatically through repeated reinforcement (64). Support to the role of caudate nucleus in memory comes from studies in PD patients, in which the dopaminergic deafferentiation of this nucleus is associated with profound changes in cognitive function and habit learning (65).

Two cognitive tests were included in our research, Y-maze task and Novel object recognition task (NOR). Firstly, Y-maze task is a type of T-maze task, which is often used in rodents for short-term spatial memory and general cognitive function in rodents. Another test used was NOR task, which is very useful to study short-term memory, intermediate-term, and long-term memory. Moreover, results of the NOR paradigm are influenced by both hippocampal and cortical lesions (66).

3.6. Tests for cognitive abilities

3.6.1. Novel object recognition

Assessments of Novel object recognition (NOR) are widely used for evaluating non-spatial working memory in rodents (67).

NOR experiments were performed in a Plexiglas cage (length 23.5 cm, width 17.5 cm, height 14 cm) with the floor covered with sawdust. Objects to be discriminated were plastic, different shape and color. Objects had no genuine significance, and had not been previously associated to rewarding or aversive stimuli. The day before the test, mice were allowed to explore the cage for 5 min, in order to acclimatize. On the testing day each mouse was placed into the box for two 3 min sessions and left to explore objects freely. During the first session (S1) two copies of the same object were present, whereas in the second session (S2) mice were exposed to a copy of the objects presented previously in S1, plus a novel object (Figure 6). S1 and S2 were separated by a 60 min interval, which was controlled precisely.

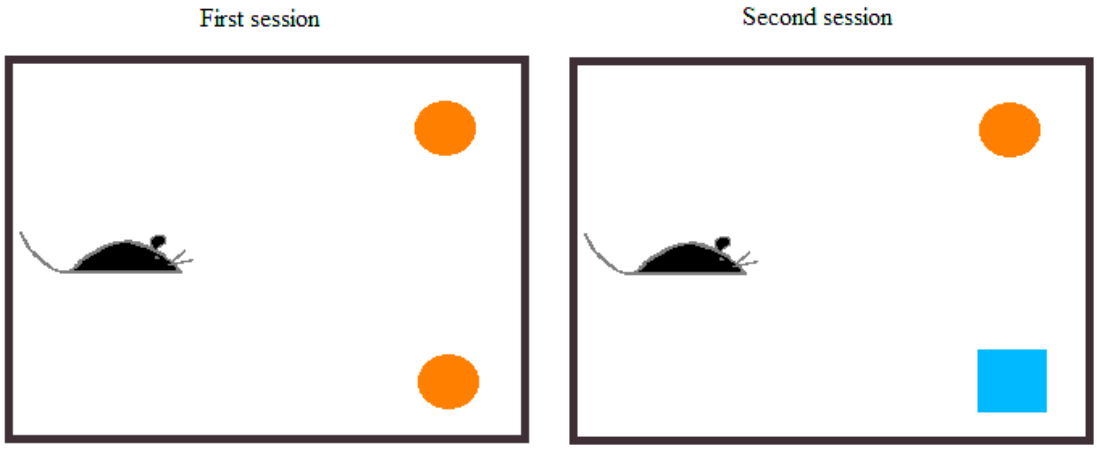

Figure 6 Schematic presentation of procedure used in the NOR task

Exploration was defined as the mice sniffing, gnawing or touching the object with the nose, whereas sitting and/or turning around the object were not considered as exploratory behaviors. To avoid the presence of olfactory cues, objects were thoroughly cleaned after each session. Moreover, the combination of objects (novel vs. old) and their

respective position (right vs. left) were counterbalanced to prevent biased preferences for particular objects or positions. Importantly, the mice performance during NOR task was videotaped and researchers always left the room, that there was any influence on exploration of the objects. Additionally, all mice were always examined on the same day, in random order that any possible errors were avoid and that they all had the same conditions during experiment.

Further, video were analyzed on the computer and the following parameters were evaluated: a) time spent by each mouse to explore the objects during S1 and S2, and b) novel object recognition (Figure 7). The latter was calculated as the percentage of time spent in exploring the novel object, with respect to the total amount of time spent in exploring the two objects during S2 (67). It was defined that one fast touch of the mouse's nose with an object means 0,5 second of exploration, accordingly two rapid touches count as a 1 second. Moreover, if the mouse smelled the object for a longer time, seconds were counted.

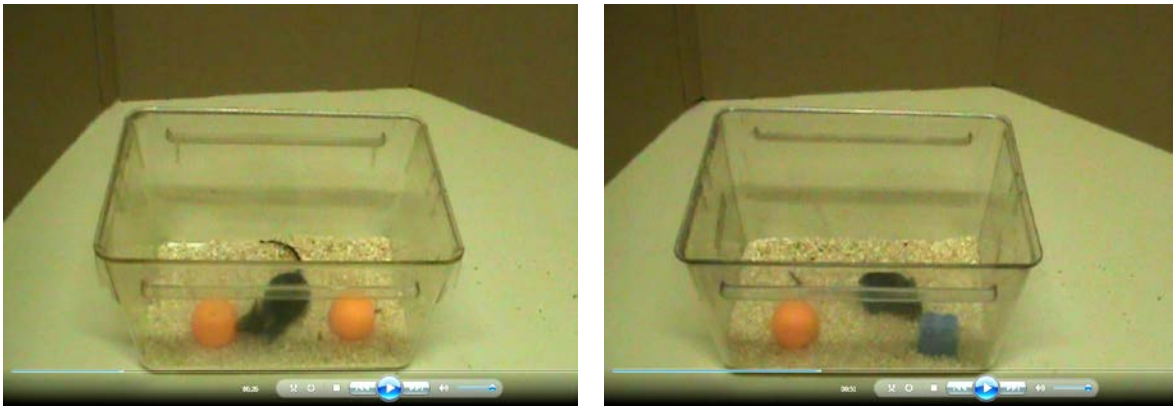

Figure 7 Photos of analysis videotaped NOR task

3.6.2. Spontaneous alternation behavior in a Y-maze

Evaluation of spontaneous alternation behavior in a Y-maze is commonly utilized to investigate short-term spatial memory and general cognitive function in rodents (68). The apparatus was made of black PVC, consisting in three equal arms (length 40 cm, width 11 cm, height 20.5 cm) as seen on Figure 8. Arms converged into a central triangular area, and the maze had its floor covered with sawdust, which was changed in between each mouse (Figure 8).

Figure 8 Y-maze equipment for the evaluation of spontaneous alternation

Mice were individually placed in the central area and in the moment when all four paws were laid on the floor, time started counting. They were left to explore the whole apparatus freely for a single 8 min trial, during which their performance was videotaped. If mice had entered into one arm just with some paws, we did not count it as an entrance. Whereas the mice entered with all four paws into an arm, the entrance was counted. Additionally, for easier counting the spontaneous alternation, we marked arms with letters A, B and C.

Video were analyzed on the computer (Figure 9), where the percentage of spontaneous alternation was calculated on the basis of the sequence of arm entries.

Figure 9 The photo of analyzing video of videotaped Y-maze

Method of analyzing the data out of videotaped Y-maze test

The spontaneous alternation was analyzed as described on the further example:

M1: BAB-CAB-ABC-ACA-CAC-BCA-CA

BAB-ABC-BCA-CAB-ABA-BAB-ABC-BCA-CAC-ACA-CAC-ACA-CAC-ACB-CBC-BCA-CAC-ACA

7/18 = 0,3889

In this case mice named M1 entered into the arms ABC in the sequence written in the line above. For analyzing the spontaneous alternation we write the line under with the following rule of making triplet. Firstly, we copy first triplet (BAB), then we take last two letters of this triplet (AB) and add next letter, which is the same time the first letter of the following triplet (C). We continue this to the end and then count the right spontaneous alternation. For right triplets we take into account triplets with all three letters in any order. Finally, we calculate the percentage of spontaneous alternation: right triplets divided to all triplets (7/18 = 0.3889).

3.7. Preparation of brain sections for immunohistochemistry

Three days after the last administration of MPTP or vehicle mice were anesthetized with chloral hydrate and transcardially perfused with 4% paraformaldehyde in phosphate buffer 0.1M (pH 7.4). Brains were isolated and put in fixing solution for 2h and later in PBS plus sodium azide.

Coronal sections (50µm thick) of mice brain were cut on a vibratome. After slice is cut, we have to take it from the vibratome's PBS solution with a very with thin paintbrush and put it into the petri dishes, also filled with PBS, doing it carefully to not damage these tiny free-floating sections.

Figure 10 Vibratome and collecting free-floating sections inside the PBS solution

After being cut they were incubated overnight with GFAP, CD11b or TH antibodies in order to analyze microglial and astroglial activation or dopaminergic degeneration.

3.7.1. Analysis of GFAP, CD11b and TH

For each mouse, three sections from the striatum and three sections from the SNc were analyzed for glial fibrillary acidic protein (GFAP) and complement receptor type 3 (CD11b) in both areas and for tyrosine hydroxylase (TH) in the striatum. Images were digitized with a video camera, captured at x20 real magnification in gray scale and evaluated with the Scion Image image-analysis program.

GFAP is an intermediate filament protein that is expressed by numerous cell types of central nervous system including astrocytes. It is involved in many important CNS processes, including cell communication and the functioning of the blood brain barrier. If

brain or spinal cord cells are injured through trauma or disease, astroglial cells react by rapidly producing more glial fibrillary acidic protein. Therefore, it is used as a marker to distinguish astrocytes from other glial cells during development.

CD11b is a marker, commonly used in immunohistochemistry. Its expression corresponds to severe activation of microglia. Thus, when high levels of CD11b are observed in the striatum and the SNc, the cells of microglia are inflamed (69).

TH is the enzyme responsible for catalyzing the conversion of amino acid L-tyrosine to L-3,4,-dihydroxyphenylalanine (L-DOPA), which is a precursor for DA. Cofactors are molecular oxygen (O2) as well as iron (Fe2+). Logically, TH is used as a marker for the degeneration of dopaminergic neurons (70).

Immunohystochemical staining procedure

Brain sections were washed in a phosphate buffered saline (PBS) between every step of immunostaining. Further, the following procedure was applied: brain sections were treated with 1% hydrogen peroxide in PBS for blocking endogenous peroxidase and after they were permeabilized with Triton in order to detect intracellular antigens (Figure 11, up). Next, blocking buffer was used (1.5% normal goat serum in PBS + 0.5% Triton) to reduce non-specific binding.

Further, brain sections were immunostained either with glial fibrillary acidic protein (GFAP), complement receptor type 3 (CD11b) or tyrosine hydroxylase (TH): for each marker primary antibody in blocking buffer were applied (monoclonal rat anti-CD11b, 1:1000, Serotec; monoclonal mouse anti-GFAP, 1:400 Sigma; polyclonal rabbit anti-TH, 1:1000, Millipore) and incubated overnight at 4°C, protected from the light. Brain sections were carefully washed with PBS and incubated with a secondary antibody in blocking buffer for 1h at room temperature. For higher visualization was used avidin-biotin-peroxidase complex (ABC) in blocking buffer. Finally, they were incubated with 0.1M phosphate buffer solution (containing diaminobenzidine-DAB, NH_4Cl and glucose). Next, solution of glucose-oxidase in H_2O was applied, because a combination of glucose oxidase and DAB highlight details immunoreactive structures in immunostained preparations. After getting colored, sections were mounted on gelatin-coated slides (Figure 11, down), dried and dehydrated with 70%, 96% and two times in 100% ethanol.

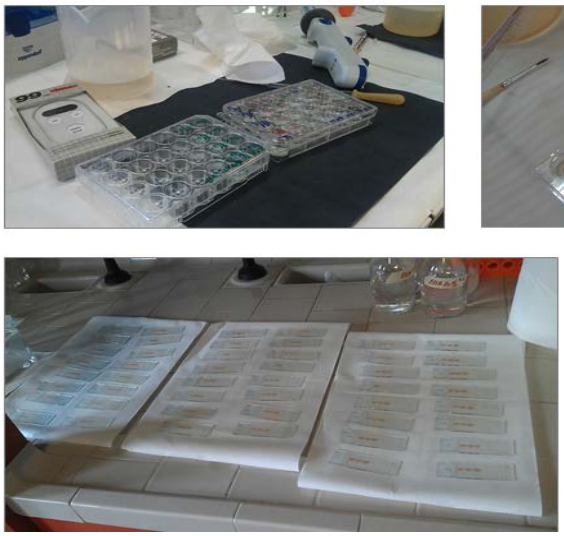

Figure 11 Coloring brain sections with markers (up left and right) and mounted sections (down).

3.8. Statistics

Statistical analysis was performed with Statistica for Windows (StatSoft, Tulsa, OK, USA). Data were statistically compared with a two-way analysis of variance, followed by Newman-Keuls post-hoc test. Results are expressed as mean ± SEM, and were considered significant at $P < 0.05$.

4 RESULTS

4.1. Novel object recognition task

Since the NOR task is used to investigate the existence of deficits in non-spatial working memory, we performed it to evaluate the effect of MDMA on non-spatial working memory of adolescent mice. Briefly, mice were videotaped and evaluated for time that they spent in exploring the novel object.

4.1.1. NOR during MDMA treatment

Mice treated with repeated MDMA (10 mg/kg, i.p., twice a week, for 9 weeks) were examined as described in materials and methods: at the beginning, in the middle, at the end of MDMA injections and after 2 weeks of MDMA washout (Figure 12).

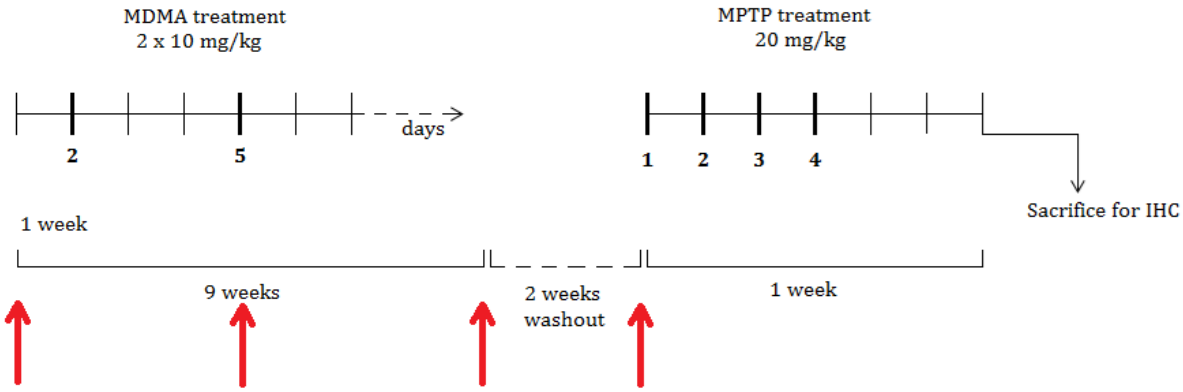

Figure 12 Treatment scheme; red arrows indicate when the NOR task was performed during MDMA treatment

The results of this cognitive test were that mice did not show any significant differences in the percentage of time spent exploring the novel object throughout the treatment. Moreover, MDMA-treated mice did not show any deficits in object exploration during the experimental trials, as they spent an amount of time in exploring the objects during the S1 and S2 trials similar to that spent by the control mice. However, when NOR was evaluated after two weeks after MDMA wash out, MDMA-treated mice spent a significant lower percentage of time ($P < 0.005$) in exploring the novel object, as compared with vehicle-treated mice (Figure 13).

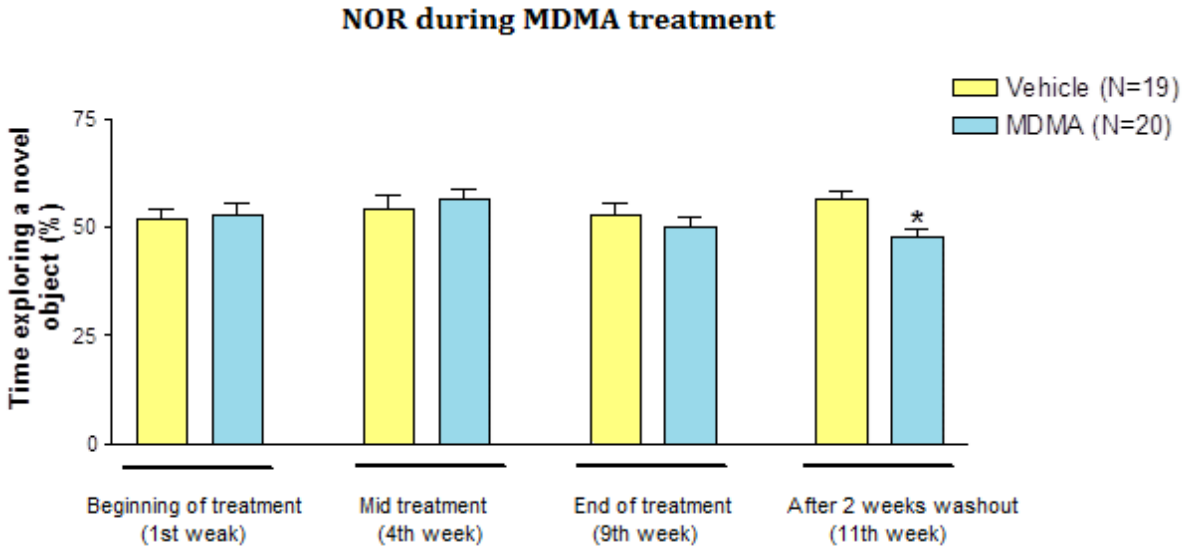

Figure 13 Effect of chronic administration of MDMA (10 mg/kg, i.p.) during late adolescence on the time spent in exploring a novel object in the NOR task.
*P < 0.005 vs vehicle-treated mice

4.1.2. NOR after MPTP

Second, to evaluate the effect of MPTP in mice pretreated with MDMA, NOR was evaluated after 24 hours from the last MPTP (20 mg/kg, i.p.) injection as indicated on the Figure 14.

Figure 14 Treatment scheme; bold red arrow indicates when the NOR task was performed after MPTP treatment; blue tiny arrows indicate when NOR task was performed during MDMA treatment;

MPTP was administrated at adult age and the time spent exploring a novel object in the NOR task was evaluated.

Both MDMA-vehicle and vehicle-MPTP mice spent a significant lower percentage of time ($P < 0.005$ for both groups) in exploring the novel object, as compared with vehicle-treated mice. Mice in the MDMA-MPTP group showed a further reduction in the percentage of time spent in exploring the novel object, as compared with MDMA-vehicle ($P < 0.05$), vehicle-MPTP ($P < 0.05$) and vehicle-treated mice ($P < 0.005$) as seen on the Figure 15.

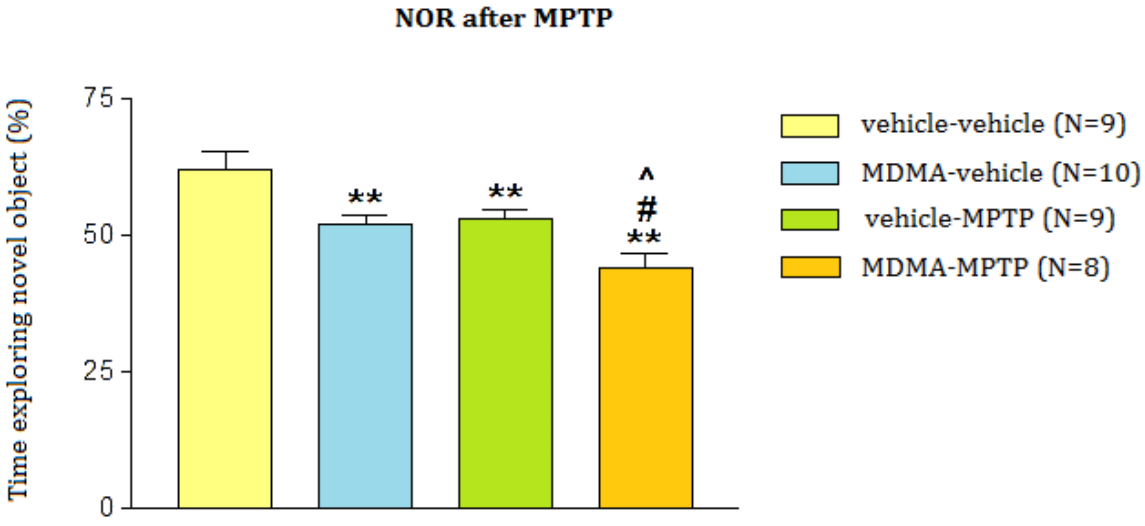

Figure 15 Effect of chronic administration of MDMA (10 mg/kg, i.p.) during late adolescence and subsequent MPTP (20 mg/kg, i.p.) administration at adult age on exploring novel object in NOR task.

**P < 0.005 vs vehicle-vehicle; #P < 0.05 vs MDMA-vehicle; ^P < 0.05 vs vehicle-MPTP by the Newman–Keuls post-hoc test.

Taken together, it was observed that MDMA-treated mice during experimental tests did not show any change in total amount of time spent in exploring the object comparing to vehicle-treated mice. Thus we could suppose that impairment in recognition of the object is not an effect caused by MDMA. Further, when mice were examined in NOR task after MPTP treatment, they showed reduced exploration of the novel object comparing to the other experimental groups.

4.2. Spontaneous alternation behavior in a Y-maze

Since the assessing of spontaneous alternation behavior in a Y-maze is often used to analyze the short-term memory and general cognitive function in rodents, we performed it to evaluate the effect of MDMA on cognitive functions of adolescent mice. Briefly, mice were videotaped and evaluated for spontaneous alternation of the entries to the arms of the Y-maze.

4.2.1. Y-maze during MDMA

Mice were treated with repeated MDMA (10 mg/kg, i.p., twice a week, for 9 weeks) as described in materials and methods: at the beginning, in the middle, at the end of the MDMA treatment and after 2 weeks of washout (Figure 16).

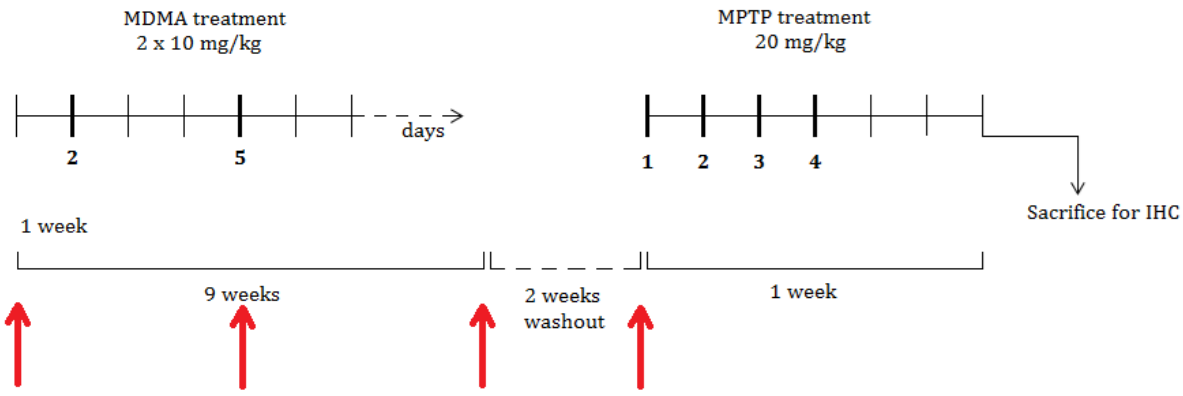

Figure 16 Treatment scheme; red arrows indicate when the Y-maze was performed during MDMA treatment

Mice did not show any significant differences in the percentage of spontaneous alternation over the course of the treatment, comparing to mice, which received just vehicle. Moreover, mice treated with MDMA did non display any deficits in spontaneous alternation from the first administration until the last administration. However, when Y-maze was evaluated after 2 weeks of MDMA wash out, there was any significant difference (Figure 17).

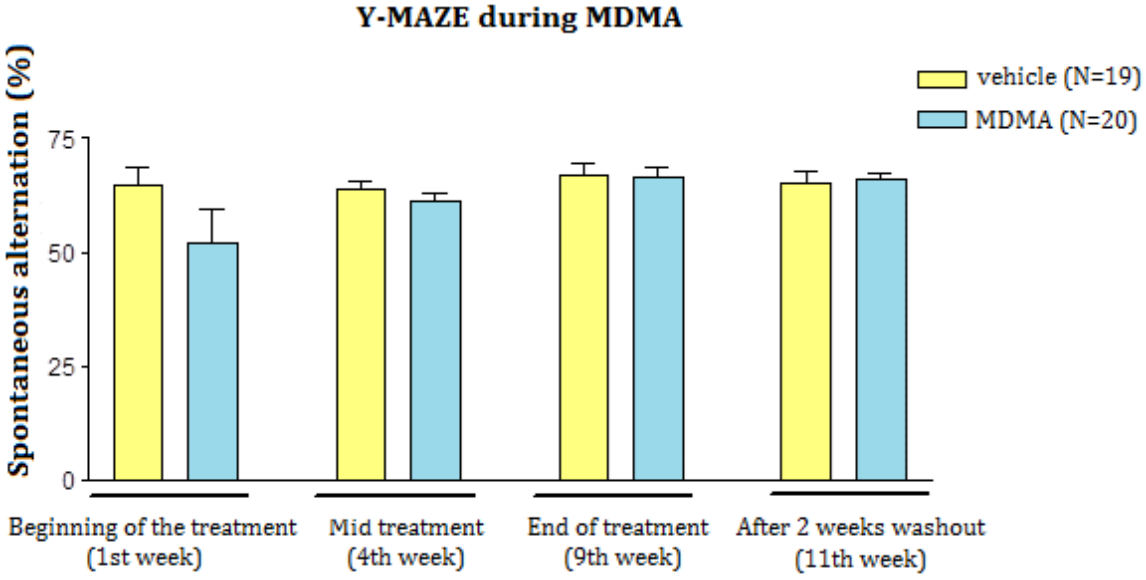

Figure 17 Effect of chronic administration of MDMA (10 mg/kg, i.p.) during late adolescence on spontaneous alternation in a Y-maze

4.2.2.Y-maze after MPTP

Similar results were observed when mice were tested again for spontaneous alternation behavior in a Y-maze 48 hours after the last injection of MPTP (Figure 18).

Figure 18 Treatment scheme; red bold arrow indicates when the Y-maze was performed after MPTP treatment; blue tiny arrows indicate when Y-maze was performed during MDMA treatment.

However, evaluation in Y-maze did not show any significant differences in the percentage of spontaneous alternation between MDMA-MPTP, MDMA-vehicle, vehicle-vehicle or vehicle-MPTP group of mice (Figure 19).

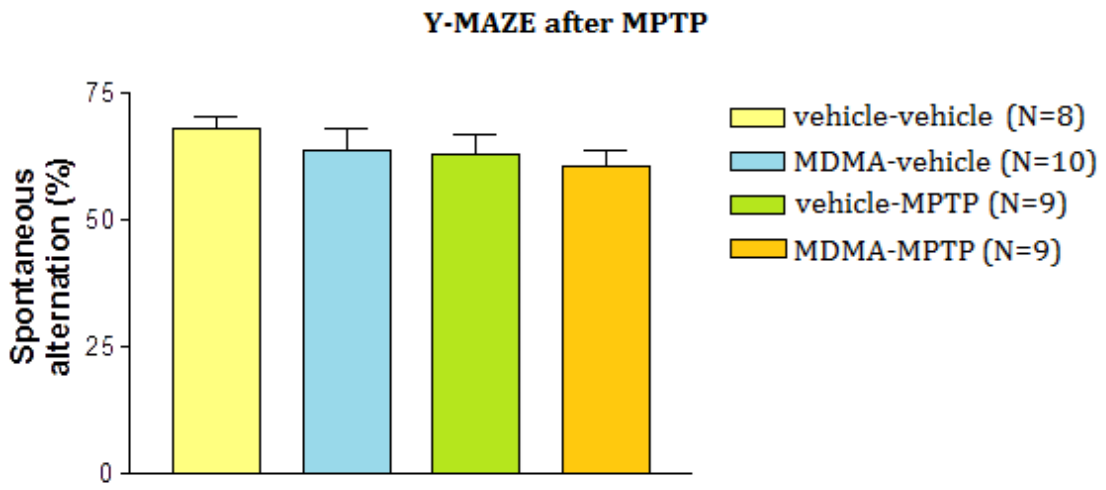

Figure 19 Effect of chronic administration of MDMA (10 mg/kg, i.p.) during late adolescence and subsequent MPTP (20 mg/kg, i.p.) administration at adult age on spontaneous alternation in a Y-maze.

To summarize, the results of spontaneous alternation in a Y-maze have not shown any deficits produced by MDMA and following MPTP treatment, neither any interactions between MDMA and MPTP (Figure 19).

4.3. Immunohistochemistry

The immunohistochemical part of the research that includes microscopy and further analysis was mainly made by my tutor Giulia Costa. Helping in sacrificing, isolating brains, cutting them on vibratome and further immunohistochemical reactions with GFAP, CD11b, and TH was also part of my work. Images are enclosed in my thesis for the clearer presentation of results obtained in entire research. According to the results obtained in NOR task and spontaneous alternation in a Y-maze, immunohistochemistry substantiates neuroinflammatory and harmful actions of MPTP in the mouse, which pre-received prolonged MDMA treatment.

4.3.1. CD11b

Chronic treatment of MDMA in the adolescence and following MPTP treatment in the mouse in adulthood induced a greater activity of microglia in the SNc and the striatum, which is seen with an elevated concentration of CD11b in both areas versus to vehicle-vehicle, MDMA-vehicle and vehicle-MPTP group (Figure 20, see page 36).

4.3.2. GFAP

Chronic treatment of MDMA in the adolescence and following MPTP treatment in the mouse in adulthood induced a greater activity of astroglia in the SNc and the striatum, which is seen with an elevated concentration of GFAP-positive cells in both areas versus vehicle-vehicle, MDMA-vehicle and vehicle-MPTP group (Figure 21, see page 37).

4.3.3. Tyrosine hydroxylase

Chronic treatment of MDMA in the adolescence and following MPTP treatment in the mouse in adulthood induced a reduction of TH-positive neurons in the SNc, which was evidently lower in MDMA-MPTP comparing to vehicle-vehicle, MDMA-vehicle and vehicle-MPTP group (Figure 22, see on page 38). Moreover, in mice pre-treated with MDMA, further MPTP treatment produced a reduction of TH-positive fibers in the striatum comparing to vehicle-vehicle, MDMA-vehicle and vehicle-MPTP group (Figure 23, see on page 39).

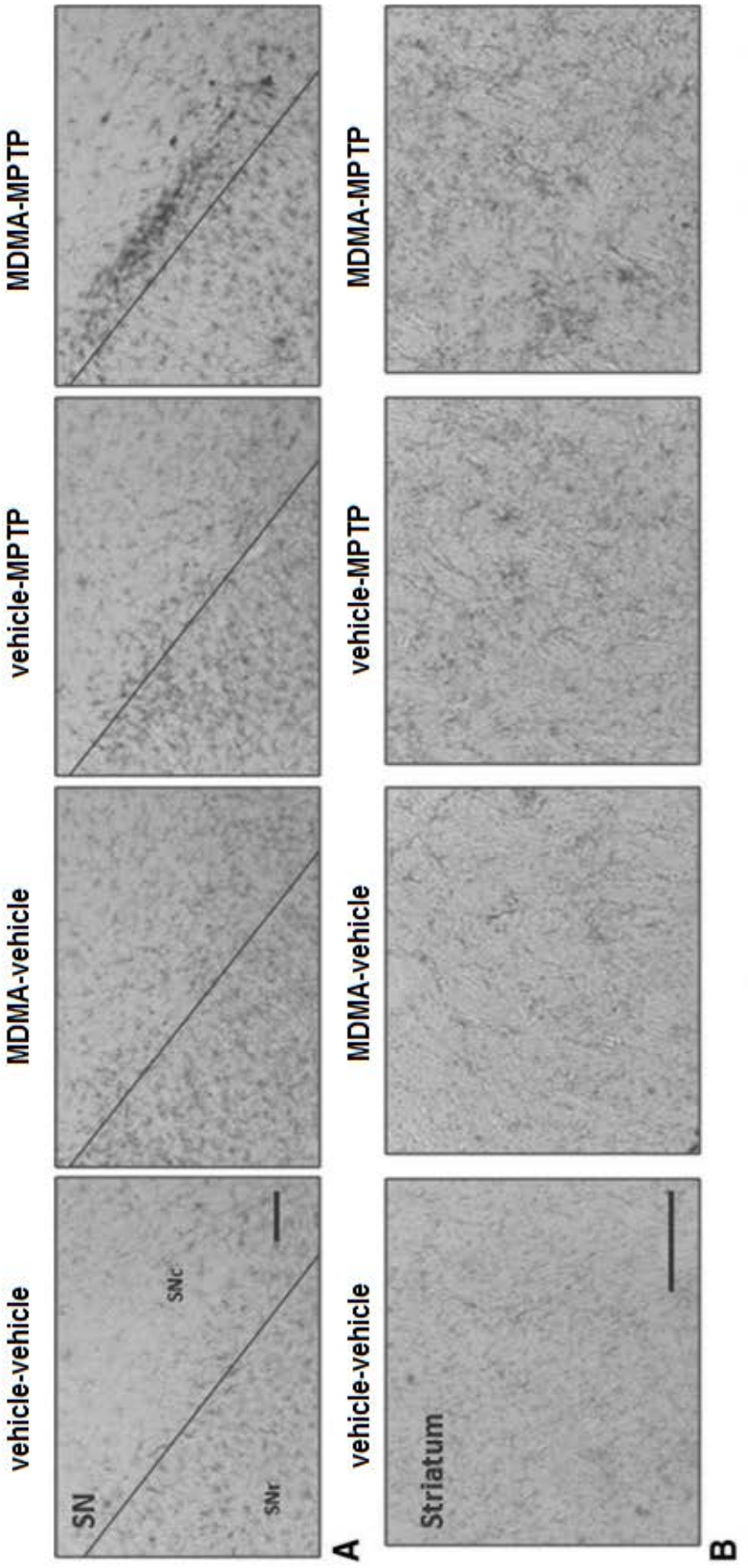

Figure 20 CD11b

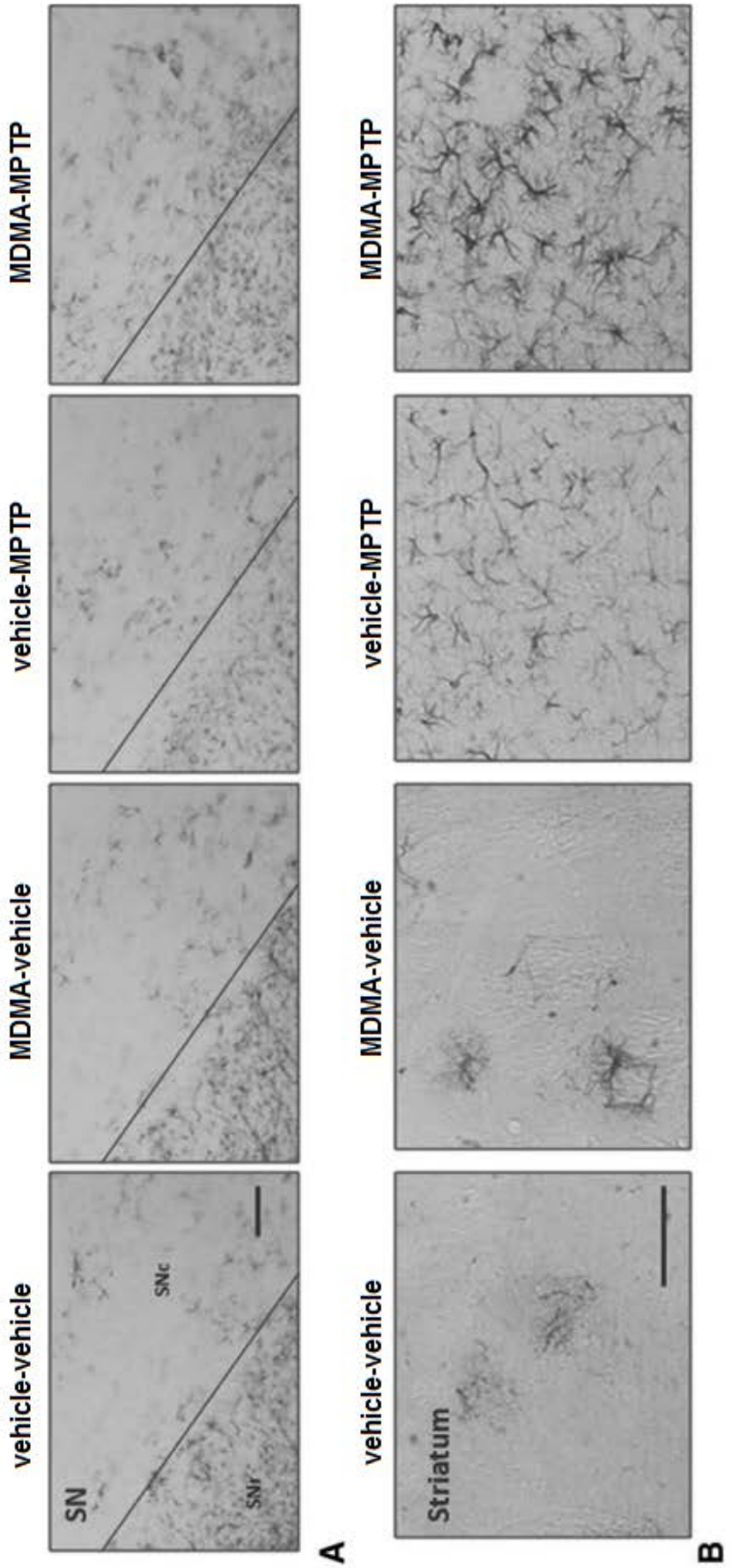

Figure 21 GFAP

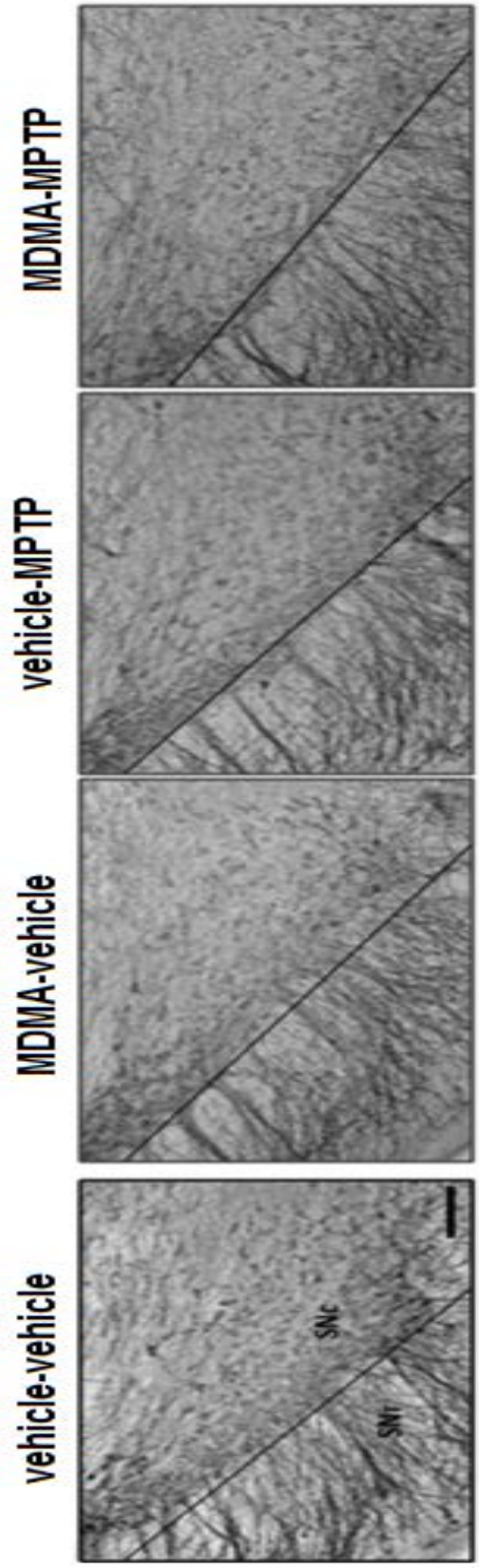

Figure 22 Tyrosine hydroxylase, SNc

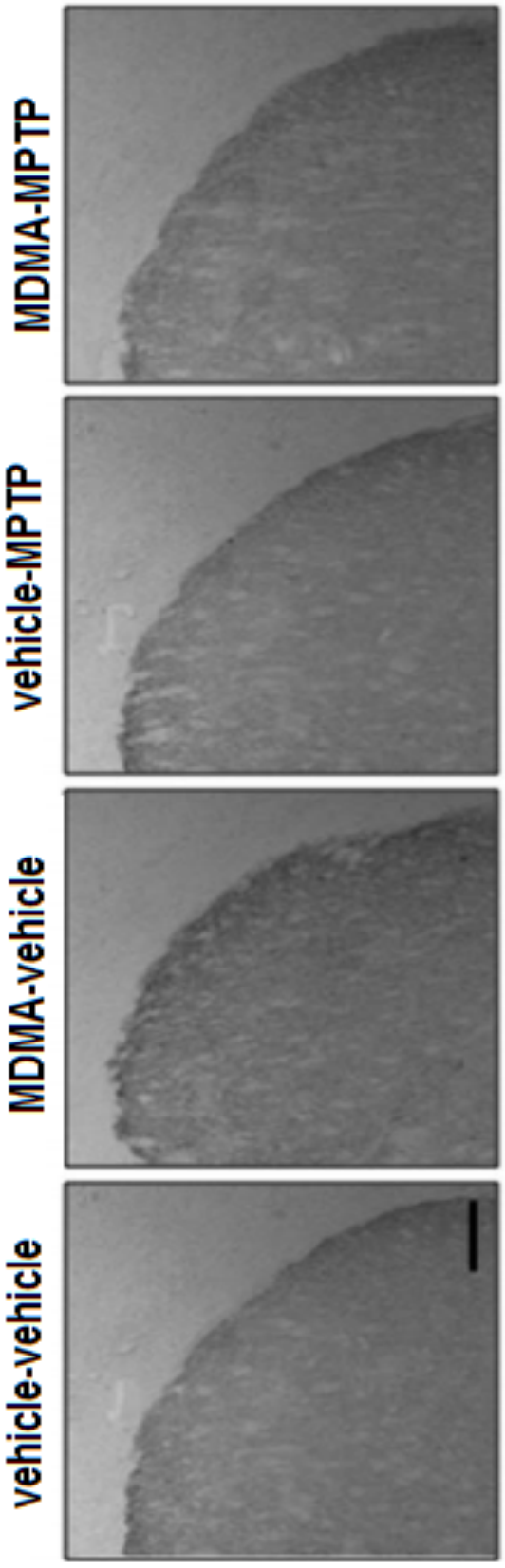

Figure 23 Tyrosine hydroxylase, striatum

5 DISCUSSION

The recent research was performed in order to assess the effect on the short term/working memory, induced by a frequent use of MDMA during the adolescence, and to clarify whether pre-exposure to MDMA may amplify the detrimental effects on cognitive function elicited by MPTP, a neurotoxin, which causes dopaminergic neuron degeneration in people, administered at adult age. Further, it was evaluated also the effect on the neuroinflammation and toxicity toward dopaminergic nigrostriatal neurons.

The investigation of the effects of MDMA on cognitive function performed in this study has a great significance, because use of this substance is still extending during young people. On the other hand, scarce information is available about MDMA producing permanent changes, especially taking into account its consumption in adolescence that is a fundamental period in cerebral development.

The findings reported by the present study point out a few relevant facts. Mice chronically treated with MDMA showed a reduction in exploration of the novel object in the NOR tasks two weeks after treatment discontinuation. Interestingly, this result is in line with findings obtained in a recent investigation by Ros-Simò et al. (52) in adult mice, where the NOR task was utilized to assess declarative memory 72 hours after only one neurotoxic dose of MDMA. Since the NOR task allows to selectively investigate the existence of deficits in working memory (67), the present results add to data obtained by Ros-Simò et al., in the way they show that repeated MDMA exposure during adolescence can affect this aspect of cognitive function. It is important to point out that observations of MDMA-treated mice during experimental trials revealed no differences in the sum of time used for exploration comparing to vehicle-treated mice. This suggests that the deficit in object recognition seen here is not induced by a lower interest for exploration produced by MDMA. Moreover, and most importantly, this study observed that administration of MDMA during adolescence seems not to change the potential of the mice for differentiating different objects. Taken together, these considerations suggest that the results obtained in the NOR task likely reflect an actual specific deficit in working memory of mice exposed to the pharmacological treatments evaluated in this study.

Systemic giving of MPTP to mice leads to reduction of DA in the striatum and losing cells in the SNc, an effect that has been very well characterized and widely used to model PD-like neurodegeneration in the mouse (53). In spite of these clear neurochemical effects,

the long-lasting behavioral effects of MPTP on the cognitive effects are not known. In this regard, the present study provides important and novel information, by showing that MPTP treatment results in cognitive decline, as revealed by a reduced exploration of the novel object in the NOR task by mice treated with MPTP. This finding is also important, as it may reproduce at the preclinical level some features of the cognitive deficits observed in the early phases of PD. Most notably, these effects of MPTP were found to be amplified in the mouse exposed to the prolonged treatment of MDMA through late adolescence. Thus, it can be hypothesized that cognitive deficits created by MDMA might generate the predisposition for a worsening of MPTP effects on cognition.

The cognitive deficits observed in this study however were narrowed to the NOR task, whilst no deficits could be observed in spontaneous alternation in a Y-maze, both during MDMA and following MPTP treatment, and no interaction between MDMA and MPTP was found. This could be due to a more marked sensitivity of the areas involved in the regulation of non-spatial working memory to the neurotoxic effects of MDMA, MPTP, and their interaction, and is in line with previous studies, which show that spontaneous alternation in a Y-maze may be not affected by submaximal neurotoxic insults (67). Nevertheless, the present results provide clear indication that MDMA abuse may amplify the detrimental effects exerted by substances that elicit dopaminergic neurotoxicity.

MDMA and other amphetamine-related drugs induce harmful effects on dopaminergic neurons (11). Neurotoxicity observed in the adolescent mouse is essential, since MDMA in people is commonly used among young individuals. Several clinical and preclinical studies show that PD is caused by numerous factors (71). Over the years, few epidemiological studies have made a correlation between abuse of amphetamine-like drugs during young age, and later development of PD. The study carried out by Garwood et al. (72) showed the results of a telephone survey conducted on patients with neurodegenerative diseases, including PD, peripheral neuropathy, and amyotrophic lateral sclerosis, who abused amphetamine-like drugs. Prolonged exposure to these substances is statistically more frequent in PD patients compared to patients with other neurodegenerative diseases. Moreover, for most individuals, exposure occurred long before diagnosis. Another study by Moszczynska et al. compared the average levels of DA in post-mortem brain tissue taken from methamphetamine users, PD patients, and healthy individuals. Methamphetamine users displayed average levels of DA that were lower in the caudate (-61%) than in the putamen (-50%) (73). Since dopaminergic neurons degenerate

with age progression, the discovery of a 50% reduction DA levels in the putamen of methamphetamine users shows neurotoxicity by this substances, which does not necessarily contradict the idea that the earlier exposure to amphetamine-related drugs may be a risk factor for the subsequent PD (62). Additionally, the results of the present study that show an amplification of cognitive deficits induced by MPTP in mice pre-treated with MDMA, are in line with these facts. Considering all obstacles for performing experiments of MDMA in people, outcomes of research on experimental animals are essential and some of them are rather appropriate to pharmacological effects in people (26).

Moreover, several studies highlighted how amphetamine-related drugs may damage dopaminergic terminals, cause a decrease of DA concentrations (13), and lead to cognitive deficits. MDMA administration to rats during first postnatal days induced impairments of sequential learning, spatial learning, and spatial memory. These effects can therefore expand to permanent damage (47). In humans however, MDMA users displayed cognitive deficits already after occasional use, which were enhanced in intensive drug users (18).

The behavioral and biochemical consequences of long-term exposure to MDMA during adolescence have not been exhaustively characterized in experimental animals, and the majority of the data available are concerned with the effects of MDMA on monoamine transporter proteins (26). Even though many studies have been performed, most of them are flawed by the lack of a real correspondence of the protocols used, the doses and habits of MDMA intake between animals and adolescent humans (26).

Several studies revealed that continious injections of MDMA to adolescent rats induced locomotor sensitization (23) and augmented the rewarding effects of cocaine in adulthood, measured by the place preference test (74). In the light of previous studies suggesting that the behavioral responses to psychostimulants are the result of neurobiological adaptations that occur primarily in the mesolimbic DA system, it is expected that exposure to MDMA during adolescence would produce long-term changes in these systems that perpetuate in adulthood. Accordingly, in our study mice exposed to chronic treatment of MDMA during their adolescence showed long-term impairments in their adulthood (75).

Moreover, a lately study in mice has revealed that the concomitant administration of MDMA and ethanol during adolescence causes long-term behavioral consequences, as evidenced by the motility test, passive avoidance test, and elevated plus maze test (two tests used to evaluate anxiety), and a change in the levels of monoamines in adulthood.

Administration of MDMA and alcohol during the young age produced a reduction of DA, 5-HT and NA in adult age and impaired behavior, evaluated with different cognitive tests (76).

Importantly, the current study has raised several important findings also in the immunohistochemical part of the research. First, mice that had been treated chronically with MDMA, after 3 weeks of the break of MDMA treatment showed reduced number of dopaminergic neurons in the SNc. We could assume that acute, repeated administration of MDMA produces dopaminergic neuron degeneration. The observation obtained in adolescent mice is important finding, since in people this drug is usually consumed among young people. Another important finding is that the effect of MPTP and MDMA appears to be additive. Namely, MPTP injections after MDMA produced an increase in dopaminergic neuron destruction comparing to the other experimental groups. We could suppose that MDMA-produced dopaminergic destruction may generate the predisposition for destruction, which can be worsened by different factors from the environment. Further, in this study was also observed that in the SNc and striatum appeared an increased astroglial and microglial activation in MPTP mice, chronically pretreated with MDMA. Interestingly, the outcomes are highly appropriate to effects of these substances in people. Therefore, we could suppose that activated glia cells appear as an answer to destructive substances and in the future leads to progression of different neurodegenerative diseases, for example PD. On the other hand, microglia produces also many ROS, which can only worsen harmful effects.

To summarize, the results suggest that MDMA may amplify the deleterious cognitive effects of the dopaminergic neurotoxin MPTP and might produce deficits in working memory. Taken together, the results of this study show that abuse of amphetamine-related drugs in adolescence increases a risk for the producing a drug-dependence, and contributes to greater CNS sensitivity to substances that lead to neurotoxicity of dopaminergic neurons in adulthood. Activities of brain areas during adolescence are importantly modulated by DA; therefore substances like MDMA, which impact these processes, can deeply disturb DA activities. Remarkably, utilizing MDMA in the mouse might be a preferential dopaminergic neurotoxin in mice (77), thus its use in this species might explain the character of this substance on DA processes in the brain areas, giving a greater conclusions of the short-term and long-term effects on cognitive functions.

6 CONCLUSIONS

In the present work the effects of MDMA (known as ecstasy) were investigated in mice and compared to already known neurotoxic substance MPTP, for which is proven to provoke a degeneration of dopaminergic neurons. Two cognitive tests were used to examine cognitive impairments: Novel object recognition and Spontaneous alternation in a Y-maze. Mice treated with MDMA spent lower amount of time exploring the novel object comparing to vehicle-treated mice, while mice treated with both MDMA and MPTP showed a further reduction in time exploring the novel object comparing to the other experimental groups. On the other hand, Y-maze test did not show any significant difference between experimental groups.

The study has proved that MDMA induces weak neurotoxicity, which is expanded by MPTP much more than when MPTP injected alone. This suggests that effects of MDMA and MPTP are additive. Effects of these two substances were observed also by neurohistochemical analysis of brain sections with following markers: CD11b, GFAP and TH. Marker CD11b showed a greater microglial activity, GFAP a greater astroglial activity, and TH indicated dopaminergic degeneration.

From the results obtained in the graduation thesis can be concluded that MDMA and other amphetamine-related drugs may create a base damage in brain areas, important for memory and learning, which can be exacerbated by different surrounding factors later in life.

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