Charles University

Third Faculty of Medicine

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Disertation work summary

Is adolescence a critical period for drug addiction in the laboratory rat?

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Prague 2024

Postgraduate study program in Biomedicine

Charles University, Prague

Field of study: Human Physiology and Pathophysiology

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Reviewers: …… ……

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Thesis summary delivery date: …….……

The defense of the Dissertation will take place on (date):

……..……

Content

Summary

Methamphetamine (MA) is a synthetic psychostimulant that is widely abused due to its rapid-onset stimulant effects. However, long-term use of MA can lead to severe impairment of central nervous system function and associated behavioral changes. MA interacts with neurotransmitters, causing permanent damage to terminal endings of neurons through oxidative stress, neuroinflammation, and apoptosis. This leads to various behavioral disorders such as depression and psychosis, contributing to the outbreak of Alzheimer's and Parkinson's diseases. Pregnant women often abuse MA, leading to negative impacts on their baby's development and behavior. Clinical studies are complicated due to the presence of other drugs and the concentration and purity of these substances. Animal studies have shown that the negative effect on cognitive functions in adults exposed to MA prenatally depends on the stage of pregnancy and neonatal administration, specifically during the first 12 postnatal days. This study aimed to observe the effects of MA on adolescent subjects exposed to MA during the first 12 days of life. The animals were exposed to the drug both directly by subcutaneous injection and indirectly via breast milk when MA was given to mothers. After weaning, behavioral tests were performed to test memory. Separation, a stress factor, had a greater negative impact on learning and memory than MA alone. The enriched environment also had a negative impact. The study measured neurotransmitter levels, growth factors, oxidative stress, and c-Fos at different stages of adolescence, including PD 28, PD 35, and PD 45. Neurotransmitter levels were affected mainly by post-weaning stress, or the pre-weaning environment, as well as growth factors. Oxidative stress levels did not change depending on MA, and c-Fos expression decreased during early and late adolescence following MA administration. In conclusion, the administration of MA during the first 12 days has a less pronounced effect in indirect administration, suggesting that the development and environment of the developing individual play a critical role in this case.

Souhrn

Metamfetamin (MA) je syntetický psychostimulant, který je široce zneužíván pro své stimulační účinky s rychlým nástupem. Dlouhodobé užívání MA však může vést k vážnému poškození funkce centrálního nervového systému a souvisejícím změnám chování. MA interaguje s neurotransmitery a způsobuje trvalé poškození terminálních zakončení neuronů oxidativním stresem, neuroinflamací a vede k apoptóze. To vede k různým poruchám chování, jako je deprese a psychóza. Těhotné ženy často zneužívají MA, a to vede k negativním dopadům na vývoj a chování jejich dítěte. Klinické studie jsou komplikované kvůli přítomnosti jiných drog a neznáme koncentraci a čistotě těchto látek. Studie na zvířatech prokázaly, že negativní vliv na kognitivní funkce u dospělých exponovaných MA prenatálně závisí na stádiu těhotenství, v kterém je MA podáván. Cílem této studie bylo pozorovat účinky MA na dospívající jedince potkanů vystavených MA během prvních 12 dnů života, Tato perioda odpovídá 3.trimestru u lidí. Zvířata byla vystavena MA přímo subkutánní injekcí, nebo nepřímo prostřednictvím mateřského mléka, když byl MA podáván matkám. Před odstavem byla zvířata vystavena obohacenému nebo standardnímu prostředí. Po odstavení byla zvířata umístněná do klecí po 4, což je pro potkany přirozené, nebo byla separována. Následně, byly v různých stádiích adolescence provedeny behaviorální testy pro testování paměti. Další sledované parametry byly hladiny neurotransmiterů, růstových faktorů, oxidativního stresu a exprese c-Fos a to konkrétně v postnatálních dnech 28, 35 a 45. Separace jako stresový faktor, měla větší negativní dopad na učení a paměť než samotný MA. Negativní vliv na tyto kognitivní funkce však mělo i obohacené prostředí. Hladiny neurotransmiterů byly ovlivněny především stresem po odstavení, nebo prostředím před odstavením, stejně tak i růstové faktory. Hladiny oxidačního stresu se také neměnily v závislosti na podávaní MA a exprese c-Fos se snížila během časné a pozdní adolescence po podání MA. Negativní účinek MA byl pozorován především u zvířat, které byly této droze vystavené přímo. Závěrem lze říct, že podávání MA během prvních 12 dnů má v nepřímém podávání méně výrazný účinek. Tento výsledek naznačuje, že v tomto případě hraje rozhodující roli vývoj a prostředí vyvíjejícího se jedince.

1. Introduction

Methamphetamine (MA) is a highly addictive and abuse-prone psychostimulant that can damage neurons, impair cognition, memory, and attention. It is lipophilic, allowing it to quickly cross the blood brain barrier and enter the brain. MA primarily enhances neurotransmitter dopamine (DA), serotonin (5HT), norepinephrine (NA-noradrenaline), and glutamate (GLU) release in the brain. High MA levels may cause tachycardia, hypertension, mental illness, and death [1]. Reactive oxygen species (ROS) formation is partly caused by MA, leading to damage to cellular macromolecules such as proteins, lipids, and DNA. Oxidative stress induced by MA results in lipid peroxidation, protein misfolding, and nuclear damage. By entering the neuronal cell, MA can displace DA from its vesicles and release it into the synaptic cleft, resulting in an increase in DA levels both inside cells and synapses [2]. The administration of MA leads to the activation of microglia dominantly in the striatum (STR) and hippocampus (HP).

Neuronal plasticity, the ability of the brain to change through growth, is crucial for successful information storage and memory formation, as well as adaptive responses resulting in various modifications of behavior [3]. Increased BDNF synthesis and release play an important role in mediating synaptic changes involved in learning and memory, which underlies behavioral and structural adaptations associated with drug addiction [4]. Critical developmental periods, which are momentary disruptions in the developmental process during specific periods of embryogenesis, can lead to significant repercussions [5].

These periods include heightened responsiveness to external stimuli, reorganization of functional systems, and alterations in an individual's interaction with its surroundings. The central nervous system (CNS) is a neural platter formed from cell precursors, and the primary neurotransmitter systems linked to the stimulating effects of drugs are the noradrenergic, dopaminergic, and serotoninergic systems. In humans, synaptic joints reach full maturity within a few more weeks, typically around 34-36 weeks of gestation. In rats, this process occurs between the last few days of prenatal development (ED 18) and PD 19-21. Neurogenesis reaches its highest point during the 40th week of pregnancy in humans and during the first and second week after birth in rats. After birth, rats experience rapid maturation of astrocytes, leading to changes in the morphology, connectivity, and electrophysiological properties of the CNS. Methamphetamine is a drug that can easily cross barriers within the mother's body, including the placenta, and enter the developing fetus. The drug concentration in the child's circulation is about 50% of the concentration in the mother's plasma, and as the drug takes effect, it slows down its breakdown in the liver, causing the medication concentration to rise. Prenatal exposure to MA in rats can take place during the whole prenatal period or during specific stages of fetal development. In rats, the drug is transmitted through breast milk during lactation, with evidence of MA in the plasma and brain of women exposed to the drug [6].

Extensive clinical and experimental investigations have examined the detrimental impact of MA on the development of children born to mothers who are addicted on drugs. Previous studies indicate that animals exposed to MA during the periods of ED 12-22 and PD 1-11, which align with the second and third trimesters, exhibited notable impairments in behavior during both their developmental stages and adulthood [7]. Experiments have shown that exposure to MA during prenatal and neonatal stages of neuroontogenetic development did not lead to drug addiction in offspring. However, exposure during pregnancy and early infancy can result in a decrease in both social and non-social behavior, and exposure of pups to MA during these stages can result in persistent cognitive deficits into adulthood. During critical developmental periods, genetic processes and environmental factors interact to stabilize specific traits of an organism.

Adolescence is a critical period for developing the brain, with 5HT levels varying in different brain regions. Stress, such as MA exposure, can cause behavioral impairments in pups, leading to memory impairments in adulthood and reduced BDNF levels. Maternal care and mother-child relationships are crucial in preventing these changes. Social isolation during adolescence can have serious consequences for development and behavior in adulthood, leading to anxiety-like behaviors and reduced cell proliferation and neurogenesis. Chronic social isolation in rats induces depression, anxiety, and psychosis-like behaviors, with altered expression of BDNF in the brain. Proper maternal care and environmental enrichment (EE) can have therapeutic potential in improving animal welfare and stimulating HP neurogenesis. EE can increase monoamine neurotransmitters in mesolimbic structures of the brain, enhancing growth factors that promote neurogenesis, including NGF. Studies have shown that EE can reduce seeking for psychostimulants, reduce the risk of relapse, and protect animals from drug addiction by sensitizing limbic structures. Rats given EE are less sensitive to the reinforcing effects of MA.

2. Aims and hypotheses

Given the recent data from Professor Šlamberová's laboratory suggesting that the early postnatal period PD 1-12, which corresponds to the human third trimester, is the most critical period for the damaging effect of MA in a period simulating human pregnancy, our aim was to monitor the effects of MA in the above period. In view of previous results from our laboratory indicating the presence of MA in the plasma and brain of the pups and in the breast milk, the drug was administered to the nursing mother and the pups were thus exposed to the drug indirectly via breast milk. Indirect administration of the drug was then compared with direct administration of MA directly to the pups during the same period (PD1-12). The aim of this project was also to investigate whether improved housing conditions (enriched environment) can enhance the negative effects of MA in early postnatal development. Therefore, we observed the effect of enriched environment (EE) in the pre-weaning period and the effect of EE vs separation in the post-weaning period. The PD 1-12 period is also a time of increased development of the hippocampus, a brain structure associated with an individual's cognitive functions. Since Clancy et al have shown that the drug affects precisely those structures undergoing development, we hypothesize that MA administration during the PD1-12 period will lead to modifications in hippocampus-related functions that will correlate with changes in hippocampal neurogenesis. And these effects of MA will also be influenced by the enriched environment and separation.

This research has two main hypotheses:

The long-term effects of early postnatal MA exposure are influenced by pre-weaning and postweaning housing conditions:

1. Enriched environment during the preweaning period has a positive effect on the long-term effects of early postnatal MA exposure.
2. The post-weaning environmental conditions influence the long-term effects of early postnatal MA exposure the following way: group housing will improve the results of early postnatal MA exposure relative to the separate housing.

Pre-weaning environmental conditions influence the long-term effects of early postnatal MA exposure

Standard enclosures are characterized by their boundary size, which restricts the animals' natural movement. In contrast, EE for laboratory animals entails their exposure to housing conditions that provide heightened stimulation of the brain's sensory, cognitive, and motor systems, as opposed to the deplorable standard housing conditions. It has been demonstrated that EE induces neural plasticity at multiple levels in the brain, including structural and circuitry modifications, enhancements in cognitive function, and predominantly positive changes in brain chemistry. Hence, the proposed project will involve a comparison of two housing conditions: conventional "maternity" cages and cages that have been enhanced with diversions such as wheel-running on a voluntary basis. The observed behavioral and structural plasticity after enrichment was found to be partially ascribed to the increased expression of neurotrophic factors, as well as crucial genes and proteins implicated in neuronal plasticity. It is not very well understood what role EE plays in terms of drug addiction since some studies reported that EE Alleviates behavioral deficits induced by MA withdrawal, and that EE significantly prevented these reinstatement effects of MA in condition place preference task, while other reported that the rewarding and neurotoxic effects of MA are not reduced by EE. However, the fact remains that the environment plays a crucial role during the development of an individual. Therefore, we hypothesize that EE will moderate the potentially adverse neurotoxic effects of MA on the rat brain.

Environmental conditions during the post-weaning period influence the long-term effects of MA exposure in the early postnatal period

In addition to the period preceding weaning from the mothers, housing conditions may also influence the neurogenesis and behavior of individuals after weaning. Rats are social creatures that have developed a hierarchy through group living. Consequently, social instability resulting from an unreliable number of animals per group leads to enduring challenges in their social conduct and stress response. Animal experimental models frequently employ social disruption to examine the impact of environmental adversity on young animals with the aim of assessing the neurobiological mechanisms that underlie psychiatric disorders [90]. Isolation and other social stressors, such as unstable hierarchies and social defeat, are frequently employed in adolescent animals. Comparing the effects of group housing (four males confined in a stable social group) and solitary housing (one male per cage) is the purpose of this study. Following weaning, the environment in which the animals were raised prior to weaning will remain unchanged (i.e., animals raised in EE will continue to be confined in groups in EE conditions during the post-weaning period).

The above-mentioned hypotheses were tested through the following methods:

A. Behavioral testing
   Our prior research has shown that rat offspring exposed to MA during the early postnatal period (PD1-12) experience deficits in learning and memory. Four kinds of assessments utilized cognitive abilities (memory and learning) in accordance with our prior findings:
   - Habituation serves as an assessment tool for non-associative learning.
   - Object Recognition Test (ORT) evaluates short-term (working) recognition memory.
   - Object Location Test (OLT) evaluates short-term (working) spatial memory.
   - Morris Water Maze (MWM) functions as a hippocampus-dependent test of special learning, encompassing reference memory and retention spatial memory.

B. Levels of neurotransmitters:
   - Levels of GLU are associated with cognitive functions and the HP, but also, MA causes excitotoxity in cells via increasing GLU levels. Therefore, GLU will be measured as one of the neurotransmitters in the proposed research.
   - Levels of NA, 5HT and DA, which are hypothesized to have a significant impact on the effects of MA were also measured. Rats prenatally exposed to MA have elevated DA levels in the mesolimbic dopaminergic system.

C. Levels of neurotrophins - BDNF and NGF. These proteins significantly contribute to neuroprotection in MA-induced brain injury. Inconsistent data exist in the scientific literature regarding their production in the HP; upregulation and downregulation differ according to the experimental protocol of MA administration. Therefore, these neurotrophins were measured.

D. Oxidative stress-Multiple studies have provided evidence that when neurotoxic concentrations of MA are administered, DA oxidation occurs, resulting in the production of reactive oxygen species derived from DA in the STR and HP. 4-hydroxynonenal and malondialdehyde were measured in HP and STR.

E. Expression of c-Fos, which is frequently observed in neurons that discharge action potentials. It has been demonstrated that psychostimulants increase c-Fos production in the mesocortical and mesolimbic reward pathways [96]. Also, upregulation of c-Fos is associated with learning and memory. Therefore, we examined the c-Fos protein in HP.

3. Material and methods

Animal Care and Treatment

- Wistar rats were purchased from Velaz and bred by Charles River Laboratories International, Inc.
- Rats were housed in a temperature-controlled room with a standard 12 h light/dark cycle.
- Rats were left undisturbed for one week before fertility determination.
- Female rats were smeared using vaginal lavage for estrous cycle phase determination.
- The day after birth, the number of pups in each litter was adjusted to 12 – ideally 8 males and 4 females.
- Pups were randomly assigned to MA-treated (MA) groups and saline (SA)-treated control groups.

Drugs

- Physiological saline (0.9 % NaCl) and d-Methamphetamine hydrochloride were purchased from Sigma-Aldrich.

Experiment Design

- Two different methods of postnatal MA administration were studied: direct and indirect.
- MA was injected at a 5 mg/ml/kg dose per day during exposure period.
- Exposure to MA or SA was performed every day during morning hours.
- Pups were exposed to a standard preweaning housing (SC) or to an enriched preweaning housing (EE) with larger cages containing toys.
- On PD 21, pups were weaned from mothers and divided into two groups: housed in groups (GH) and housed separately (SH).

Behavioral Testing

- Animals were exposed to MA before behavioral testing.
- After the last day of exposure, animals were left undisturbed until weaning on PD 21.
- Animals remained in this environment until the behavioral tests were completed.
- Animals (n=8) were tested during PD 28-32 (Habituation test), PD 35-38 (Object Recognition Test and object location test), and PD 40-51 (Morris Water Maze).

Habituation was evaluated by comparing distance moved between the first and fourth day of exposure to the Open field arena.

Object Recognition and Object Location Tests

- Object Recognition Test (ORT) measures the exploration of novel versus familiar objects, a component of recognition memory.
- The test consists of habituation, training, and testing.
- Animals were habituated to the black empty square arena for three days, each day for 10 minutes.
- Training involved placing the rat into the arena for 5 minutes to explore two identical objects placed in the arena.
- Testing involved placing one of the original objects (beer cans) and one new object of similar size (glass jar) on the same places as during the training.
- Object Location Test (OLT) measures the exploration time of two same objects (beer cans) but one is placed to a novel location.

Morris Water Maze

- The MWM is a circular pool with 2 m diameter filled with water, with four start positions marked on the edges of the pool.
- The swimming of the animals is automatically captured by a camera placed above the pool and evaluated by EthoVision 14 program.

- One animal is tested for 12 days in total, with the first six days performing a learning task, the 8. day "probe" test, and the 12. day memory test.

Learning Test

- Rats were placed in a pool eight times a day for six consecutive days.
- The platform was identical during the whole experiment, with a maximum time limit of 60 seconds.
- The learning test evaluated distance moved, search error, time taken to find the platform, and velocity of swimming.
- Strategies of looking for the platform were evaluated: thigmotaxis and scanning.
- Thigmotaxis was used during the first days of learning, where animals were not properly oriented in the pool.
- Scanning was used when animals were already orienting in space and were familiar with the position of the hidden platform.

Memory Test

- The test was performed on the 12th day, testing long-term spatial memory.
- Spatial memory involves the ability to remember locations, routes, and spatial relationships in their environment.
- The hidden platform was returned to its original position during the learning phase.

Brain Sample Collection and Analyses

- HP (hippocampus) and STR (striatum) were collected for molecular analyses.
- Brain tissues were anesthetized intraperitoneally and given an intracardial perfusion of heparinized saline.
- Samples were homogenized for neurotransmitter detection, BDNF and NGF detection, and oxidative stress detection.
- Nuclear extracts of HP were used for c-Fos detection.
- Protein concentrations were estimated using the BCA method with Bicinchoninic Acid Kit for Protein Determination.

ELISA

- ELISA procedures were used for the detection of catecholamines (DA, NA), 5HT, GLU, BDNF, NGF, 4HNE, and TBARS.
- The color reaction during ELISA is illustrated in Figure 9.
- Calibration curve-4 parameters are used for calculation of neurotransmitters concentrations.
- CAT Reasearch ELISA kits were used for DA and NA detection.
- Serotonin Research ELISA kits were used for 5HT detection.
- Glutamate ELISA kits were used for GLU detection.

Neurotrophin Detection and Oxidative Stress Analysis

- BDNF and NGF ELISA Kits used for BDNF and NGF detection.
- Samples diluted 20-times and analyzed according to manufacturer's instructions.
- Protein concentrations estimated using Bradford method.

Oxidative Stress Estimation

- TBARS Lipid Peroxidation (MDA) Assay Kit used for thiobarbituric acid reactive substances estimation.
- 4-HNE analyzed using ELISA kits from Cusabio.
- Proteins in homogenates estimated using Bicinchoninic Acid Protein Assay Kit from Sigma-Aldrich.

c-Fos Activation Detection

- c-Fos Transcription Factor Assay Kits used to detect activation of c-Fos.
- Protein concentration determined by BCA method after nuclear extract preparation.
- Samples diluted with ENE2 extraction buffer to maximum 15 mg protein per well.

- Absorbances compared to absorbance of well containing AP-1 mutated oligonucleotide, AP-1 wild-type oligonucleotide, and K-562(TPA) nuclear extract stimulated with TPA.

Statistical Analysis of Results

- Determined distribution and variance of data in individual subgroups to determine if a parametric test can be used.
- Three-way ANOVA used for data analysis for all experiments.
- Tukey post hoc test used for multiple comparisons between groups.
- Differences considered significant if $p < 0.05$.

4. Results

4.1 Behavioral testing

4.1.1 Learning

Direct exposure

First parameter examined during the learning task was distance moved during 6 consequent days of this trail. Distance swam was significantly impaired by postweaning housing since separated animals swam longer distance than grouped animals [$F_{(1,56)} = 15.96$, $p=0.0001$]. Interaction between all three observed factors was significant [$F_{(1,56)} = 10.66$, $p=0.002$]. Separated animals swam longer distance in all days in comparison with grouped animals. On the first and last day of learning, EE exposed animals swam significantly longer distance than animals with standard housing. Velocity of swimming was altered by both preweaning since EE animals swam more quickly than SC [$F_{(1,56)} = 8.50$, $p=0.005$] and postweaning housing [$F_{(1,56)} = 13.99$, $p=0.0004$] where separated animals swam more quickly than grouped animals. Also, interaction between treatment and preweaning housing was significant since [$F_{(1,56)} = 6.46$, $p=0.01$]. MA treatment significantly decreased velocity in SC animals but in EE exposed animals. SA/SC/GH treated grouped controls were significantly quicker than MA/SC/GH animals. Latency to discover hidden platform was significantly altered by postweaning housing, since separated animals were significantly slower in discovering hidden platform than grouped [$F_{(1,56)} = 8.34$, $p=0.005$]. Interaction between all observed factors was also significant since [$F_{(1,56)} = 9.5$, $p=0.003$]. SA/EE/SH animals showed significantly increased latency to hidden platform however, this phenomenon was also present in MA/SC/GH animals, suggesting importance of some stress factor in term of learning alteration (MA or separation). Search error was significantly altered by postweaning housing [$F_{(1,56)} = 6.9$, $p=0.01$] as well as interaction between treatment, preweaning and postweaning housing [$F_{(1,56)} = 7.51$, $p=0.008$]. Separated control animals showed significantly increased search error than grouped animals as well as MA exposed standard grouped. Thigmotaxic strategy was also altered by postweaning housing [$F_{(1,56)} = 10.51$, $p=0.001$] as well as interaction between treatment, preweaning and postweaning housing [$F_{(1,56)} = 9.40$, $p=0.003$]. Separated animals used this strategy significantly longer than grouped animals and as in previous cases, thigmotaxis was mostly used by separated control animals exposed to EE as well as grouped animals raised in standard housing and exposed to MA. Scanning was altered by interaction between treatment and preweaning housing [$F_{(1,56)} = 5.71$, $p=0.02$]. This strategy was used more by control animals exposed with standard preweaning housing and MA treated animals exposed to EE.

Indirect exposure

Parameters within learning test after indirect exposures were not significantly altered by any observed factor. Distance was not significantly altered by any of observing factors, neither velocity, latency however, MA exposed animals showed higher latency than controls. Search error displayed higher tendency of EE exposed animals to have higher search error than standard animals during all days of learning [$F_{(1,56)} = 3.54$, $p=0.004$]. This situation was similar in case of thigmotaxis and scanning.

4.1.2 Memory

Direct exposure

Distance was significantly altered by treatment [$F_{(1,56)} = 9.54$, $p=0.003$] and preweaning housing [$F_{(1,56)} = 11.258$, $p=0.001$]. MA exposed animals swam significantly less distance than controls and EE exposed animals swam significantly more distance than standardly raised animals. Velocity was significantly altered by all three factors [$F_{(1,56)} = 8.17$, $p=0.006$; $F_{(1,56)} =$

16.261, p=0.002; $F_{(1,56)} = 8.68$, p=0.004]. MA exposed animals were significantly slower than controls, EE exposed animals were significantly quicker than standardly raised animals and separated animals were significantly quicker than grouped. Latency was not significantly altered by any factor, neither search error, thigmotaxis, and scanning.

Indirect exposure
Distance moved was significantly altered by preweaning housing [$F_{(1,56)} = 4.32$, p=0.04]. EE exposed animals swam longer distance than standardly raised. Velocity, latency, neither search error was not significantly altered by any factor. Thigmotaxis significantly altered by interaction between treatment and postweaning housing [$F_{(1,56)} = 6.48$, p=0.013]. Separated MA treated animals used this strategy most along with grouped controls. Scanning was significantly altered by preweaning housing [$F_{(1,56)} = 5.98$, p=0.02], since EE exposed animals used this strategy eminently more than standardly raised animals.

4.2 Immunoanalyses

4.2.1 Catecholamines

Hippocampus

PD 28
Levels of DA in HP were highest in MA/EE/GH animals, and within SA treated animals, these levels were similar. Differences in NA levels were not statistically significant. Indirectly exposed animals had significantly higher levels of NA within MA/ EE/GH in comparison with MA/ SC/SH (p=0.0411) and preweaning housing had significant impact on these results [$F_{(1, 54)} = 7.444$, p=0.0111].

PD 35
Levels of DA in HP on this PD were lower in EE GH animals among both treatment groups, which differ from previous PD situation. Levels of NA were significantly higher in SA/ SC/SH animals in comparison with SA/ EE/GH, (p=0.0363) which is opposite phenomenon described on previous PD, SA/SC/SH showed significantly higher levels of NA in comparison with SA/EE/SH (p=0.0407). In this case, interaction between treatment and preweaning housing had significant impact on these results [$F_{(1, 54)} = 5.070$, p=0.0324]. Similarly, as on previous PD within indirect exposure, preweaning housing had significant impact on levels of NA [$F_{(1, 54)} = 11.61$, p=0.002]. SA/SC/SH animals had significantly higher levels of NA in comparison with SA/EE/GH (p=0.0135).

PD 45
Levels of DA within SA treated animals got to similar levels, however within MA treated animals, these levels remained in similar ration as on previous PD. Treatment [$F_{(1, 54)} = 11.53$, p=0.0021] as well as preweaning housing [$F_{(1, 54)} = 19$, p=0.0002] had significant impact on levels of NA. MA/SC/SH animals had significantly higher levels in comparison with SA/SC/SH (p=0.0305), as well as SA/EE/GH (p=0.001) and MA/EE/GH (p=0.048). Within indirectly exposed animals we obtained significant differences in levels of DA and NA as well. Preweaning housing had significant impact on differences in levels of NA [$F_{(1, 54)} = 5.745$, p=0.023], and these levels were significantly higher in MA/SC/SH animals in comparison with MA/EE/GH (p=0.016). Interaction between treatment and preweaning housing had significant impact on levels of DA [$F_{(1, 54)} = 9.374$, p=0.005] and as in case of NA, levels of DA were significantly higher in MA/SC/SH in comparison with MA/EE/GH (p=0.021).

Striatum

PD 28

Preweaning housing had significant influence on DA levels in striatum [$F_{(1, 54)} = 91.05$, p=0.0001]. SA/SC/SH animals had significantly lower levels than SA/EE/GH (p=0.0013) as well as MA/EE/GH (p=0.0007). MA/SC/SH animals had significantly lower levels than MA/EE/GH (p=0.005) and SA/EE/GH (p=0.009). Levels of DA were also significantly lower in SA/SC/GH in comparison with SA/EE/GH (p=0.0002), significantly lower in MA/SH/GH in comparison with MA/EE/GH (p=0.001) as well as in MA/SC/SH than MA/EE/SH (p=0.0001). Levels of NA were significantly influenced by preweaning housing [$F_{(1, 54)} = 12$, p=0.0019] and these levels were significantly higher in MA/EE/GH in comparison with SA/SC/SH (p=0.038) and significantly higher in MA/SC/SH than MA/EE/SH (p=0.0012). Within indirectly exposed animal, preweaning housing had significant impact in differences of levels of DA [$F_{(1, 54)} = 15.47$, p=0.0007]. SA/SC/SH animals had significantly lower levels of DA in comparison with SA/EE/GH (p=0.0128) and MA/ SC/SH (p=0.0139). NA levels were influenced by preweaning housing as well DA [$F_{(1, 54)} = 7.444$, p=0.011] and MA/EE/GH had significantly higher levels of DA in comparison with MA/SC/SH (p=0.041).

PD 35

Treatment [$F_{(1, 54)} = 22.12$, p=0.0001] and preweaning housing [$F_{(1, 54)} = 5.326$, p=0.0289] had significant influence on DA levels as well as interaction between these factors [$F_{(1, 54)} = 6.636$, p=0.0158]. MA/EE/GH animals had significantly higher levels of DA in comparison with SA/SC/SH (p=0.002), SA/EE/GH (p=0.0001) and MA/EE/GH (p=0.0081). These levels were also significantly higher in MA/EE/GH than. MA/EE/SH (p=0.0055), and MA/SC/GH (p=0.0001). Levels of NA were influenced by treatment [$F_{(1,54)} = 14.83$, p=0.0008] and interaction between treatment and preweaning housing [$F_{(1, 54)} = 5.03$, p=0.033]. MA/EE/GH animals had significantly higher NA levels in comparison with SA/SC/SH (p=0.0402) and SA/EE/GH (p=0.0014). Also, these levels were significantly higher in MA/EE/GH in comparison with SA/EE/GH (p=0.0124) as well as in SA/EE/SH than SA/EE/GH (p=0.0128). Indirectly exposed animal levels of NA did not show any significant differences between groups and DA levels were significantly impacted by interaction between treatment and preweaning housing [$F_{(1, 54)} = 8.329$, p=0.0073]. MA/EE/GH had significantly higher than MA/SC/SH animals (p=0.0455).

PD 45

Within directly as well as indirectly exposed animals, no significant differences were obtained in levels of DA and NA.

4.2.2 Neurotrophins

BDNF

Direct exposure

On PD 28 there were no significant differences between groups. On PD 35 several factors of significance were found: treatment [$F_{(1,56)} = 12.41$, p=0.0009], preweaning housing [$F_{(1,56)} = 31.20$, p=0.0001], interaction between preweaning housing and postweaning housing [$F_{(1,56)} = 5.030$, p=0.0289], and interaction between all factors [$F_{(1,56)} = 6.588$, p=0.0130]. In multiple comparison analysis we acquired differences between these groups: levels of BDNF in SA/SC/SH animals were significantly lower than in MA/SC/SH (p=0.0005). This levels in MA/SC/SH were significantly higher than MA SC/GH (p=0.0069), MA EE/SH (p=0.0001) as well as MA/EE/GH (p=0.0004)**.** On PD 45 the following significant factors were: treatment [$F_{(1,51)} = 5.495$, p=0.0230], treatment × housing [$F_{(1,51)} = 4.624$, p=0.0360] and interaction of all

three factors [$F_{(1,51)}$ =5.290, p=0.0256]. Levels of BDNF in MA/SC/SH were significantly higher than in SA/SC/SH (p=0.0030), MA SC/GH (p=0.0301) and MA/EE/GH (p=0.0481).

Indirect exposure

According to statistical analyses, our results show, that indirect exposure of MA did not eminently alter BDNF levels and we did not obtain significant differences.

NGF

Direct exposure

On PD 28 after direct exposure, there were no significant differences between groups. On PD 35, factor of significance was preweaning housing [$F_{(1,56)}$ =8.626, p=0.0048]; levels of NGF levels in SA/SC/SH were significantly higher than in SA/EE/GH (p=0.0040), which was more apparent in SA group. On PD 45 the only factor of significance was preweaning housing [$F_{(1,55)}$ =27.45, p=0.0001]; there are significantly lower NGF levels in SA/SC/SH (p=0.0059) than in SA/EE/GH (p=0.0040).

Indirect exposure

On PD 28 factors of significance were preweaning housing [$F_{(1,56)}$ =17.55, p=0.0001] and interaction between treatment and preweaning housing [$F_{(1,56)}$ =19.31, p=0.0001]. There were significantly higher levels in SA/EE/SH in comparison to MA/EE/SH (p=0.072), significantly higher levels in MA/SC/SH in comparison to MA/EE/GH (p=0.0009) as well as significantly higher levels in MA/SC/SH in comparison to MA/EE/SH (p=0.0008) and MA/EE/GH (p=0.0006).On PD 35 the only factor of significance was preweaning housing [$F_{(1,56)}$ =19.48, p=0.0001]. We obtained significantly higher levels of NGF in SA/SC/SH in comparison to SA/EE/SH (p=0.0009) as well as SA/EE/GH (p=0.0018). On PD 45, there were none significant differences.

4.2.3 Oxidative stress

Levels of 4HNE

PD 28

In terms of direct exposure, interaction between all factors was significant [$F_{(1, 57)}$ = 5.473, p=0.0228] in HP. Levels of 4HNE were significantly higher in SA/SC/SH than MA/SC/SH (p=0.0131), significantly lower in SA/EE/SH than MA/EE/SH (p=0.0417) as well as in SA/SC/GH than SA/SC/SH (p=0.0363), significantly higher in SA/SC/SH than SA/EE/SH (p=0.0152) and significantly lower in MA/SC/SH than MA/EE/SH (p=0.0322). In STR, there were not any significant differences. In terms of indirect exposure, preweaning housing [$F_{(1, 54)}$ = 7.740, p=0.0074] and interaction between treatment and preweaning were significant in HP [$F_{(1, 54)}$ = 4.191, p=0.0455]. Levels of 4HNE were significantly higher in SA/SC/GH than SA/EE/GH (p=0.0181) as well as in SA/SC/SH than SA/EE/SH (p=0.0264). In STR there were not any significant differences.

PD 45

In terms of direct exposure, there were not any significant difference in HP. In STR, preweaning housing [$F_{(1, 60)}$ = 3.302, p=0.0742], interaction between treatment and preweaning housing [$F_{(1, 60)}$ = 6.956, p=0.0106] as well as interaction between all factors were significant [$F_{(1, 60)}$ = 4.245, p=0.0437]. Levels of 4HNE were significantly higher in SA/SC/GH than MA/SC/GH (p=0.0102) and then SA/SC/SH (p=0.0077) and significantly lower in MA/SC/GH than MA/EE/GH (p=0.0085). In terms of indirect exposure, preweaning housing was significant factor in HP [$F_{(1, 48)}$ = 4.063, p=0.0494]. Levels of 4HNE were significantly higher in MA/SC/GH than MA/SC/SH (p=0.0421) and significantly lower in MA/SC/SH than

MA/EE/SH (p=0.0092). In STR, treatment [$F_{(1, 49)} = 7.006$, p=0.0109] and preweaning housing were significant [$F_{(1, 49)} = 14.11$, p=0.0005]. Levels of 4HNE were significantly higher in SA/SC/SH than SA/EE/SH (p=0.0205).

Levels of TBARS

PD 28

In terms of direct exposure, there were not any significant differences between groups in HP neither STR. Within indirect exposure in HP, treatment [$F_{(1, 55)} = 6.33$, p=0.0148], preweaning housing [$F_{(1, 55)} = 10.12$, p=0.0024] and interaction between treatment and postweaning housing were significant [$F_{(1, 55)} = 14.34$, p=0.0004]. Levels of TBARS were significantly lower in SA/SC/SH than MA/SC/SH, 0.0021, and SA/EE/SH (p=0.0292). In STR, factor of significance was preweaning housing [$F_{(1, 56)} = 14.89$, p=0.0003]. Levels of TBARS were significantly lower in MA/SC/GH than MA/EE/GH (p=0.0103).

PD 45

In terms of direct exposure, there were not any significant differences between groups in HP. In STR, all factors were significant [$F_{(1, 60)} = 11.29$, p=0.0014], [$F_{(1, 60)} = 30.47$, p=0.0001], [$F_{(1, 60)} = 8.182$, p=0.0058] as well as interaction between preweaning housing and treatment [$F_{(1, 60)} = 4.531$, p=0.0374], and all factors [$F_{(1, 60)} = 5.958$, p=0.0176]. Levels of TBARS were significantly lower in SA/SC/SH than MA/SC/SH (p=0.0006) than SA/SC/GH (p=0.0024), significantly higher in SA/SC/GH than SA/EE/GH (p=0.0016), significantly higher in MA/SC/GH vs. MA/EE/GH (p=0.133), and MA/SC/SH than MA/EE/SH (p=0.0017).In terms of indirect exposure, in STR, interaction between preweaning and postweaning housing was significant [$F_{(1, 51)} = 7.605$, p=0.0081]. Levels of TBARS were significantly lower in SA/EE/GH than MA/EE/GH (p=0.0106) and significantly lower in SA/EE/GH than SA/EE/SH (p=0.0397).

5. Discussion

The study investigates the long-term effects of early postnatal MA administration on various behavioral and molecular parameters, as well as the role of the individual's environment during development. The study hypothesizes that EE during the preweaning period have a positive effect on the long-term effects of early postnatal MA exposure. Postweaning environmental conditions, such as group housing or separation, also influence the long-term effects of early postnatal MA exposure.

Firstly, our study found that MA-treated pups on PD 12 showed significantly decreased levels of DA in HP and STR, indicating that subcutaneous MA exposure during this period significantly alters DA neurotransmission. Other neurotransmitters, such as NA, GLU, and 5HT, were not significantly altered. The behavioral testing outcomes were tested using four types of tests: habituation, ORT, OLT, MWM - learning and memory tests.

5.1 Learning and memory

Our study found that MA exposure during the neonatal stage significantly impaired spatial learning, which relies on the HP. The study also found that separation had the greatest influence on learning, with MA/EE/SH swimming the greatest distance on the first day and SA/EE/SH swimming the least. The study also observed that separation had the greatest influence on learning, with EE having no beneficial effects. Separated animals were exposed to EE only during the postweaning period, while grouped animals were exposed to EE only during the postweaning period. Two strategies were observed during the test: thigmotaxis and scanning. The time spent in thigmotaxis was highest in SA/EE/SH and MA/EE/SH, while the least in SA/EE/GH and MA/EE/GH on the first day of learning. The scanning strategy was mostly used by MA/SC/SH, SA/EE/SH, and MA/EE/SH, and least used by the sixth day, SA/EE/GH on the last day of learning. Separated animals may have higher motivation, but their ability to learn was poorest among all animals. This may be due to anxiety-driven motivation among separated animals, while animals housing in EE cages in groups were exposed to various social and sensory stimulants, potentially causing their lack of motivation. This study investigates the impact of social separation on learning ability in animals. Control separated animals performed best in almost all parameters but had the worst results at the end of the test. During the first four days, animals exposed to EE in combination with MA learned the worst, but this trend changed positively at the end of the testing. Indirect exposure to MA significantly impaired learning ability, while the role of MA in this case is disputed. The effect of EE did not show to be beneficial. In terms of memory recall, animals exposed to EE generally swam a greater distance than animals in standard cages. Swimming speed was the fastest in separated animals exposed to EE in both treatments. Latency to the platform was affected by EE in controls, where EE animals searched for the platform significantly longer, but in MA this phenomenon was not as intense. Among animals exposed to MA, animals with a standard environment housed in groups showed the lowest error. The use of the scanning strategy, like other parameters, was influenced by EE, but only in controls. Thigmotaxis was most used in controls exposed to EE and separation in controls, and this difference was striking especially when compared to the same group within MA. Indirect exposure to MA also showed that social separation stress in combination with MA improved these processes. EE in combination with MA did not show any differences, but within the animals exposed to MA that were after weaning in groups, there was a slight improvement. Strategies and search error were also apparently impaired by EE, but stress from separation also improved performance of control animals. MA treatment has been shown to have negative effects on cognitive abilities during specific developmental periods, such as PD 6-15 and PD 11-20 [1]. Studies have found that MA doses of 10-25 mg/kg had a greater impact during these periods, while PD 1-10 or PD 21-30 were less affected or not

affected at all. Exposure to MA during the early postnatal period has been found to be detrimental, possibly due to the smaller dose used in this study and the fact that animals were exposed only once a day [2-6]. Other study reported that EE exposure to obese rats with cognitive deficits has been observed to increase the volume of HP and neuron number in the CA1 subfield of the HP [7]. However, the effect of EE was opposite, suggesting that the correct determination of the critical window period for EE interventions in restoring cognitive functions is crucial. EE exposure during adolescence has been shown to alleviate memory impairment, decrease BDNF levels, and anxiety-like behavior induced by experimental depletion of 5HT [8]. Studies have also shown that EE exposure during the juvenile period improves selective attention, increases foraging-like behavior, and reduces anxiety levels. Postweaning EE reverses maternal separation, improving passive avoidance memory and increasing nociceptive response against thermal stimulus in both sexes [9, 10]. In adulthood, social discrimination is impaired in deprived male and female rats in the three-chamber social approach task. However, after 24 hours of isolation, these animals showed shorter latencies to engage in social play behavior [11].

5.2 Catecholamines

The study investigates the effects of MA on neurotransmitters, specifically DA and NA. Directly exposed animals showed no significant difference in dopamine levels within PD 28 but increased in STR in EE animals in both treatments. Indirectly exposed animals showed similar increases in dopamine levels in HP and STR, but in MA, these values were significantly higher in MA/EE/GH.

Several studies have shown that MA exposure has some effect on DA neurotransmission, with different forms of administration having similar effects [12-14]. Studies have shown that MA effect is age-dependent and reversible, with MA exposure 4-times a day in a dose of 10mg/kg causing a significant decrease in striatal activity of DA transporters [15, 16]. Noradrenaline levels in PD 28 in directly exposed animals in HP were not significantly different except for EE/GH in MA, while in STR these values were significantly higher in EE than in the standard environment within both treatments. In indirectly exposed animals, NA levels in HP were increased in EE animals within MA but partly also in SA. At PD 35 in directly exposed animals, NA levels in HP were significantly increased in SA/SC/SH but not within MA. In STR, these values were high in SA/EE/SH and decreased in SA/EE/GH, unlike MA, where these values were highest in EE/GH. At PD 45 in directly exposed animals, NA levels in HP were significantly lower in EE animals in both treatments. In STR, these differences were not visible, and NA levels were increased only in SA/EE/GH. In MA, these differences were significant. Long-term administration of high doses of MA to rhesus monkeys has been reported to deplete NA in the frontal cortex and midbrain and DA in the caudate nucleus. However, recent research has shown that these depletions are not irreversible and can remain as long as twelve months past the repeated injection period [17-19]. Experiments have shown that amphetamine and DA compete at the same binding site and are transported. Postweaning EE partially buffered these changes, providing evidence on reversibility of these alterations following EE. Studies have also shown that EE exposure causes decreased D2 receptor expression in HP and suppresses D2 dopamine receptor expression in rats with traumatic brain injury [20].

5.3 Serotonin

Serotonin levels have also been observed, with studies showing that EE significantly increases the muted 5HT in SA pups after separation and restores the inhibition of 5HT by MA. The timing and method of MA administration are crucial due to the different susceptibility of 5HT neurons during postnatal development. Interestingly, social isolation during adolescence leads to vulnerability to cocaine seeking behavior and alters behavioral responses to cocaine later in

adulthood [21]. Conversely, EE stimulates various biochemical and functional changes in the HP, especially network connectivity and the developing of new neurons in the dentate gyrus of mice exposed to an EE compared with standard housing environment. In conclusion, MA, preweaning, and postweaning environments all play a role in affecting serotonergic neurotransmission in various brain regions [22, 23].

5.4 Glutamate

In term of GLU, we found that GLU levels were higher within MA administered grouped animals compared to separated animals, both exposed to EE. The effect of EE on neurotransmitter levels has not been sufficiently documented, especially in connection with MA and adolescent brain. GLU transporters were less susceptible than 5HT transporters to the effects of MA treatment. Other study found that social isolation during adolescence leads to a decrease in GLU presynaptic neurotransmission, in ventral HP and nucleus accumbens [24].

5.5 Neurotrophins

Adolescent social isolation also leads to vulnerability to cocaine reinstatement compared to animals isolated in adulthood. Our study also found that exposure to EE did not lead to an increase in BDNF levels in either the control group or the group exposed to MA. However, social separation following weaning did decrease BDNF levels compared to animals in conventional housing. Direct exposure to MA counteracted this impact. The other study reported that exposure to EE resulted in a more robust dorsal hippocampal BDNF response and elevated serum BDNF levels [25]. Animals exposed to EE had a greater brain weight compared to rats kept in isolation, suggesting a resilient phenotype in response to stressful scenarios. In conclusion, the study highlights the importance of understanding the effects of MA on neurotransmitters and neurotrophins in adolescent animals.

5.6 Oxidative stress and c- Fos

Oxidative stress plays a significant role in the neurotoxicity caused by MA, as demonstrated by De Vito's research in 1989 [26]. MA can be reduced by pre-treating with antioxidants, with doses ranging from 0.25 to 10 mg/kg. In experimental research, adult rats are commonly administered doses of 10 mg/kg and above. Studies have shown that repeated doses of MA increase levels of lipid peroxidation products, such as MDA and HNE, which can provide valuable information about oxidative damage in the brain. The effect of MA exposure on rats during the preweaning period (PD 1-20) is like that of developing human fetus during the second half of gestation [27]. Brain structures that form higher cognitive functions develop during this developmental window and are susceptible to the damaging effects of drugs. Recent research has investigated the impact of repeated neonatal MA administration on striatal and hippocampal monoamines and peroxidation of lipids at the threshold of adolescence, and in combination with emotional stress applied after weaning [14, 28, 29]. Direct injection and indirect administration have been shown to trigger corticosterone release, which has detrimental effects on the immature HP. The study focuses on the juvenility phase in rat ontogeny, which corresponds to early human adolescence. Manipulation of social experiences during this period in rats has been shown to impair cognitive, behavioral, learning, and emotional balance later in adulthood. The adolescent brain is more responsive than the adult brain when confronted to a stressor, and daily isolation during this period results in a robust corticosterone release. In our series, brain monoamines were not affected by MA, but the immediate stress effect of separation was evident on enhanced NA concentrations in HP and STR. This was manifested only in the group of not injected pups who remained undisturbed until the weaning. The study found that immature male rats treated with MA or exposed to stress do not exhibit enhanced lipid peroxidation in striatum and hippocampus. This result does not exclude any harmful oxidative attack to brain during the periadolescent period, however, there are no conclusive data in the

literature yet, showing at which level MA produces oxidative stress in immature brain. Activation of c-Fos in HP was lowered by direct MA exposure on PD 28 and eminently by separation within indirectly exposed animals. This suggests that c-Fos induction in response to toxic doses of MA might be involved in protective mechanisms against drug-induced neurotoxicity [30, 31].

6. Conclusion

Our study led to several interesting results:

1. MA exposure during PD 1-12 promote less severe alteration in terms of indirect exposure via breastfeeding then the direct ones.
2. Housing in EE surprisingly worsened learning and memory functions, however caused elevated 5HT levels in HP as well as DA in STR in PD 28-old animals.
3. Social separation during postweaning period significantly worsened learning and memory abilities regardless of the treatment in comparison with group housing.
4. Animals exposed to EE and subsequently exposed to separation had impaired cognitive function, leading us to speculate that animals exposed to EE cope worse with stressful situations than animals that were not exposed to EE.

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Maronek M, Gromova B, Liptak R, Klimova D, CechovaČechová B, Gardlik R. Extracellular DNA is Increased in Dextran Sulphate Sodium-Induced Colitis in Mice. Folia Biol (Praha). 2018;64(5-6):167-172. PMID: 30938673. **IF =0.6**

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