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

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Dissertation work

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Prague 2024

## DECLARATION OF AUTHENTICITY

I declare that I prepared my dissertation work independently and precisely cited all scientific articles and literature used in my thesis. I also declare that the work has not been used to obtain any other or the same title. I hereby agree to upload the electronic version of my thesis to the database of the interuniversity project Thesis.cz for continuous monitoring of thesis similarity.

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# CONTENT

**DECLARATION OF AUTHENTICITY** ..... **2**

**IDENTIFICATION RECORD** ..... **3**

**ACKNOWLEDGEMENT** ..... **4**

**CONTENT** ..... **5**

**ABBREVIATIONS** ..... **7**

**ABSTRACT** ..... **8**

**ABSTRAKT** ..... **10**

**1. INTRODUCTION** ..... **12**

1.1 METHAMPHETAMINE NEUROTOXICITY ..... 12

1.2 CRITICAL DEVELOPMENTAL PERIODS ..... 16

1.3 METHAMPHETAMINE AND ITS IMPACT ON DEVELOPMENT IN RATS ..... 18

1.4 THE ROLE OF UPBRINGING AND ENVIRONMENT ON THE DEVELOPING BRAIN ..... 20

**2. AIMS AND HYPOTHESESIS** ..... **23**

**3. METHODS** ..... **27**

3.1. ANIMAL CARE ..... 27

3.2 DESIGN OF THE EXPERIMENT ..... 27

3.3. BEHAVIORAL TESTING ..... 31

*3.3.1. Habituation* ..... *31*

*3.3.2. Object recognition and object location tests* ..... *32*

*3.3.3. Morris Water maze* ..... *33*

*Learning test* ..... *34*

*Memory test* ..... *35*

3.4. BRAIN SAMPLE COLLECTION AND ANALYSES ..... 36

*3.4.1 CATECHOLAMINES* ..... *39*

*3.4.2 SEROTONIN* ..... *39*

*3.4.3 GLUTAMATE* ..... *40*

*3.4.4 NEUROTROPHINS* ..... *40*

*3.4.5 OXIDATIVE STRESS* ..... *40*

*3.4.6 C-FOS* ..... *41*

STATISTICAL ANALYSES OF RESULTS ..... 42

**4. RESULTS** ..... **43**

4.1 Levels of neurotransmitters after direct exposure to MA on PD 12 ..... 43
4.2 Behavioral testing ..... 44
*4.2.1 Habituation ..... 44*
*4.2.2 Object recognition test ..... 45*
*4.2.3 Object location test ..... 51*
*4.2.3 Morris Water Maze ..... 57*
4.3. Immunoanalyses ..... 69
*4.3.1 Neurotransmitters ..... 69*
*4.3.1.1 Catecholamines ..... 69*
*4.3.1.2 Glutamate ..... 76*
*4.3.1.3 Serotonin ..... 78*
*4.3.1.4 Neurotrophins ..... 84*
*4.3.2 c-fos in hippocampus ..... 89*
*4.3.3 Oxidative stress ..... 91*

**5. DISCUSSION ..... 98**

5.1 Levels of neurotransmitters on PD 12 ..... 98
5.2. Behavioral testing outcomes ..... 99
*5.2.1 Habituation (PD 28-31) ..... 99*
*5.2.2 ORT and OLT (PD 31-32) ..... 103*
*5.2.3 Morris Water Maze ..... 106*
5.3 Molecular analyses ..... 113
*5.3.1 Neurotransmitters ..... 113*
*5.3.2 Neurotrophins ..... 119*
*5.3.3 Oxidative stress ..... 120*
*5.3.4 Activation of c-fos ..... 123*

**6. CONCLUSION ..... 125**

**AUTHOR'S PUBLICATIONS ..... 126**

**CONFERENCES ..... 127**

**REFERENCES ..... 130**

# ABBREVIATIONS

MA - methamphetamine

SA - saline

CNS – central nervous system

DA - dopamine

NA -noradrenaline

5HT - serotonin

GLU -glutamate

BDNF- brain-derived neurotrophic factor

NGF – nerve growth factor

HP -hippocampus

STR - striatum

4HNE -4-hydroxynonenal

TBARS- thiobarbituric acid reactive substances

ROS – reactive oxygen species

MWM – Morris water maze

ORT – object recognition test

OLT – object location test

PD – postnatal day

LTP – long-term potentiation

CA1 – cornu ammonis

## ABSTRACT

Methamphetamine (MA) is a synthetic psychostimulant that is one of the most abused drugs in the world. It is a member of the amphetamine group, and its popularity is based on its numerous rapid-onset stimulant effects, such as increased alertness, increased energy, decreased appetite, etc. However, in the long term, this drug has very serious consequences for the mental and physical health of individuals. Long-term use of MA causes severe impairment of central nervous system (CNS) function and associated behavioral changes. By virtue of its chemical similarity to monoamine neurotransmitters, MA interacts with their respective receptors and transporters. This phenomenon causes permanent damage to the terminal endings of neurons through the development of oxidative stress, neuroinflammation, and subsequently apoptosis. Thus, MA use causes various behavioral disorders, such as depression and psychosis and contributes to the outbreak of Alzheimer's and Parkinson's diseases. The use of such drugs can have a very serious impact during critical developmental periods, which are periods in which certain parts of the brain and the whole body are undergoing intensive development. Disrupting these processes can have irreversible consequences. These drugs are very often abused by pregnant women, who starve them for their stimulating effects. It has been observed that the use of MA by pregnant women has a negative impact on the development of the baby and its behavior. However, clinical studies are complicated because women who abuse this drug often abuse other drugs as well, and we do not know the concentration and purity of these substances, as well as how long they have abused this substance before and after pregnancy. Not only does MA cross the placenta, but it is also found in breast milk. However, the environment and upbringing of the individual are also important and can affect development positively or negatively. Animal studies are, therefore, useful. In our laboratory, it has been found that the negative effect on various cognitive functions in an adult exposed to MA prenatally depends on the stage of pregnancy at which the substance is administered. It has also been found that neonatal administration of this substance, specifically during the first 12 postnatal days, has a negative impact on the cognitive function of the individual. This period

in the rat corresponds to the third trimester in man. Therefore, in this study, we decided to observe the effects of MA on adolescent subjects who were 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 the mothers. The animals were exposed to an enriched environment during development. After weaning, we performed several behavioral tests, which were mainly used to test memory. Animals were grouped or separated after weaning. Separation is a stress factor that can also negatively affect the development of an individual. The results of behavioral tests showed us that separation has a greater negative impact on learning and memory than MA alone, but surprisingly, the enriched environment also had a negative impact in this case. In the next part of the experiment, we measured the levels of neurotransmitters, growth factors, as well as oxidative stress and c-fos at different stages of adolescence, namely in PD 28, PD 35 and PD 45. Neurotransmitter levels were also 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. C-fos expression was decreased during early and late adolescence following MA administration. Our results suggest that the administration of MA during the first 12 days has a less pronounced effect in the case of indirect administration and that the development and environment of the developing individual plays a critical role in this case.

# ABSTRAKT

Metamfetamin (MA) je syntetický psychostimulant, který je jednou z nejčastěji zneužívaných drog na světě. Patří do skupiny amfetaminů a jeho obliba je založena na četných rychle nastupujících stimulačních účincích, jako je zvýšená pozornost, zvýšená energie, snížená chuť k jídlu atd. Z dlouhodobého hlediska má však tato droga velmi závažné důsledky pro duševní a fyzické zdraví osob. Dlouhodobé užívání MA způsobuje závažné poškození funkce centrálního nervového systému (CNS) a s tím související změny chování. Na základě své chemické podobnosti s monoaminovými neurotransmitery MA interaguje s jejich příslušnými receptory a transportéry. Tento jev způsobuje trvalé poškození terminálních zakončení neuronů prostřednictvím rozvoje oxidačního stresu, neurozánětu a následně apoptózy. Užívání MA tak způsobuje různé poruchy chování, jako jsou deprese, psychózy, a přispívá ke vzniku Alzheimerovy a Parkinsonovy choroby. Užívání těchto léků může mít velmi závažný dopad v kritických vývojových obdobích, což jsou období, kdy určité části mozku a celého těla procházejí intenzivním vývojem. Narušení těchto procesů může mít nevratné následky. Tyto drogy jsou velmi často zneužívány těhotnými ženami, které si je oblíbily pro jejich stimulační účinky. Bylo zjištěno, že užívání MA těhotnými ženami má negativní vliv na vývoj a chování dítěte.

Bylo zjištěno, že užívání MA těhotnými ženami má negativní vliv na vývoj a chování dítěte. Klinické studie jsou však komplikované, protože ženy, které tuto drogu zneužívají, často zneužívají i jiné drogy a neznáme koncentraci a čistotu těchto látek, stejně jako to, jak dlouho tuto látku zneužívaly před těhotenstvím a po něm. MA nejenže prochází placentou, ale nachází se také v mateřském mléce. Důležité je však také prostředí a výchova jedince, které mohou vývoj pozitivně či negativně ovlivnit. Užitečné jsou proto studie na zvířatech. V naší laboratoři bylo zjištěno, že negativní vliv na různé kognitivní funkce u dospělého jedince vystaveného prenatálně působení MA závisí na fázi těhotenství, ve které je látka podávána. Bylo také zjištěno, že neonatální podání látky, konkrétně prvních 12 postnatálních dnů, má negativní vliv na kognitivní funkce jedince. Toto období u potkanů odpovídá třetímu trimestru u člověka. Proto jsme se v této studii rozhodli sledovat účinky MA na dospívající jedince, kteří byli vystaveni MA během prvních

12 dnů života. Zvířata byla vystavena působení drogy jak přímo subkutánní injekcí, tak nepřímo prostřednictvím mateřského mléka, kdy byl MA podáván jejich matkám. Zvířata byla během vývoje vystavena obohacenému prostředí. Po odstavu jsme provedli několik behaviorálních testů, především k testování paměti. Po odstavu byla zvířata rozdělena do skupin nebo oddělena. Oddělení je stresovým faktorem, který může negativně ovlivnit i vývoj jedince. Výsledky behaviorálních testů nám ukázaly, že separace měla větší negativní vliv na učení a paměť než samotná MA, ale překvapivě v tomto případě mělo negativní vliv i obohacené prostředí. V další části experimentu jsme měřili hladiny neurotransmiterů, růstových faktorů a také oxidačního stresu a c-fos v různých fázích dospívání, konkrétně v PD 28, PD 35 a PD 45. V dalších fázích experimentu jsme měřili hladiny neurotransmiterů, růstových faktorů a také oxidačního stresu a c-fos. Hladiny neurotransmiterů byly rovněž ovlivněny především stresem při odstavu nebo prostředím před odstavem a také růstovými faktory. Hladiny oxidativního stresu se v reakci na MA nezměnily. Exprese C-fos byla po podání MA snížena během časné a pozdní adolescence. Naše výsledky naznačují, že podávání MA během prvních 12 dnů má méně výrazný účinek v případě nepřímého podávání a že rozhodující roli v tomto případě hraje vývoj a prostředí vyvíjejícího se jedince.

# 1. INTRODUCTION

## 1.1 Methamphetamine neurotoxicity

The psychostimulant methamphetamine (MA) is highly addictive and abuse-prone psychostimulant and second most common illegal drug worldwide [1]. Chronic MA use damages neurons and impairs cognition, memory, and attention [2]. It is lipophilic substance which means that it can quickly cross the blood brain barrier and therefore enter the brain [3].

In brain, it mostly enhances neurotransmitter dopamine (DA), serotonin (5HT), norepinephrine (NA-noradrenaline), and glutamate (GLU) release [4]. This is due to fact that MA can interact with respective neurotransmitter receptors. These receptors and also transporters are located as integral proteins on cell surfaces or vesicular membranes [2]. It also blocks monoamine oxidase, which results in reducing monoamine breakdown [5].

High MA levels may cause tachycardia, hypertension, and mental illness, as well as death [5]. Acute and continuous use of the drug may cause severe neurotoxic events due to oxidative stress and energy metabolism changes [3, 6].

Several studies have shown that MA is responsible for neurotoxicity of neurons, and this is partly caused by reactive oxygen species (ROS) formation [7]. The given text is a list containing the numbers 9 and 10. Formation of these species causes damage to cellular macromolecules such as proteins, lipids, and DNA, leading to the eventual impairment of biological functions [3].

Oxidative stress induced by MA results in lipid peroxidation, protein misfolding, and nuclear damage [8]. By entering the neuronal cell, MA can displace DA from its vesicles and subsequently release it into the synaptic cleft. As a result, there is an increase in DA levels both inside the cells and synapses. The auto-oxidation of DA and leads to an elevation in its metabolism, which may produce several ROS, including hydrogen peroxide ($H_2O_2$). Excessive DA concentrations lead to increase of its metabolism and undergo oxidation of quinones and semiquinones are created [6].

MA also hinders the functioning of mitochondrial complex II, leading to an elevation in oxidative stress and an accumulation of impaired mitochondria [8]. Disturbed terminals of DA and 5HT by MA can induce the activation of inflammatory responses. The activation of microglia leads to these harmful outcomes [9, 10].

The administration of MA leads to the activation of microglia dominantly in the striatum (STR and hippocampus (HP). It is not clear how these mechanisms work, but it is hypothesized that dopaquinones, which are a derivative of DA, serve as the principal stimulants for microglia [11]. Microglia activation results in elevated levels of many neurotoxic substances, such as proteinases, pro-inflammatory cytokines, and ROS, ultimately causing neuroinflammation [11].

Furthermore, active microglia are responsible for releasing a significant amount of excitotoxic GLU, which serves as a mediator connecting excitotoxicity and neuroinflammation, ultimately leading to neurodegenerative processes [12]. This neurotransmitter is believed to have a substantial impact on the progression of neuroinflammation in neurons. Repeated administration of MA leads to the activation of GLU receptors, resulting in the release of GLU [12]. This action triggers the activation of the transcription factor NF-PB and then lead to neuroinflammation by generating inflammatory mediators such as interleukin-1β, tumor necrosis factor-alpha, and interleukin-6 [13]. These cytokines can elevate extracellular GLU levels by inhibiting GLU absorption from microglial cells and enhancing GLU release from these cells. This leads to the formation of a feed-forward loop that enhances neurotoxicity [14, 15].

GLU is the most abundant excitotoxic neurotransmitter within the brain however it has several crucial functions especially in HP. MA exposure alters the functional and structural plasticity of hippocampal neurons. Acute and systemic MA treatment reduces long-term potentiation (LTP) of CA1 pyramidal neurons through activation of D1 receptors and increases baseline excitatory synaptic transmission [16], as well as long-lasting morphological changes in CA1 neurons [16-18]. Acute MA exposure reduces excitability of dentate gyrus neurons, whereas repeated exposure to MA increases excitability of these neurons [16].

All these events lead to the degradation of cytoskeletal proteins, DNA damage, and the malfunction of cellular organelles such as mitochondria and endoplasmic reticulum, among other consequences. An overabundance of GLU triggers many subsequent signaling pathways, including an elevation in $Ca^{2+}$ influx, resulting in an escalation of intracellular $Ca^{2+}$ concentration [19]. NMDA receptors and metabotropic glutamate receptors become active in the presence of an excessive accumulation of GLU [20].

Endoplasmic reticulum stress triggers apoptosis, leading to cell death, via activating death receptors and involving mitochondrial dependent cell death pathways [21]. The neurotoxic impact of MA on human brains is like that reported in animal models. Repeated administration of a large amount of MA leads to a long-term decrease in DA levels and the quantity of DA absorption sites in the STR [22].

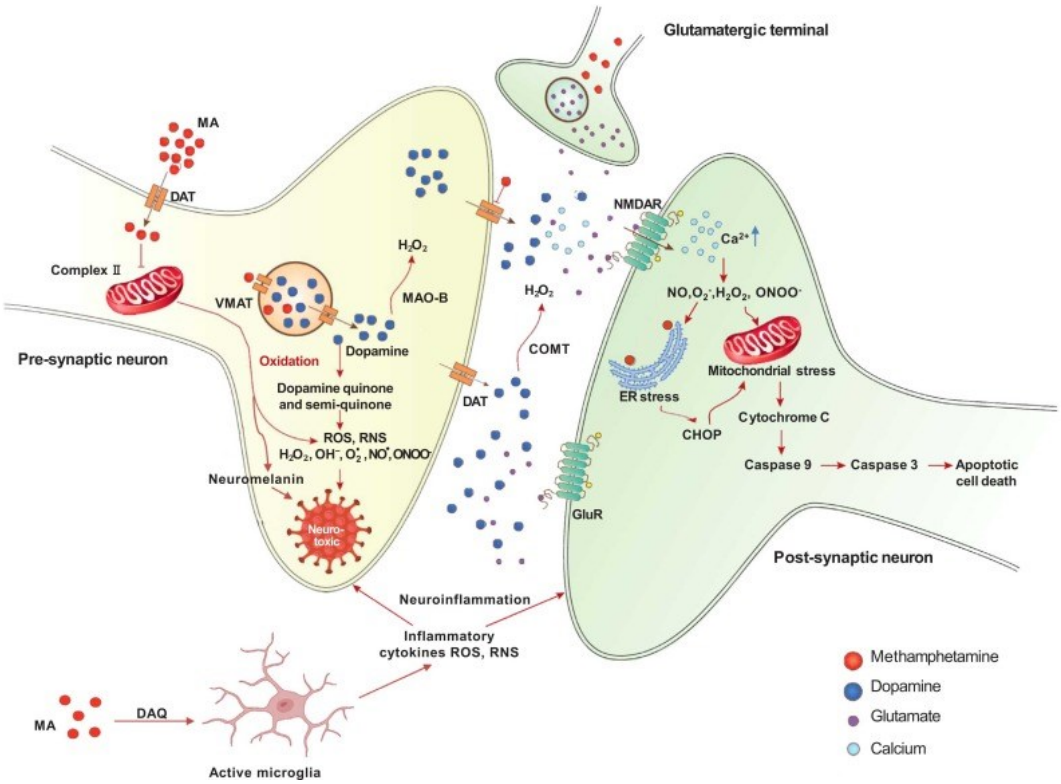


***Figure. 1 Mechanism of action of methamphetamine.***

*Figure shows main mechanisms of action of MA on the outside and inside of the neuron. These mechanisms include interaction of MA with DA transporters and receptors secretion of these neurotransmitter into synaptic cleft. At the same time, there is an increased concentration and excretion of GLU, which leads to excitotoxicity. These processes lead to the emergence of various forms of oxidative*

*stress, which damage cell and activate glial cells. These cells also secrete excitotoxic amounts of GLU as well as inflammatory cytokines, which leads to inflammation. Eventually all these processes lead to activation of apoptotic cell death cascades and therefore to neurodegeneration of nerve tissue. This Fig. was taken from the publication by Clancy et al. (2007)[23].*

Several studies have demonstrated that injecting toxic amounts of MA can change the activity of certain transcription factors in different parts of the brain [24]. Several transcription factors, such as c-fos, fosB, Fra-2 etc., have been identified. Deng et al. (1999) demonstrated that c-fos plays a role in MA-induced cell death. They found that METH-neurotoxicity was significantly worsened in mice with one or both copies of the c-fos gene knocked out [25]. The researchers also demonstrated that c-fos knock-out mice displayed increased DNA breakage in nondopaminergic cells in the dorsal STR [25].

These data indicate that the stimulation of c-fos after administering hazardous levels of MA may serve to stimulate the activation of protective mechanisms, such as the synthesis of antioxidant enzymes or brain-derived neurotrophic factor (BDNF), in order to reduce the harmful effects of MA on the brain [26]. Neurotrophins are important regulators of neural survival, development, function, and plasticity [27]. Nerve growth factor (NGF) and BDNF are proteins which serve as potential therapeutic options to increase neural repair and recovery as they promote neuroprotection and regeneration. These proteins are abundantly expressed in the neocortex and HP during development, but their expression continues in adulthood, as reported from animal studies [28, 29].

Development of addiction is associated with alteration of structural and functional changes which together belong to processes of neuronal plasticity. Neuronal plasticity, as ability of brain to change through the growth is necessary for successful information storage and memory formation as well as for adaptive responses resulting in various modifications of behavior. These processes are facilitated by increased BDNF synthesis and release [30, 31].

Link between BDNF and neuronal plasticity is well established and play important role in mediating synaptic changes involved in learning and memory, which underlies behavioral and structural adaptations associated with drug addiction. A study conducted by Schweppe et al. (2020) reported that rats exposed to high doses

of MA showed a decrease in BDNF and its receptor TrkB in STR and HP. This reduction persisted for up to 75 days following the administration of the drug [32].

## 1.2 Critical developmental periods

The term "critical developmental period" has been present in the field of experimental psychology since 1921. It describes a momentary disruption in the developmental process during specific periods of embryogenesis, which can lead to significant repercussions [33]. The originator of the initial idea additionally contended that the significance of the pace of growth extends beyond embryogenesis and encompasses postnatal ontogenesis as well [33]. Scott (1962) supported the concept that postnatal development and growth are reliant on organized mechanisms that can only be affected during critical development periods [34]. In the 1960s and 1970s, Křeček (1978) conducted studies on ontogenetic physiology to determine criteria for identifying critical periods in development and investigating the potential for inducing permanent developmental changes through external intervention [35].

According to his idea, there may be a delay between the intervention and the observed change, meaning that the changes may not be immediately apparent when the stimulus is applied. In addition, critical periods can have varying durations and do not occur simultaneously for all functions. Furthermore, a single function might have many critical periods [35].

During critical developmental phases, there are three significant changes that occur:

a) heightened responsiveness to external stimuli,
b) reorganization of functional systems, and
c) alterations in an individual's interaction with its surroundings [35].

According to these standards, there are three primary stages of postnatal development: infancy (from birth to weaning, for rats up to 20-25 days), juvenile (from weaning to sexual maturation), and adulthood (after reaching maturity) [36]. The synchronization of functional system development and their mutual inductive activity is a crucial aspect of the formation of highly specialized tissues and organ structures. Nerve cells establish their own characteristics during development, forming organized and precise connections between synapses. These connections

are shaped by both genetic factors and the surrounding environment, affecting both the structure and function of the cells [37]. The central nervous system (CNS) is a neural platter formed from cell precursors [38]. During the early phase of embryogenesis, the ectoderm undergoes significant changes, particularly in the gastrular region. This process occurs during the second week of gestation in humans and on embryonal day (ED) 5-6 in rats. During neurulation, the lateral edges of the organism start to rise around GD 18, resulting in the formation of neural valves [39]. As development progresses, the neural valves gradually move closer to each other in the middle line until they fuse together, forming a neural tube [38]. The neural tube is closing on ED 27-28 in humans and rats on ED 12. Neuroepithelial cells start to gather, giving rise to neurons and glia cells through mitotic division. Signals from surrounding structures play a crucial role in the differentiation of neuronal populations in the CNS [4, 39, 40]. Cell proliferation processes exhibit similar structures in both rats and humans, although they occur at different time intervals. The primary set of genes that control the development of brain neurons are known as homeotic genes, which contain transcription factors that play a role in the differentiation of genes and growth factors, which are important for the development of neurons in brain [41]. The primary neurotransmitter systems linked to the stimulating effects of drugs are the noradrenergic, dopaminergic, and serotoninergic systems [23]. Noradrenergic neurons are found in the brain stem, specifically in the locus coeruleus and reticular formation of the medulla and pons. They play a crucial role in the subsequent development of the brain [42, 43]. Dopaminergic neurons have a crucial role in regulating motor activity and cognitive function, with cells found in various regions of the brain, including the prefrontal cortex. There are two primary types of dopaminergic receptors: D1 and D2, along with three additional types: D3, D4, and D5. The activation of D1 receptors plays a role in the formation of memory [4]. Serotoninergic neurons are found in various regions of the brain, including the midbrain, pineal gland, substantia nigra, hypothalamus, and brain stem. They possess extensive projections that allow for the coordination of intricate sensory and motor patterns of behavior. Serotonin plays a crucial role in influencing neuronal proliferation, differentiation, and synaptogenesis [44]. Cells that produce 5HT are among the earliest cells to develop

in the neurotransmitter 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. Following childbirth, there is a decrease in the growth of neurons [39]. The central nervous system undergoes significant development and maturation by the age of 2.5 years. After birth, rats experience a rapid maturation of astrocytes, leading to changes in the morphology, connectivity, and electrophysiological properties of CNS [38, 45]. According to a study conducted by Bockhorst et al. (2008), the most substantial alterations in the gray matter of rats happen within the initial 5 days after birth [46]. In conclusion, the development of the CNS is influenced by various factors, which will be discussed will be discussed further in this thesis.

### 1.3 Methamphetamine and its impact on development in rats

MA easily crosses all barriers within the mother's body, including the placenta, and enters the developing fetus [47]. The drug concentration in the child's circulation is about 50% of the concentration in the mother's plasma [48]. As the drug takes effect, it slows down its breakdown in the liver, causing the medication concentration to rise. The physiological changes that occur in plasma volume during pregnancy can have an impact on the half-life and distribution volume of drugs. Drug sensitivity and metabolism can be altered during pregnancy. A study conducted by White et al. (2011) explores the variations in the elimination rate of MA metabolite in pregnant females at different stages of gestation. In the later stages of gestation (ED 21), the drug is eliminated at a slower rate in the mother's body compared to the early stages (ED 39-41) [49].

Prenatal exposure to MA in rats can take please during the whole prenatal period or during specific stages of fetal development [50]. Rice and Barone (2000) conducted a comparison between the prenatal phase of rat development (ED 1-21) and the first and second trimesters of human gestation. They found that ED 1-9 in rats is equivalent to the first trimester in humans, whereas ED 10-21 corresponds to the second trimester. Several experimental investigations have been conducted to

examine the effects of prenatal exposure to MA, which is delivered continuously during the entire gestation period (ED 1-22 in rats) [38].

Most drugs that are abused can pass into breast milk, resulting in the infant being exposed to the drug after birth while breastfeeding (Fox 1965). Nevertheless, there were disparities in the way breastfeeding duration is perceived in humans and rodents. The breastfeeding phase in humans begins at birth and lasts until approximately 2.5 years of age. In rats, it starts at birth and ends at weaning, which occurs around PD 21 [38, 50].

The extended maturation time of the brain, in contrast to other organs, highlights the distinctions between humans and rats. The development of the neural system in humans is particularly important during the later stages of pregnancy, specifically the second and third trimesters. In contrast, in rodents, the maturation of the nervous system largely occurs within the first two to three weeks following birth [38].

In humans, the drug is transmitted to the offspring through the placenta, whereas in rats, it occurs through breast milk during lactation. Rambousek et al. (2014) provided evidence of the existence of MA in the plasma and brain of women who were exposed, as well as in the breast milk obtained from the stomach of their infants [51].

Drug transmission through breast milk was verified by the detection of MA and in the serum (0.1-1 ng/ml) and brains (1-10 ng/g) of offspring whose mothers were exposed to a dosage of 5 mg/ml/kg of MA from postpartum until weaning. There is a limited number of clinical investigations that elucidate the transfer of MA through breast milk. Several empirical investigations have also documented that a key detrimental effect of consuming MA while breastfeeding is a decrease in interaction between the mother and offspring [52].

Extensive clinical and experimental investigations have thoroughly examined the detrimental impact of MA on the development of children born to mothers who are addicted on drugs [52]. Nevertheless, the specific stage of human gestation that is most vulnerable to the detrimental impacts of this medication remains uncertain. Previous studies from our laboratory 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 [53, 54] and adulthood [55-57].

The study from our laboratory by Hrebíčková (2017) investigated the effects of prenatal and neonatal exposure to MA on active drug search, behavior, and learning in adult offspring from affected mothers. Results showed 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. Exposure of pups to MA during these stages can result in persistent cognitive deficits into adulthood. Direct exposure to MA through breast milk or directly to pups had a greater impact on animal behavior. To provide a more comprehensive understanding of the effects of neonatal exposure to MA during PD 1-11, additional experiments will be conducted to analyze MA concentrations in the brain, serum, and breast milk following both direct and indirect exposure. The crucial time for the impact of MA on adult animal behavior is during the later stages of prenatal development and the early postnatal period in laboratory rats, which roughly aligns with the second and third trimesters of prenatal development in humans [56].

### 1.4 The role of upbringing and environment on the developing brain

During critical developmental periods, genetic processes and environmental factors interact to stabilize certain specific traits of an organism [58]. Major developmental processes occur during adolescence, which is typically the period between 12 and 20 years of age in humans, and postnatal days (PDs) are 28–45 in rodents, however the boundaries of this period are still unclear [59]. During adolescence 5HT levels vary differently in different brain regions. There is an increase in serotonergic activity in the prefrontal cortex, but a decrease in serotonergic activity in the HP [60]. Major developmental changes are triggered by some kind of stress that is associated with changes in the reward circuit's neural pathways.

Probably most crucial aspect of development is maternal care and mother-child relationship. According to clinical and experimental studies, any separation of the developing individuals from their mother causes significant changes in developmental patterns, such as an increased risk for addiction later in life [61, 62].

Also, this form of stress causes disruption in the hypothalamus-pituitary-adrenal axis [63, 64]. According to study by both prenatal and neonatal MA exposure causes severe behavioral impairments, since MA-exposed mothers showed lack in maternal behavior towards their pups [65]. Some of our previous studies also showed that proper maternal care can reverse those effect and pups raised by control mother during lactation had better results in behavioral testing [37, 66, 67].

There have been reports of reductions in 5HT release, reduced 5HT tissue concentrations, and reduced synaptic activity in critical corticolimbic structures after exposure to stress in rodents [68, 69]. Early postnatal stress in humans leads to significant memory impairments in adulthood and it is associated with reduced BDNF levels [70, 71].

In rats, it leads to decreased synaptogenesis of HP, as well as decreased levels of BDNF, and long-term potentiation and memory defects [72]. Not only is maternal separation a major source of stress, but social isolation during adolescence can have serious consequences for development and behavior in adulthood. Rodent animal models reported, that is deprivation of social contact for approximately 1–3 weeks leads to anxiety-like behaviors and reduction in cell proliferation and neurogenesis are observed [60, 73].

Social isolation elicits chronic stress since rats naturally live in groups and preventing them of social contacts and interaction for a longer time deprives them of important stimuli and represents a significant stressor. Chronic social isolation induces a variety of symptoms in rats, including depression, anxiety, and psychosis-like behaviors [74]. There has been reported an altered expression of BDNF in the brain of rats housed in social isolation [75, 76].

Majority of studies across all age groups (post-weaning, adolescent, adult) reported a decreased expression of BDNF in HP. This supports the evidence that chronic stress downregulates hippocampal BDNF expression in rats, in line with the findings from other chronic stress paradigms [77]. Social isolation is a good laboratory tool to induce stress, since in animals usually involve depriving them of social contact with their own species and studying how their brains and behaviors change during and after social isolation. These effects of social isolation on the brain and behavior are significant, especially during development. Moreover,

animal models of social deprivation demonstrated that neurobiological mechanisms and development are deprived of stimuli that are crucial for their maintenance and development [60].

As mentioned above, role of proper maternal care on pup development is crucial environmental factor and indicates significant importance of the environment during several critical developmental periods. While a stressful situation can have a negative effect on an individual's development, environmental enrichment (EE), on the other hand, can have therapeutic potential. In laboratory condition, EE aims to improve the welfare of the animal by including social or nonsocial features and EE stimulates HP neurogenesis [78].

The act of socializing itself can increase the level of monoamine neurotransmitters in mesolimbic structures of the brain. Studies reported that effect of EE on brain plasticity and behavior in adolescent rodents were more significant compared with those before weaning due to the more complex neural circuits and the approaching maturity of the nervous system [79]. Previous studies have also shown that EE can enhance the growth factors that promote neurogenesis, including an NGF and that EE significantly induced neurogenesis of HP in adult mice [80]. Other study reported that rats housed in group exposed 30 days to EE had significantly higher levels of NGF mRNA than rats housed individually in single cages without stimulus-enrichment [81]. Studies have also shown that EE has beneficial effects on these diseases [82]. Moreover, EE exposure can also reduce the seeking for psychostimulants and reduce the risk of relapse, as well as protect animals from drug addiction by sensitizing limbic structures [83]. In addition, rats given EE are less sensitive to the reinforcing effects of MA [84].

# 2. AIMS AND HYPOTHESESIS

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 (PD 1-12).

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 (2007) have shown that the drug affects precisely those structures undergoing development, we hypothesize that MA administration during the PD 1-12 period will lead to modifications in hippocampus-related functions that will correlate with changes in hippocampal neurogenesis [23]. 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 post-weaning 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 postweaning 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 subsequent to 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 [85]. 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 [86], and that EE significantly prevented these reinstatement effects of MA in condition place preference task [87], while other reported that the rewarding and neurotoxic effects of MA are not reduced by EE [88]. However, the fact remains that the environment plays a crucial role during the development of an individual [89-91]. 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 [92]. 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 [92]. 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 (PD 1-12) experience deficits in learning and memory [57, 93]. 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 [94]. 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 [95].

C. Levels of neurotrophins - BDNF and NGF. These proteins significantly contribute to neuroprotection in MA-induced brain injury [96]. 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 [7, 94]. 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 [97]. It has been demonstrated that psychostimulants increase c-Fos production in the mesocortical and mesolimbic reward pathways [98]. Also, upregulation of c-Fos is associated with learning and memory [99]. Therefore, we examined the c-Fos protein in HP.

# 3. METHODS

## 3.1. Animal care

Adult female and male Wistar rats were purchased from Velaz (Prague, Czech Republic) and bred by Charles River Laboratories International, Inc. Rats were housed in a temperature-controlled (22–24°C) room using a standard 12 h light/dark cycle with light on 6 am. Animals were left undisturbed for one week before fertility determination. Food and water were available *ad libitum* during that period. For determination of estrous cycle phase female rats were smeared using vaginal lavage. At the estrous phase females were housed overnight with sexually matured males [55]. Determination of fertilization was performed by smearing of females for presence of sperm. 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 (Czech Republic).

## 3.2 Design of the experiment

In this experiment, we studied the effect of two different methods of postnatal MA administration:

- direct – subcutaneous administration to pups on postnatal days (PD) 1–12
- indirect – subcutaneous administration to mothers on PDs 1–12, so that pups received the drug via maternal breast milk (intact).

MA was injected at a 5 mg/ml/kg dose per day during exposure period, and control SA rats were given the same volume of SA. In contrast to indirectly exposed animals, directly exposed animals were not administered SA, they were exposed to SHAM injection as in our previous studies [100]. Exposure to MA or SA was performed every day during morning hours. During the period before weaning (PD 1–21), pups were exposed to a standard preweaning housing (i.e., standard cages SC; L:39 x W:24 x H:18) used in our laboratory or to an enriched preweaning

housing (EE) with larger cages (L:51 x W:42 x H:41) containing various toys such as tunnels and wheels, which provide sensory and cognitive stimulation and therefore may promote neurogenesis of brain [101]. Pups were divided into groups according to the preweaning housing in which they were raised. On PD 21, the pups were weaned from mothers and divided into two different groups:

- housed in groups (GH) – housed in groups of 4 (natural for rats as social animals) (L:51 x W:42 x H:41)
- housed separately (SH)– rats were housed separately, one animal per cage (L:39 x W:24 x H:18) which is thought to be a stressor [102].

Difference between EE cages and standard cages are shown in *Fig. 2*.

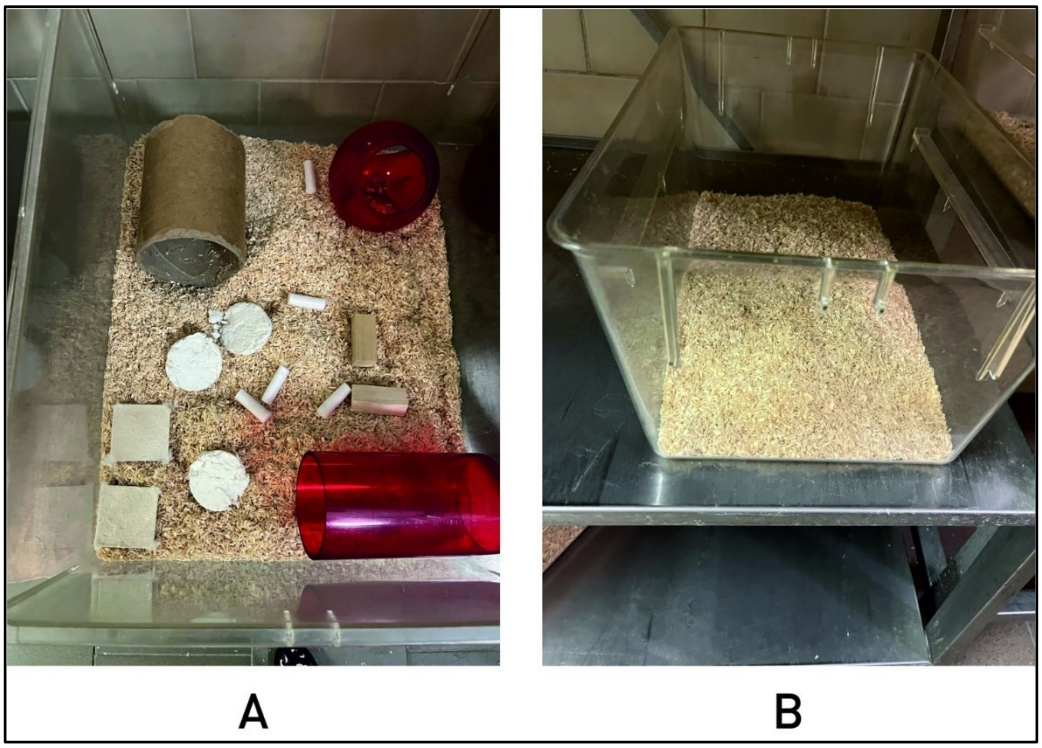


***Figure 2. Preweaning housing conditions***
*Fig. A shows EE conditions and Fig. B shows standard conditions. Enriched environment consisted of bigger rat cages, and various toys such as tunnels and many other objects made with natural materials and suitable for chewing.*

In total, 1536 male rat pups were used in this study, female rats were used in other experiments, eight male rats were used per group, divided according to:

age when sacrificed

- PD 28 - early adolescence
- PD 35 - mid-adolescence
- PD 45 - late adolescence

treatment – MA vs. SA

drug application – direct vs. indirect

housing before weaning – standard preweaning housing (SC) vs. enriched preweaning housing (EE)

housing postweaning – group housing (GH) vs. social separation (SH)

Before behavioral testing, animals were exposed to MA as described above. After the last day of exposure, animals were left undisturbed until weaning on PD 21. On PD 21, animals were weaned from mothers and divided into cages according to the respective groups:

- 1 animal in a cage – separate housing - without toys - 4 animals – grouped housing with environment according to environment which they were in before weaning (EE or SC). To avoid the cage effect, a maximum of 2 animals of a given cage were used in the behavioral test and the rest of the animals were used for further analyses. Animals remained in this environment until the behavioral tests were completed.

***Table 1. Experimental groups of this work***

*SA – saline, MA – methamphetamine – postnatal day, EE – enrich preweaning housing, SC – standard housing – group housing, SH – social separation. Number of animals per groups was 8.*

| PD | Treatment PD 1-12 | Form of treatment | Preweaning housing | Postweaning housing | Number of animals per group |
|---|---|---|---|---|---|
| 28 | SA/MA | Direct | EE/SC | GH/SH | 8 |
| 28 | SA/MA | Indirect | EE/SC | GH/SH | 8 |
| 35 | SA/MA | Direct | EE/SC | GH/SH | 8 |
| 35 | SA/MA | Indirect | EE/SC | GH/SH | 8 |
| 45 | SA/MA | Direct | EE/SC | GH/SH | 8 |
| 45 | SA/MA | Indirect | EE/SC | GH/SH | 8 |

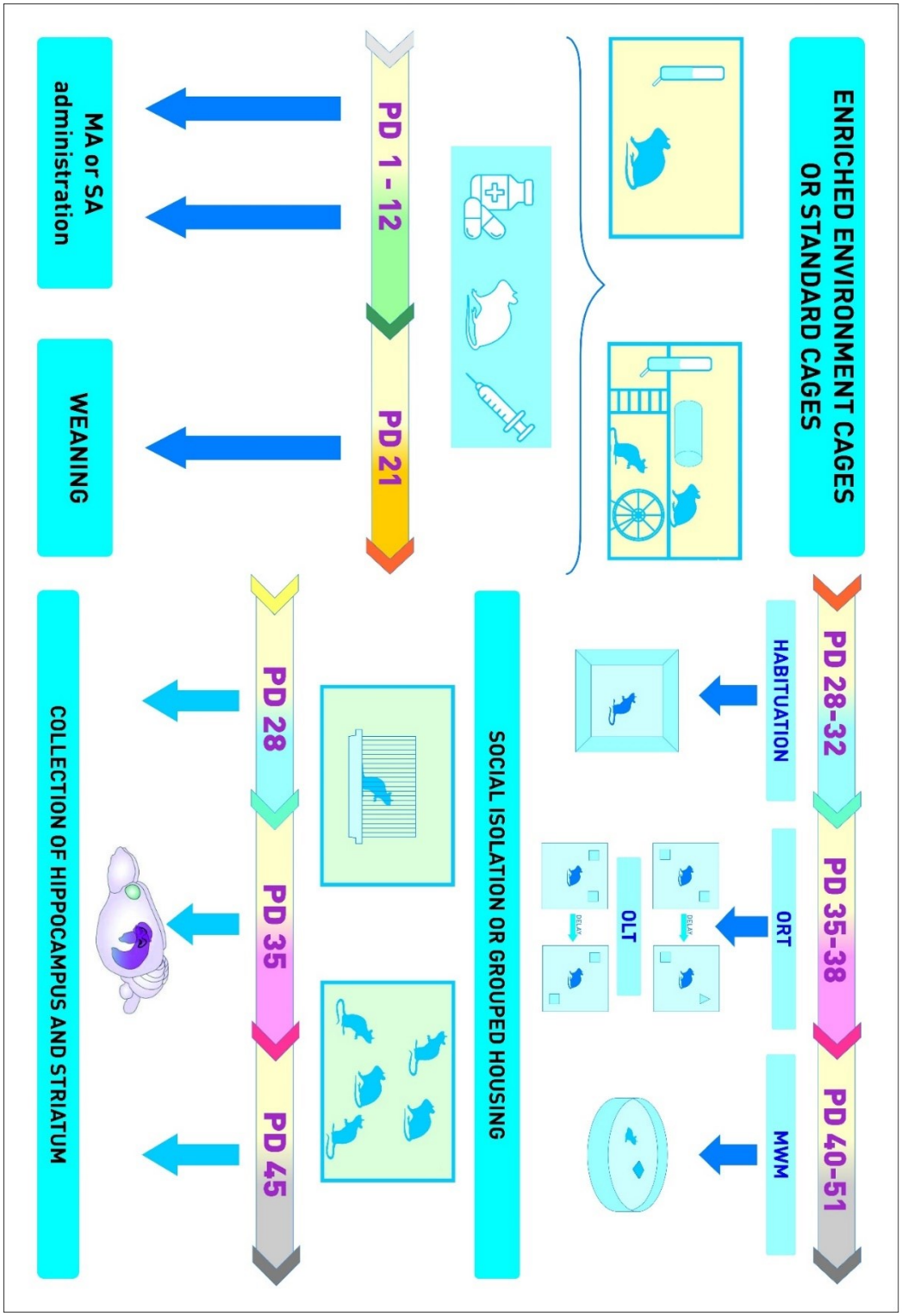


***Figure 3. Timeline of the experiment***

*Figure shows timeline of the experiment in terms of MA exposure, housing conditions, behavioral tests and brain sample collection.*

### 3.3. Behavioral testing

Animals (n=8) were tested during the following time: PD 28-32 (Habituation test), PD 35-38 (object recognition test -ORT and object location test -OLT), PD 40-51 (Morris Water Maze-MWM). The same animals (n=8) were tested in all behavioral tests. Timeline of experiments including behavioral tests is shown in *Fig. 2*.

#### 3.3.1. Habituation

Habituation to a novel preweaning housing in rodents is commonly defined as a change in exploratory or locomotor activity over time (within-session) or with repeated exposures (between-session) [103]. While numerous neuroactive substances are known to influence habituation, neurotransmitters that play particularly important roles are 5HT, DA, and GLU[104]. Animals were tested for 4 consecutive days (one 10 min session each day) in the Open field arena. Habituation was evaluated by comparing distance moved between the first and fourth day of exposure to the Open field arena (100, 101). Arena setting is shown in *Fig. 4*.

***Figure 4. Habituation in Open Field arena***
*The animals were placed in an empty arena for 10 minutes for 4 consecutive days. We monitored the distance that this animal traveled during this test.*

### 3.3.2. Object recognition and object location tests

Object Recognition Test (ORT) measures the exploration of novel versus familiar object, which is a component of recognition memory (102, 103).

The test consists of three parts: habituation, training, and testing.

Habituation: Animals were habituated to the black empty square arena (70×70×60 cm) for three days, each day for 10 minutes. (Described above)

Training: On the fourth day the animal was trained: the rat was placed into the arena for 5 min to explore two identical objects placed in the arena. After 5 min the rat was removed and put back into the cage. Arena and the objects were then cleaned of potential odors by disinfectant.

Testing: In the testing phase, rat was placed to the box, where one of the original objects (beer cans) and one new object of similar size (glass jar) – see *Fig.* 5, were placed on the same places as during the training. An experienced researcher, blind to pharmacological intervention and to the group of animals measured the time spent exploring of both objects (the familiar and the new one). The following elements of behavior were considered as the exploration of the subject: sniffing to the object, close circumvention of the object with the sniffing or observation, rearing to the object, with or without sniffing.

These procedures were performed also 15 and 30 minutes after first trial.

Evaluation of exploration time was performed by EthoVision 14 program (Noldus Information Technology, Netherlands).


***Figure 5. Object recognition test***

*Animals were put in same arena as during habituation. During training phase, animals were introduced to the objects for 5 minutes. During testing phase, animals were obligated to explore objects, one of which was exchanged for a similar object,*

*for one minute. Fig. A demonstrates two similar objects in default position and Fig. B demonstrates change of the second object.*

Object Location Test (OLT) works on the same rules as ORT and contains the same three parts (habituation, training, testing) and evaluation is identical. The difference is that OLT measures the exploration time of two same objects (beer cans), but one is placed to a novel location – see *Fig.* 6. It assesses especially spatial memory and discrimination. In this test, were tested animal in 1., 15. And 30-minute like in previous test (102, 103). Procedure of this test is shown in *Fig. 5* and *Fig. 6*.

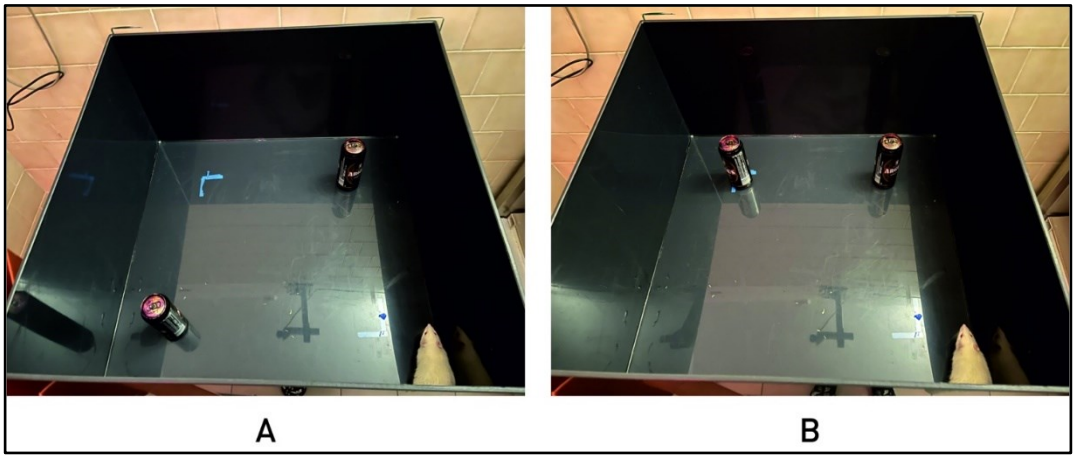


***Figure 6. Object location test***
*Animals were put in same arena as during habituation. During training phase, animals were introduced to the objects for 5 minutes. During testing phase, animals were obligated to explore objects, one of which's location was changed, for one minute. Fig. A demonstrates two similar objects in default position and Fig. B demonstrates change of the second object. Fig. A demonstrates two same objects in default position and Fig. B demonstrates same objects, however second object with changed location.*

### 3.3.3. Morris Water maze

This test is commonly used in evaluation of cognitive functions such as learning and memory in laboratory animal models (104).

MWM is the circular pool with 2 m diameter filled with water (usually 15 cm under upper edge of pool) with steady temperature (18-20°C). On the edges of pool, there are 4 start positions marked: N (north), S (south), E (east) and W (west). These positions divide pool to 4 quadrants. In quadrant N-E, the platform made by transparent plastic was placed (diameter of 13 cm) and it is 1-2 cm covered under the water level. In the room, where this test is performed, there are several pictures

placed on the wall, which serve as orientation points for animal's navigation in space. Swimming of the animals is automatically captured by camera place above the pool and evaluated by EthoVision 14 program (Noldus Information Technology, Netherlands). One animal is tested for 12 days in total. First six days, learning task was performed. On the 8. day „probe "test was performed and memory test on the 12. day. This design has been used at our laboratory for a long time and was created based on the recommendations of dr. Mikulecká from the Institute of Physiology AV ČŘ (88, 104).

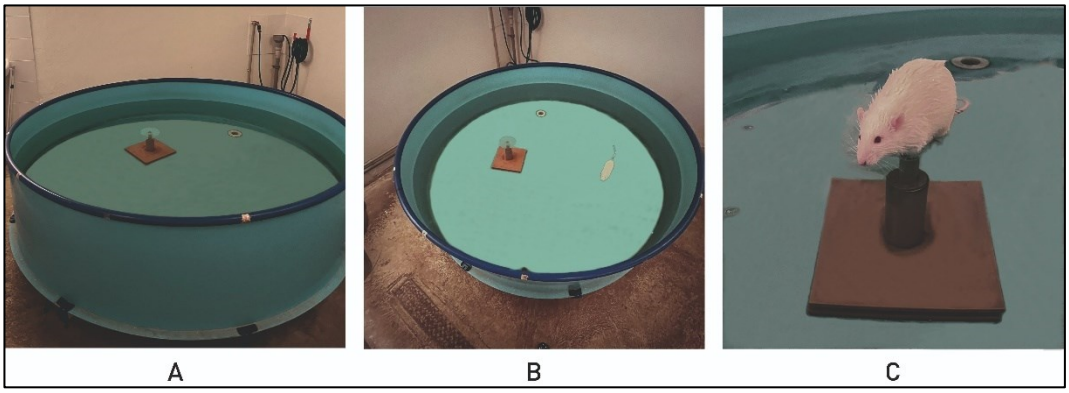


***Figure 7. Morris Water Maze***
*The animals were placed in the pool a total of eight times a day - once from each side of the world during 6 consecutive days in the learning test. Memory test was identical, but animals were tested only on 12$^{th}$ day. Fig. A shows setting of the pool with hidden platform. Fig. B shows rat during learning test. Fig. C shows rat on hidden platform.*

**Learning test**

During learning task, animals were learning to find hidden platform for 6 consecutive days in shortest time possible. Position of the platform was identical during whole experiment. Every animal performed test 8 times each day – 2 time from 4 start positions (N, S, E, W). Maximal time limit for finding the platform was 60 seconds. In case, that animal did not find the platform for 60 seconds, it was led manually to it. Between individual swim tasks, animal was let on hidden platform for 30 seconds for better mapping of the preweaning housing and for resting as well. In learning task, we evaluated these parameters: distance moved till animal found the platform (cm), search error – the sum of direct distances from the platform measured repeatedly during the search (cm), the time it took the animal to find the platform (s) and velocity of swimming (cm/s). We also evaluated strategies of

looking for platform – thigmotaxis and scanning. Thigmotaxis is swimming along the edges of the pool and scanning is non-organized swimming in center of pool and accidental platform finding. According to Janus et al. (2004), these swimming strategies are important signs of an animal's ability to show spatial learning [105]. See in *Fig. 6*. In our model, we were evaluating a long-term spatial memory. Swimming strategies – thigmotaxis and scanning represent good observing parameter of learning ability. Thigmotaxis is used during first days of learning, when animals are not yet properly oriented in the pool and tend to move around walls of the pool, where scanning strategy represents phenomenon when animals is already orienting in space and tend to swim closer around the hidden platform, which leads to animal randomly finding the hidden platform. In case of animal already knowing the exact position of the hidden platform, these strategies are not present anymore, since this animal swim straight to the platform.

**Memory test**

This test was performed on 12.day and we tested long-term spatial memory. Spatial memory in rats involves the ability to remember locations, routes, and spatial relationships in their environment [106]. It includes spatial working memory for temporary storage and manipulation of spatial information over short intervals and spatial reference memory for long-term retention of spatial information. Spatial memory heavily relies on HP and other brain regions like the entorhinal cortex for encoding and retrieval of spatial information [107, 108]. The hidden platform was returned to its original position during learning phase. Animals had to find the hidden platform in shortest time possible. Position of the platform was the same for whole experiment. Every animal performed test 8 times – 2x from all starting positions (100µl per vial). Maximum time limit for finding the platform was 60 seconds. In memory test we evaluated same parameters as in learning test including strategies – thigmotaxis and scanning (s)

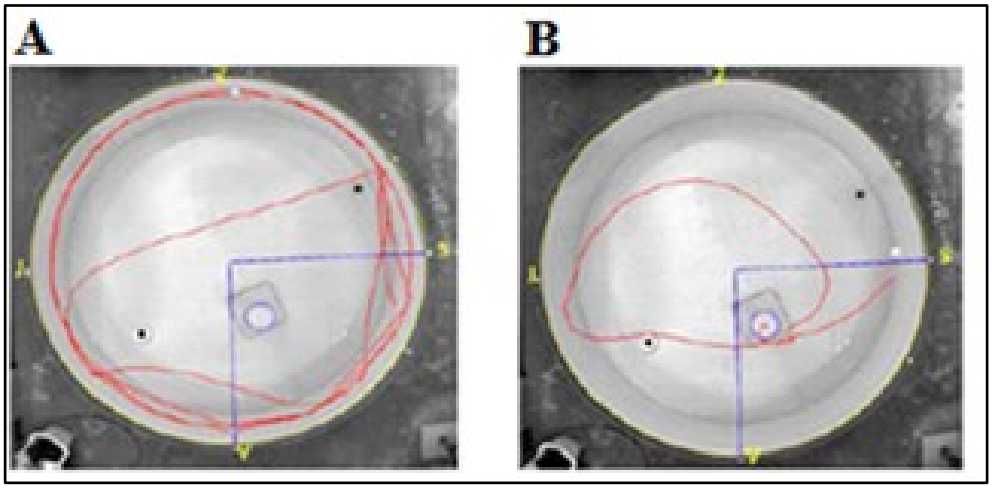


***Figure 8. Strategies in MWM***
*Strategies which were tested in these tests were thigmotaxis and scanning. Thigmotaxis is swimming near the walls of the pool, which means that the animal is not oriented in space and is not familiar with the location of the hidden platform. Scanning is uncoordinated swimming near a hidden platform, when a platform is accidentally found. This strategy usually occurs from day 3 of learning and points to the fact that the animal is already orienting itself in the pool space and is familiar with the position of the hidden platform. And yet, if the animal knows the location of the hidden island well enough, it does not use these strategies and, after being placed in the pool, swims straight to the platform. Fig. A represents thigmotaxis strategy and Fig. B represents scanning strategy. Picture was taken from Hrebičková et al. (2017) [56].*

### 3.4. Brain sample collection and analyses

For molecular analyses, HP (hippocampus), STR (striatum), were collected. On the respective days (PD 28, 35, and 45), the animals were anesthetized intraperitoneally with an overdose of chloralhydrate (400 mg/kg, Sigma-Aldrich) and given an intracardial perfusion of heparinized saline. Brain tissues were removed, snap frozen on dry ice, and stored at −80°C for further processing.

Within processing of HP and STR for neurotransmitter detection, the samples were homogenized in physiological saline containing 1 mM EDTA and 4 mM sodium metabisulfite (Sigma Aldrich, Saint-Louis, Missouri, USA) for a final concentration of 100 mg/ml. The homogenates were centrifuged at 10,000g in a cooled microcentrifuge (4°C) for 10 minutes; the supernatants were aliquoted (100µl per vial) and stored frozen at −80 °C until assayed [109].

During processing of HP and STR for BDNF and NGF detection, the samples were homogenized in phosphate buffered saline (Sigma Aldrich, Saint-Louis, Missouri,

USA) containing cOmplete™ Protease Inhibitor Cocktail (Roche) for a final concentration of 100 mg/ml. The homogenates were centrifuged at 10,000g in a cooled microcentrifuge (4°C) for 10 minutes; the supernatants were aliquoted (100µl per vial) and stored frozen at −80 °C until assayed.

Sample processing for oxidative stress detection consisted of several steps. Sample were homogenized in phosphate buffered saline (Sigma Aldrich, Saint-Louis, Missouri, USA) containing cOmplete™ Protease Inhibitor Cocktail (Roche) for a final concentration of 100 mg/ml. The homogenates were centrifuged at 10,000g in a cooled microcentrifuge (4°C) for 10 minutes; the supernatants were aliquoted (100µl per vial) and stored frozen at −80 °C until assayed.

Nuclear extracts of HP were used for c-fos detection. For extraction, Nuclear Extraction Kit (ab221978, Abcam, Cambridge, UK) was used. After extraction, the protein concentrations were estimated using the BCA method with Bicinchoninic Acid Kit for Protein Determination (BCA1-1KT, Sigma Aldrich, Saint-Louis, Missouri, USA) and then stored at −80 °C until assayed.

**Enzyme-linked immunosorbent assay (ELISA)**

Enzyme-linked immunosorbent assay (ELISA) is method that is considered as gold standard of immunoassays. It is very sensitive method used to quantified and detect various type of substances. ELISA is usually performed in 96-well polystyrene plates [110].

There are four major types of ELISA: direct ELISA, where plate is pre-coated with antigen and requires screening antibody, indirect ELISA, with precoated plate as well and screening antibody or antigen, sandwich ELISA with antibody precoated plate and requires screening antigen and competitive ELISA requiring only screening antibody. The primary detection antibody is a specific antibody that only binds to the protein of interest, while a secondary detection antibody is a second enzyme-conjugated antibody that binds a primary antibody that is not enzyme-conjugated. Coating of antibodies takes from 60 to 120 minutes at room temperature. Other steps of this method require chemicals such as wash buffer, and substrate – chromogen. Substrate is crucial for detection since it generates a color and the most used substrate is horseradish peroxidase (HRP) [111].

The substrate for HRP is hydrogen peroxide and results in a blue color change. Usually, detection takes about 15-30 minutes in the dark at room temperature. Another crucial step of procedure is “wash” of the plate using a buffer, such as phosphate-buffered saline (PBS) and a non-ionic detergent, to remove unbound material. The wells are usually washed three or more times during each wash step. Final read is performed by spectrophotometer. Usual wavelength for ELISA reading is 450 nm. Spectrophotometer measures color changes in individual well. Concentration of substance of measurement is calculated according to standards diluted to desired concentration. Depending on the assay, a linear curve, or a curve of 4 parameters are most often used for calculation (105, 106).

In this work, ELISA procedures were used for detection of concentration of catecholamines (DA, NA), 5HT, GLU, BDNF, NGF, 4HNE and TBARS. ELISA-related semi quantitative method was used for detection of c-fos activation.

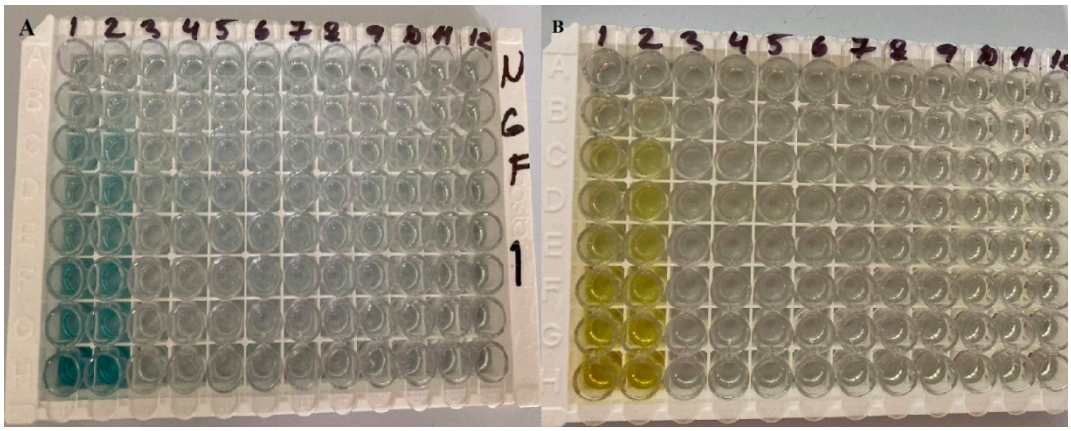


***Figure 9. Color reaction during ELISA***

*Fig. A demonstrates color changes after samples incubation with substrate. When the concentration of antigens in the sample is elevated, the concentration of enzyme-labeled antigens bound to antibodies is reduced, resulting in a lighter hue.* In contrast, a low quantity of antibody-bound enzyme-labeled antigen results in a larger concentration and a darker hue. *Fig. B demonstrates color changes after reaction with substrate was stopped before absorbance reading.*

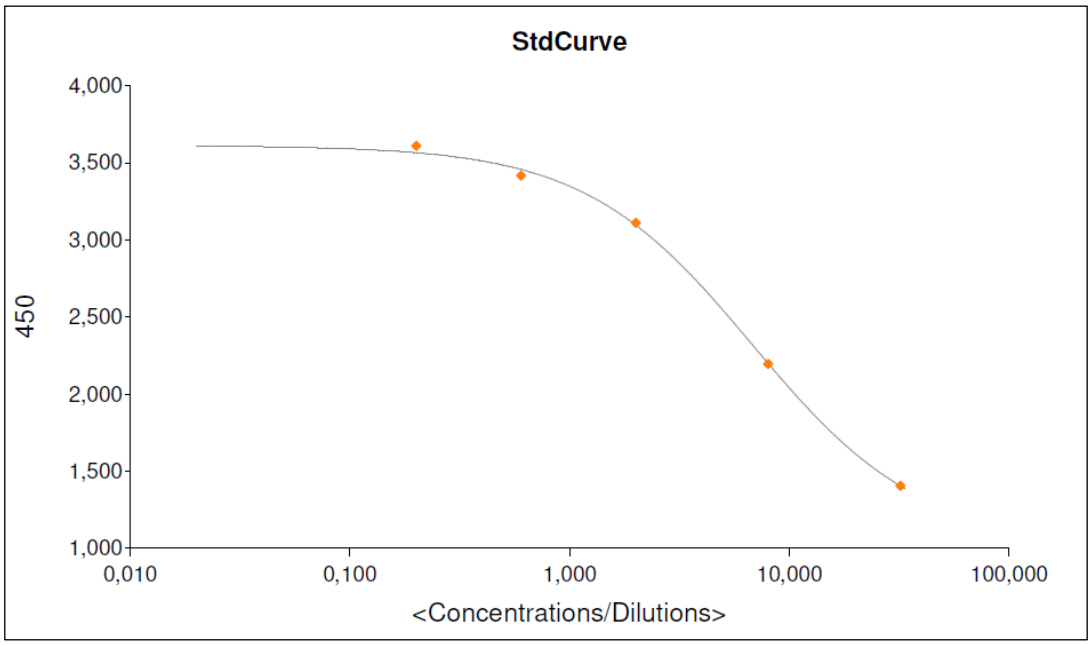


***Figure 10. Calibration curve-4 parameters***

*Figure demonstrates example of 4 parameter curve used for calculation of neurotransmitters concentrations. This picture was taken directly from GEN 4 program.*

### 3.4.1 CATECHOLAMINES

2 CAT Reasearch ELISA kits (BA E-5500R, NLDN Labor Diagnostika Nord GmbH & Co.KG, Nordhorn – Germany) were used for detection of DA and NA. Prior to assaying, we performed a serial dilution of selected samples using ultra-pure distilled water as the diluent. For determination of DA, HP samples were diluted 1.66-times and STR samples 15-times. For NA determination HP samples were diluted 1.66-times andSTR samples 7.5-times. Analyses were performed according to the manufacturer´s instructions. The absorbance was read at 450 nm on an 800™ TS microplate Absorbance Reader (BioTek). Protein concentrations were estimated using the Bradford method.

### 3.4.2 SEROTONIN

Serotonin Research ELISA kits (BA E-8200R, NLDN Labor Diagnostika Nord GmbH & Co.KG, Nordhorn – Germany) were used for 5HT detection. Prior to assaying, we performed a serial dilution of selected samples using BAE-5941 as the diluent. For determination of 5HT, HP and STR samples were diluted 20-times, and

plasma samples were 5- times diluted. Analyses were performed according to the manufacturer´s instructions. The absorbance was read at 450 nm on an 800™ TS microplate Absorbance Reader (BioTek). Protein concentrations were estimated using the Bradford method.

### 3.4.3 GLUTAMATE

Glutamate ELISA kits (BA E-2400R, NLDN Labor Diagnostika Nord GmbH & Co.KG, Nordhorn – Germany) were used for GLU detection. Prior to assaying, we performed a serial dilution of selected samples using BAE-5941 as the diluent. For determination of GLU, HP and STR samples were diluted 5-times. Analyses were performed according to the manufacturer´s instructions. The absorbance was read at 450 nm on an 800™ TS microplate Absorbance Reader (BioTek). Protein concentrations were estimated using the Bradford method.

### 3.4.4 NEUROTROPHINS

For detection of BDNF, Rat BDNF (Brain Derived Neurotrophic Factor) ELISA Kits (E-EL-R1235, Elabscience Biotechnology Inc., Houston, Texas, USA) were used and Rat NGF (Nerve Growth Factor) ELISA Kits (E-EL-R0652, Elabscience Biotechnology Inc., Houston, Texas, USA) were used for NGF detection. Prior to assaying, we performed a serial dilution of selected samples using as the ultra-pure distilled water as diluent. Assays used HP and STR samples diluted 20-times, and analyses were performed according to the manufacturer´s instructions. The absorbance was read at 450 nm on an 800™ TS microplate Absorbance Reader (BioTek). Protein concentrations were estimated using the Bradford method and expressed in mg.

### 3.4.5 OXIDATIVE STRESS

For estimation of thiobarbituric acid reactive substances TBARS Lipid Peroxidation (MDA) Assay Kit (Abcam, Cambridge, UK) was used. The homogenate was adjusted to the final concentration of 10% with the use of 2% solution of butylhydroxytoluene in dd$H_2O$, deproteinized with 2N $HClO_4$ and the 10 000 g supernatant was used for the reaction with thiobarbituric acid (TBA) to

give the MDA-TBA adduct. The final product was quantified fluorometrically (Ex/Em= 532/553 nm). The results are expressed as nmol MDA /100µg proteins. 4-HNE was analyzed with the use of ELISA kits from Cusabio, (Wuhan, PRC). The samples of 10 000 g supernatant were dilute 200 times (0.1 % homogenate) with sample diluent provided by the kit. The analysis proceeded according to manufacturer instruction; the results were red at 450 nm. and are expressed as ng of 4-HNE/mg proteins.

Proteins in the homogenates were estimated using Bicinchoninic Acid Protein Assay Kit from Sigma-Aldrich (Darmstadt Germany).

### 3.4.6 C-FOS

c-Fos Transcription Factor Assay Kits (Colorimetric) (ab207194, Abcam, Cambridge, UK) were used to detect activation of c-fos. Protein concentration determination by BCA method was performed after nuclear extract preparation. According to manufacturer instruction, samples were diluted with ENE2 extraction buffer (ab203377) to maximum 15 mg protein per well and the procedure proceeded according to manufacturer instruction. The absorbance was read at 450 nm on an 800™ TS microplate Absorbance Reader (BioTek). Since semi quantitative method do not contain any standards for calibration curve and concentration calculation, absorbances of samples were compared to absorbance of well containing AP-1 mutated oligonucleotide, as positive control, AP-1 wild-type oligonucleotide as negative control and K-562(TPA) nuclear extract stimulated with TPA (12-*O*-tetradecanoylphorbol-13-acetate) as another positive control. **See** *Fig.* 11, which represents a comparison between the manufacturer's control values and our values.

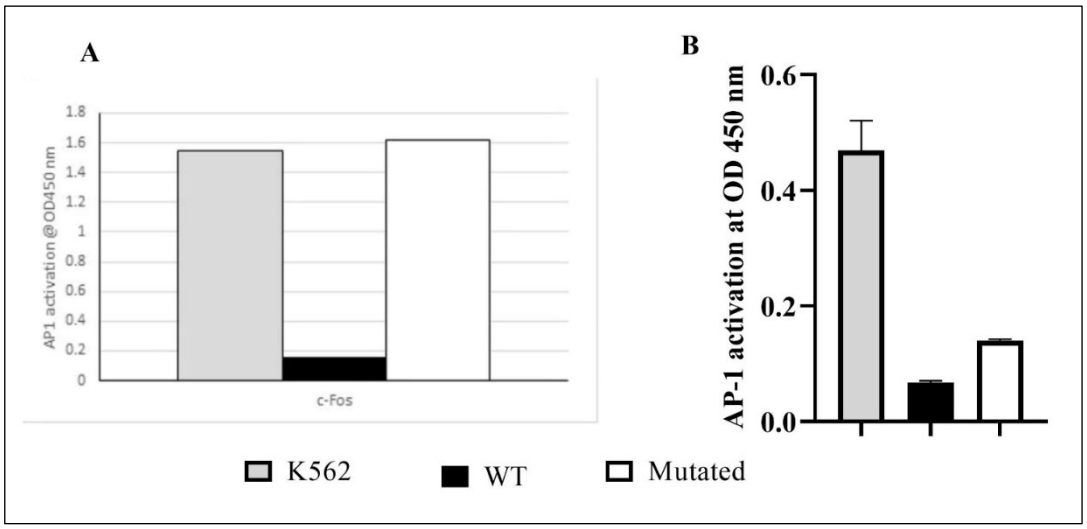


***Figure 11. Demonstration of results of c – fos control OD.***

*Fig. A shows ratios between respective control samples demonstrated by manufacturer. Fig. B shows rations between respective control samples measured in our experiment.*

**Statistical analyses of results**

Firstly, we determined the distribution and variance of the data in the individual subgroups to find out if we can use a parametric test. Three- way ANOVA (treatment x preweaning housing x postweaning housing) was used for data analysis for all experiments, however for habituation, ORT, OLT learning and memory we used analysis with repeated measures (since these tests were performed in several trials/days). The Tukey post hoc test was used for multiple comparisons between groups. For statistical analyses Tibco Statistica software version 13.5.0and Graph Pad Prism 8 were used. Differences were considered significant if $p < 0.05$. All values stated in the results are means ± SEM.

# 4. RESULTS

## 4.1 Levels of neurotransmitters after direct exposure to MA on PD 12

In terms of levels of DA in STR (t=2.666, df=13, p=0.02) and HP (t=3.735, df=13, p=0.001)., we observed significantly lower levels in MA treated animals. Levels of GLU and 5HT were not significantly altered by MA.

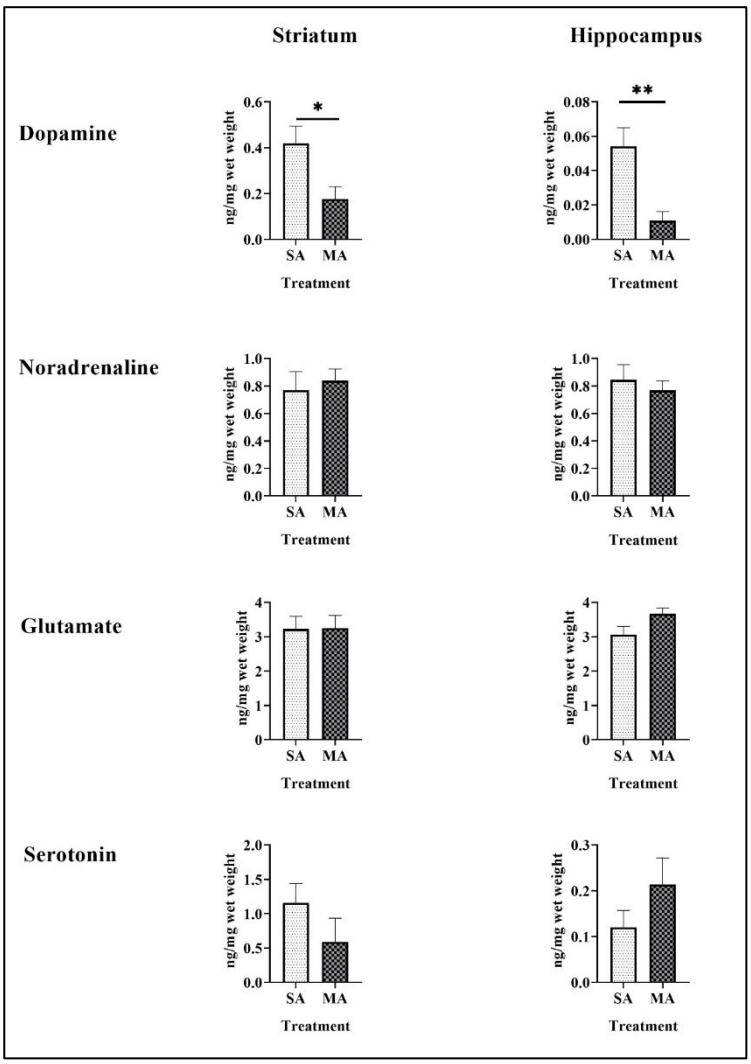


***Figure 12. Levels of neurotransmitters in hippocampus and striatum on PD 12 after direct exposure.***

*Levels are expressed in ng /mg of wet weight. Fig. shows significant differences in levels of DA in both HP and STR according to MA exposure since these levels are eminently lower. Values are ± SEM. n=8. * p<0.05, ** p<0.01, *represents effect of treatment.*

## 4.2 Behavioral testing

### 4.2.1 Habituation

**Direct exposure**

During this test, the distance moved was measured for four consecutive days. Within animals directly exposed, postweaning housing had significant impact on distance moved since separated animals moved longer distance than grouped [$F_{(1,56)} = 4.95$, $p=0.03$]. Also, interaction between trials and postweaning housing was significant [$F_{(3,168)} = 5.71$, $p=0.0001$], where EE animals explored arena more during all trials than animals with standard environment. *(Table 2).* Sidak post hoc test revealed that animals exposed to EE moved longer distance in comparison to SC during first day of trial ($p=0.020$).

**Indirect exposure**

Within indirectly exposed animals, we obtained similar results. There were no significant differences between groups affected by treatment or preweaning housing. Factors which had significant impact on results of this test were interactions between preweaning and postweaning housing [$F_{(1,56)} = 4.88$, $p=0.03$], interaction between trials and postweaning housing [$F_{(3,168)} = 4.25$, $p=0.06$] and interaction between treatment, postweaning housing and trials [$F_{(3,168)} = 2.95$, $p=0.03$]. These interactions resulted in longer distance moved within separated animals moved significantly longer distance than grouped animals, however grouped EE animals moved longer distance than separated which directly suggest impact of environmental alterations on cognitive functions. *(Table 2)*.

***Table 2. Habituation***

*Table displays distance moved during four consequent days within habituation test in cm. Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **Habituation** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Direct** | **Distance moved (means ± SEM)** | | | | | | | |
| **Days** | **SA/SC/GH** | **SA/SC/GH** | **SA/SC/GH** | **SA/SC/GH** | **MA/SC/GH** | **MA/SC/GH** | **MA/SC/GH** | **MA/SC/GH** |
| **1** | 1855,19 ±106,96 | 1890,99 ±157,38 | 1893,93 ±177,42 | 2071,02 ±87,11 | 1604,85 ± 93,33 | 1826,42 ±117,45 | 1927,33 ±134,58 | 2208,05 ±57,22 |
| **2** | 1271,61 ±132,03 | 1425,86 ±184,71 | 1119,21 ±134,11 | 1513,72 ±148,36 | 1003,63 ±61,07 | 1334,45 ±88,00 | 1382,05 ±111,64 | 1292,77 ±66,90 |
| **3** | 1164,29 ±94,48 | 1170,99 ±108,24 | 933,46 ±104,64 | 1103,88 ±124,12 | 670,10 ±89,71 | 1112,94 ±102,46 | 858,93 ±107,68 | 900,32 ±82,97 |
| **4** | 1116,17 ±158,85 | 1067,04 ±144,86 | 928,67 ±116,72 | 1057,34 ±97,08 | 821,90 ±73,01 | 942,94 ±159,16 | 967,06 ±95,03 | 880,71 ±111,75 |
| **Indirect** | **Distance moved (means ± SEM)** | | | | | | | |
| **Days** | **SA/SC/GH** | **SA/SC/SH** | **SA/EE/GH** | **SA/EE/SH** | **MA/SC/GH** | **MA/SC/SH** | **MA/EE/GH** | **MA/EE/SH** |
| **1** | 1319,87 ±240,16 | 2055,48 ±126,86 | 1627,97 ±195,86 | 1777,58 ±105,18 | 1963,43 ±85,17 | 2320,97 ±156,61 | 1739,33 ±141,61 | 1684,60 ±80,88 |
| **2** | 1145,14 ±183,63 | 1245,19 ±144,94 | 1306,82 ±149,95 | 1129,20 ±148,06 | 1303,08 ±50,30 | 1421,78 ±203,66 | 1257,07 ±111,21 | 1264,54 ±75,01 |
| **3** | 922,31 ±76,74 | 1011,49 ±123,36 | 1047,24 ±114,74 | 634,40 ±92,36 | 941,59 ±107,26 | 1225,79 ±168,73 | 812,27 ±119,59 | 822,52 ±127,70 |
| **4** | 900,98 ±87,08 | 880,04 ±161,86 | 972,59 ±74,40 | 674,17 ±114,93 | 985,93 ±111,71 | 1038,27 ±191,49 | 922,89 ±92,97 | 984,46 ±141,07 |

### 4.2.2 Object recognition test

**Direct exposure**

Distance moved in this test was significantly altered by preweaning housing [$F_{(1, 56)} = 9.60$, $p=0.0377$] since EE exposed animals moved longer distance than SC. Interaction between all considered factors [$F_{(2, 112)} = 1.0080$, $p=0.03$] was significant as well. Velocity was altered by interaction between delay, treatment and postweaning housing [$F_{(2, 112)} = 1.94$, $p=0.03$]. Duration around familiar object was significantly altered by preweaning [$F_{(1, 56)} = 6.60$, $p=0.01$] housing since animals exposed to EE spent significantly more time around familiar object than SC. Also,

postweaning housing had significant impact on this parameter since separated animals spent more time around familiar object than grouped animals [$F_{(1, 56)} = 4.62$, $p=0.03$]. Duration around novel object was altered by interaction between delay, treatment and preweaning housing [$F_{(2, 112)} = 3.41$, $p=0.04$]. Animals within MA/SC spent more time exploring novel object during second trial, while SA/EE exposed animals spent more time exploring this object during second trial. Differences in MA exposed animals were not as visible as in SA animals. Recognition index was not significantly altered by any factor. Differences were visible only in terms of respective delays [$F_{(2, 112)} = 4.55$, $p=0.01$] (*(Table 3.a, Table 3.b, Table 3.c)*.

***Table 3.a ORT after direct exposure***

*Table shows results from ORT test within animals directly exposed to MA. Parameters presented in this table are: distance moved in cm during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), velocity of movement in cm/s during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay) and recognition index in s (RI – ratio between time spent around novel object in comparison to total time of exploring both objects) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| ORT DIRECT | PARAMETERS (means ±SEM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| GROUPS | Distance 1.min | Distance 15.min | Distance 30.min | Velocity 1.min | Velocity 15.min | Velocity 30.min | IR 1.min | IR 15.min | IR 30.min |
| SA/SC/GH | 174.75 ±21.39 | 120.29 ±19.20 | 93.96 ±17.69 | 6.22 ±0.80 | 6.38 ±0.66 | 4.02 ±0.70 | 0.52 ±0.28 | 1.72 ±0.95 | 0.92 ±0.56 |
| SA/SC/SH | 174.69 ±19.53 | 143.91 ±26.45 | 180.40 ±22.55 | 6.06 ±0.71 | 5.50 ±0.88 | 6.38 ±0.80 | 2.36 ±0.79 | 2.18 ±0.72 | 4.29 ±1.21 |
| SA/EE/GH | 217.91 ±54.08 | 251.54 ±53.80 | 169.00 ±46.92 | 6.99 ±1.24 | 7.36 ±0.92 | 4.59 ±0.78 | 3.14 ±1.340 | 4.86 ±1.06 | 2.36 ±1.32 |
| SA/EE/SH | 179.48 ±53.89 | 259.37 ±56.45 | 223.15 ±57.16 | 4.60 ±0.68 | 5.93 ±0.63 | 5.43 ±0.91 | 5.14 ±3.51 | 4.10 ±1.08 | 6.74 ±3.08 |
| MA/SC/GH | 172.53 ±22.41 | 165.43 ±14.30 | 116.66 ±23.41 | 5.71 ±0.74 | 5.61 ±0.46 | 4.01 ±0.79 | 4.03 ±1.94 | 3.14 ±1.16 | 0.39 ±0.27 |
| MA/SC/SH | 152.39 ±21.97 | 157.02 ±14.36 | 120.29 ±13.42 | 5.32 ±0.74 | 5.86 ±0.61 | 5.05 ±0.39 | 1.40 ±0.58 | 6.21 ±2.58 | 4.10 ±2.31 |
| MA/EE/GH | 222.08 ±45.26 | 238.22 ±49.03 | 200.32 ±28.90 | 4.90 ±0.61 | 5.15 ±0.63 | 5.08 ±0.90 | 2.91 ±1.27 | 4.24 ±1.57 | 1.42 ±0.47 |
| MA/EE/SH | 276.07 ±57.67 | 189.94 ±42.24 | 177.05 ±41.63 | 6.34 ±0.90 | 4.39 ±0.69 | 3.82 ±0.67 | 5.95 ±2.09 | 3.13 ±1.21 | 3.35 ±1.79 |

***Table 3.b ORT after direct exposure***

*Table shows results from ORT test within animals directly exposed to MA. Parameters presented in this table are: duration of exploring familiar (old) object in s during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), duration of exploring novel object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), frequency of exploring familiar object during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **ORT DIRECT** | **PARAMETERS (means ±SEM)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **GROUPS** | **Duration-old object 1.min** | **Duration-old object 15.min** | **Duration-old object 30.min** | **Duration-new object 1.min** | **Duration-new object 15.min** | **Duration-new object 30.min** | **Frequency- old object 1.min** | **Frequency- old object 15.min** | **Frequency- old object 30.min** |
| **SA/SC/GH** | 0.58 ±0.38 | 1.22 ±0.83 | 0.85 ±0.48 | 2.89 ±2.37 | 1.03 ±0.86 | 0.91 ±0.65 | 1.13 ±0.52 | 1.63 ±0.68 | 1.00 ±0.73 |
| **SA/SC/SH** | 3.08 ±1.07 | 1.63 ±0.69 | 3.42 ±1.15 | 6.32 ±1.84 | 2.78 ±0.97 | 4.05 ±1.21 | 5.13 ±1.53 | 1.75 ±0.59 | 4.25 ±1.45 |
| **SA/EE/GH** | 4.88 ±1.90 | 3.86 ±1.06 | 3.16 ±1.11 | 3.26 ±1.48 | 4.17 ±1.34 | 1.46 ±0.88 | 3.38 ±1.24 | 3.88 ±1.06 | 2.13 ±0.74 |
| **SA/EE/SH** | 9.75 ±5.84 | 4.76 ±1.40 | 6.75 ±2.84 | 2.87 ±1.00 | 8.35 ±4.73 | 4.66 ±1.74 | 1.50 ±0.71 | 3.75 ±1.19 | 4.75 ±2.45 |
| **MA/SC/GH** | 4.04 ±1.72 | 2.39 ±1.07 | 1.10 ±0.70 | 3.08 ±1.93 | 5.31 ±2.61 | 0.15 ±0.13 | 3.75 ±0.96 | 2.50 ±0.95 | 1.63 ±0.93 |
| **MA/SC/SH** | 0.71 ±0.50 | 5.47 ±2.50 | 3.79 ±2.19 | 2.09 ±0.96 | 4.93 ±2.98 | 1.97 ±1.34 | 2.25 ±1.57 | 2.38 ±0.71 | 3.13 ±0.85 |
| **MA/EE/GH** | 2.95 ±1.35 | 3.60 ±1.45 | 1.60 ±0.65 | 3.55 ±1.31 | 1.98 ±0.90 | 1.56 ±0.92 | 3.75 ±1.39 | 3.88 ±1.25 | 4.13 ±1.53 |
| **MA/EE/SH** | 5.36 ±1.96 | 2.56 ±1.08 | 5.42 ±2.50 | 2.84 ±0.95 | 3.51 ±1.22 | 0.74 ±0.42 | 3.875 ±0.833 | 2.75 ±1.05 | 3.88 ±2.13 |

***Table 3.c ORT after direct exposure***

*Table shows results from ORT test within animals directly exposed to MA. Parameters presented in this table are: frequency of exploring novel object during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), latency to familiar object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), latency to novel object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| ORT DIRECT | PARAMETERS (means ±SEM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| GROUPS | Frequency-new object 1.min | Frequency-new object 15.min | Frequency-new object 30.min | Latency -old object 1.min | Latency -old object 15.min | Latency -old object 30.min | Latency-new object 1.min | Latency -new object 15.min | Latency -old object 30. min |
| SA/SC/GH | 2.75 ±1.90 | 1.25 ±0.73 | 1.00 ±0.63 | 9.82 ±6.29 | 8.87 ±7.16 | 5.57 ±5.11 | 2.22 ±1.38 | 6.77 ±3.83 | 7.22 ±6.05 |
| SA/SC/SH | 5.63 ±2.46 | 2.63 ±0.78 | 5.88 ±2.40 | 3.34 ±1.42 | 7.57 ±4.16 | 15.88 ±5.52 | 9.34 ±3.67 | 5.96 ±2.20 | 12.78 ±4.95 |
| SA/EE/GH | 3.25 ±1.25 | 3.88 ±0.67 | 1.25 ±0.56 | 14.83 ±7.02 | 9.55 ±4.00 | 5.65 ±3.11 | 7.99 ±3.69 | 7.15 ±1.61 | 10.19 ±4.88 |
| SA/EE/SH | 5.62 ±1.74 | 3.13 ±0.61 | 3.75 ±1.45 | 13.04 ±5.00 | 7.99 ±5.41 | 15.20 ±6.28 | 10.59 ±4.33 | 19.81 ±6.64 | 4.91 ±2.54 |
| MA/SC/GH | 2.50 ±1.09 | 3.88 ±1.37 | 0.38 ±0.26 | 4.78 ±3.06 | 11.08 ±3.59 | 3.84 ±2.70 | 6.07 ±1.95 | 6.33 ±2.20 | 13.14 ±8.65 |
| MA/SC/SH | 2.63 ±0.93 | 5.63 ±2.63 | 0.88 ±0.23 | 14.12 ±7.65 | 10.71 ±6.16 | 13.69 ±5.76 | 17.55 ±5.72 | 11.18 ±4.12 | 22.69 ±9.50 |
| MA/EE/GH | 4.13 ±1.46 | 3.00 ±1.24 | 1.25 ±0.59 | 13.83 ±4.97 | 10.55 ±6.21 | 11.50 ±5.24 | 10.37 ±3.39 | 6.62 ±2.55 | 15.64 ±6.62 |
| MA/EE/SH | 4.25 ±0.98 | 3.88 ±1.16 | 1.13 ±0.58 | 4.42 ±2.94 | 6.12 ±2.17 | 5.99 ±3.35 | 16.44 ±6.55 | 7.35 ±3.47 | 9.84 ±4.93 |

## Indirect exposure

Distance moved was altered by interaction between delay, treatment and postweaning housing [$F_{(2, 112)} = 3.18$, $p=0.04$]. Velocity was not altered by any factor. Duration around familiar object was significantly altered by interaction between treatment and postweaning housing [$F_{(1, 56)} = 6.66$, $p=0.01$] as well as interaction between delay, treatment and postweaning housing [$F_{(2, 112)} = 3.39$, $p=0.04$]. MA exposed animals in groups spend significantly more time between around familiar object than separated animals, however also than SA exposed grouped animals. Recognition index was significantly altered by interaction

between treatment and postweaning housing [$F_{(1, 56)} = 5.36$, $p=0.02$]. MA exposed animals in groups had significantly higher recognition index than separated animals but also than SA exposed grouped animals *(Table 4.a, Table 4.b, Table 4.c). (Table 4.a, Table 4.b, Table 4.c).*

***Table 4.a ORT after indirect exposure***

*Table shows results from ORT test within animals in directly exposed to MA. Parameters presented in this table are: distance moved in cm during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), velocity of movement in cm/s during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), recognition index in s (RI – ratio between time spent around novel object in comparison to total time of exploring both objects) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| ORT INDIRECT | PARAMETERS (means ±SEM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **GROUPS** | **Distance 1.min** | **Distance 15.min** | **Distance 30.min** | **Velocity 1.min** | **Velocity 15.min** | **Velocity 30.min** | **IR 1.min** | **IR 15.min** | **IR 30.min** |
| **SA/SC/ GH** | 189.50 ±31.19 | 179.39 ±21.53 | 154.96 ±29.16 | 6.24 ±0.94 | 5.91 ±0.64 | 5.17 ±0.91 | 1.13 ±0.70 | 2.87 ±1.62 | 1.96 ±1.31 |
| **SA/SC/ SH** | 171.89 ±23.79 | 164.12 ±24.33 | 113.76 ±21.58 | 5.78 ±0.82 | 5.43 ±0.79 | 3.70 ±0.71 | 2.39 ±0.58 | 2.77 ±0.77 | 1.19 ±0.64 |
| **SA/EE/ GH** | 140.86 ±34.48 | 123.59 ±29.86 | 124.13 ±25.18 | 5.84 ±1.62 | 5.26 ±1.28 | 5.17 ±1.15 | 0.26 ±0.17 | 0.41 ±0.28 | 2.22 ±1.14 |
| **SA/EE/ SH** | 185.88 ±37.03 | 204.79 ±19.17 | 107.98 ±19.50 | 7.64 ±1.79 | 8.03 ±0.85 | 4.70 ±0.77 | 3.28 ±1.90 | 2.96 ±1.24 | 0.85 ±0.33 |
| **MA/SC/ GH** | 169.25 ±22.91 | 182.04 ±18.22 | 136.86 ±15.44 | 5.75 ±0.85 | 6.30 ±0.67 | 4.83 ±0.63 | 4.17 ±1.78 | 3.26 ±1.37 | 2.69 ±1.14 |
| **MA/SC/ SH** | 180.29 ±26.15 | 183.84 ±29.21 | 133.48 ±22.16 | 6.04 ±0.89 | 6.18 ±0.99 | 4.55 ±0.74 | 1.37 ±0.38 | 0.95 ±0.50 | 1.90 ±0.52 |
| **MA/EE/ GH** | 152.21 ±18.66 | 167.07 ±21.32 | 123.40 ±21.78 | 6.02 ±0.82 | 7.09 ±1.03 | 5.46 ±0.97 | 1.83 ±0.84 | 1.76 ±0.59 | 3.41 ±1.73 |
| **MA/EE/ SH** | 164.72 ±31.23 | 140.22 ±26.46 | 146.61 ±26.58 | 6.94 ±1.57 | 5.99 ±1.22 | 6.13 ±1.20 | 0.93 ±0.42 | 1.13 ±0.73 | 3.20 ±1.61 |

***Table 4.b ORT after indirect exposure***

*Table shows results from ORT test within animals in directly exposed to MA. Parameters presented in this table are: duration of exploring familiar (old) object in s during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), duration of exploring novel object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), frequency of exploring familiar object during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| ORT INDIRECT | PARAMETERS (means ±SEM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| GROUPS | Duration -old object 1.min | Duration -old object 15.min | Duration -old object 30.min | Duration -new object 1.min | Duration -new object 15.min | Duration -new object 30.min | Frequency - old object 1.min | Frequency - old object 15.min | Frequency - old object 30.min |
| SA/SC/ GH | 1.37 ±0.66 | 2.25 ±1.53 | 1.46 ±1.21 | 4.68 ±2.38 | 2.04 ±1.32 | 3.01 ±1.48 | 2.13 ±0.88 | 2.75 ±1.15 | 2.75 ±1.68 |
| SA/SC/ SH | 1.52 ±0.52 | 1.90 ±0.72 | 0.56 ±0.56 | 2.45 ±1.31 | 1.57 ±0.61 | 1.60 ±0.76 | 2.63 ±0.87 | 2.13 ±0.88 | 0.00 ±0.00 |
| SA/EE/ GH | 0.07 ±0.06 | 0.49 ±0.33 | 1.95 ±0.95 | 2.42 ±1.61 | 0.65 ±0.43 | 0.55 ±0.32 | 0.25 ±0.16 | 1.25 ±0.65 | 1.50 ±0.57 |
| SA/EE/ SH | 2.52 ±1.84 | 2.35 ±1.13 | 1.01 ±0.50 | 1.24 ±0.67 | 2.63 ±1.99 | 2.13 ±0.97 | 1.75 ±0.75 | 3.00 ±1.10 | 1.63 ±0.87 |
| MA/SC/ GH | 4.78 ±1.86 | 3.51 ±1.11 | 2.40 ±0.96 | 7.27 ±2.36 | 6.08 ±4.59 | 9.25 ±5.00 | 4.63 ±1.73 | 3.75 ±0.82 | 2.75 ±0.86 |
| MA/SC/ SH | 0.62 ±0.27 | 1.25 ±0.50 | 1.15 ±0.40 | 3.55 ±2.64 | 0.30 ±0.28 | 0.92 ±0.58 | 1.75 ±0.65 | 2.50 ±0.95 | 1.88 ±0.64 |
| MA/EE/ GH | 5.01 ±2.64 | 1.53 ±0.61 | 3.21 ±1.56 | 2.96 ±2.30 | 1.76 ±0.62 | 0.76 ±0.47 | 2.63 ±1.09 | 3.13 ±1.04 | 1.88 ±0.83 |
| MA/EE/ SH | 0.43 ±0.28 | 1.86 ±1.02 | 2.45 ±1.54 | 3.30 ±2.20 | 1.86 ±1.32 | 2.99 ±1.13 | 1.000 ±0.627 | 2.00 ±0.91 | 2.75 ±1.03 |

***Table 4.c ORT after indirect exposure***

*Table shows results from ORT test within animals in directly exposed to MA. Parameters presented in this table are: frequency of exploring novel object during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), latency to familiar object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), latency to novel object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning).*

| ORT INDIRECT | PARAMETERS (means ±SEM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| GROUPS | Frequency -new object 1.min | Frequency -new object 15.min | Frequency -new object 30.min | Latency -old object 1.min | Latency -old object 15.min | Latency -old object 30.min | Latency -new object 1.min | Latency -new object 15.min | Latency -old object 30. min |
| SA/SC/GH | 2.63 ±1.30 | 2.38 ±1.32 | 2.75 ±1.19 | 9.67 ±5.65 | 7.98 ±4.52 | 11.09 ±7.13 | 3.55 ±2.03 | 11.21 ±4.13 | 2.85 ±1.62 |
| SA/SC/SH | 2.13 ±0.48 | 2.38 ±0.65 | 1.25 ±0.53 | 13.09 ±5.04 | 5.20 ±2.12 | 0.00 ±0.00 | 7.62 ±1.89 | 11.75 ±5.43 | 4.96 ±3.68 |
| SA/EE/GH | 1.63 ±0.93 | 0.50 ±0.33 | 1.63 ±0.65 | 0.78 ±0.60 | 2.63 ±1.72 | 12.09 ±5.75 | 3.94 ±2.36 | 6.38 ±5.34 | 5.59 ±4.24 |
| SA/EE/SH | 1.88 ±0.69 | 3.25 ±1.58 | 1.88 ±0.69 | 5.66 ±3.47 | 6.41 ±2.00 | 11.05 ±5.44 | 14.62 ±6.13 | 14.87 ±6.78 | 11.89 ±6.17 |
| MA/SC/GH | 3.88 ±1.03 | 2.00 ±0.63 | 2.38 ±0.87 | 7.91 ±2.93 | 13.13 ±5.14 | 13.55 ±6.20 | 11.84 ±3.15 | 20.91 ±6.05 | 1.64 ±0.83 |
| MA/SC/SH | 1.38 ±0.38 | 0.38 ±0.18 | 2.00 ±0.80 | 6.72 ±2.40 | 8.51 ±3.34 | 7.31 ±2.70 | 17.26 ±5.54 | 11.63 ±7.25 | 8.57 ±4.14 |
| MA/EE/GH | 2.63 ±1.40 | 2.13 ±1.08 | 0.63 ±0.18 | 12.10 ±7.28 | 7.51 ±2.91 | 19.02 ±7.97 | 15.71 ±6.16 | 18.71 ±4.68 | 19.81 ±7.67 |
| MA/EE/SH | 2.38 ±1.43 | 1.75 ±1.15 | 4.00 ±1.64 | 11.76 ±6.25 | 19.19 ±6.25 | 15.42 ±5.72 | 6.25 ±2.92 | 4.65 ±2.58 | 6.03 ±4.01 |

### 4.2.3 Object location test

**Direct exposure**

Distance moved was significantly altered by interaction between delay, preweaning and postweaning housing [$F_{(2, 112)} = 3.54$, $p=0.03$]. Velocity was significantly altered by interaction between delay, treatment and preweaning housing [$F_{(2, 112)} = 4.77$, $p=0.01$] as well as interaction delay, preweaning and postweaning housing [$F_{(2, 112)} = 3.69$, $p=0.03$]. Duration around familiar object was not altered by any factor, while duration around novel object was altered by preweaning housing [$F_{(1, 56)} = 6.16$, $p=0.02$]. Animals exposed to EE spent significantly less time around novel object than animals raised in standard conditions. Recognition index was not altered by any factor *(Table 5.a, Table 5.b, Table 5.c).*

***Table 5.a OLT after direct exposure***

*Table shows results from OLT test within animals directly exposed to MA. Parameters presented in this table are: distance moved in cm during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), velocity of movement in cm/s during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), recognition index in (s) (RI – ratio between time spent around relocated object in comparison to total time of exploring both objects) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| OLT DIRECT | PARAMETERS (means ±SEM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **GROUPS** | **Distance 1.min** | **Distance 15.min** | **Distance 30.min** | **Velocity 1.min** | **Velocity 15.min** | **Velocity 30.min** | **IR 1.min** | **IR 15.min** | **IR 30.min** |
| **SA/SC/ GH** | 338.30 ±48.43 | 244.65 ±40.10 | 221.98 ±31.90 | 6.04 ±0.87 | 4.36 ±0.76 | 4.20 ±0.73 | 6.75 ±2.33 | 8.83 ±4.45 | 0.51 ±0.19 |
| **SA/SC/ SH** | 419.24 ±50.80 | 288.03 ±61.50 | 298.39 ±40.42 | 7.27 ±0.91 | 5.43 ±1.19 | 6.19 ±0.84 | 4.97 ±2.18 | 2.70 ±1.25 | 6.33±2. 96 |
| **SA/EE/ GH** | 198.11 ±31.74 | 330.48 ±57.45 | 245.06 ±39.08 | 4.06 ±0.76 | 5.62 ±0.98 | 4.25 ±0.67 | 4.23 ±3.31 | 5.38 ±2.08 | 1.67± 1.14 |
| **SA/EE/ SH** | 389.49 ±36.05 | 403.19 ±37.91 | 196.12 ±26.75 | 6.66 ±0.64 | 6.97 ±0.68 | 3.58 ±0.61 | 4.61 ±1.61 | 8.72 ±3.53 | 0.95 ±0.40 |
| **MA/SC/ GH** | 330.84 ±60.08 | 303.91 ±41.10 | 254.35 ±43.32 | 5.86 ±1.11 | 5.32 ±0.75 | 4.86 ±0.88 | 4.70 ±1.64 | 3.81 ±1.39 | 2.87 ±1.02 |
| **MA/SC/ SH** | 311.29 ±49.84 | 300.16 ±59.27 | 259.73 ±33.53 | 5.58 ±0.94 | 5.66 ±1.20 | 5.10 ±0.84 | 7.69 ±3.71 | 2.71 ±1.17 | 5.50 ±2.95 |
| **MA/EE/ GH** | 307.71 ±50.55 | 229.48 ±29.15 | 326.39 ±45.63 | 5.24 ±0.87 | 4.91 ±0.74 | 5.62 ±0.79 | 7.31 ±6.12 | 11.96 ±6.81 | 6.27 ±3.76 |
| **MA/EE/ SH** | 409.53 ±49.28 | 285.33 ±49.87 | 275.47 ±57.60 | 7.15 ±0.93 | 5.07 ±0.90 | 4.84 ±1.02 | 6.27 ±2.62 | 5.95 ±4.34 | 5.55 ±3.78 |

***Table 5.b OLT after direct exposure***

*Table shows results from OLT test within animals directly exposed to MA. Parameters presented in this table are: duration of exploring original (old) object in s during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), duration of exploring relocated object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), frequency of exploring original object during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **OLT DIRECT** | **PARAMETERS (means ±SEM)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **GROUPS** | **Duration-old object 1.min** | **Duration-old object 15.min** | **Duration-old object 30.min** | **Duration-new object 1.min** | **Duration-new object 15.min** | **Duration-new object 30.min** | **Frequency- old object 1.min** | **Frequency- old object 15.min** | **Frequency- old object 30.min** |
| **SA/SC/GH** | 5.75 ±2.33 | 7.95 ±4.42 | 2.55 ±2.54 | 14.24 ±4.75 | 9.41 ±4.50 | 7.11 ±3.39 | 2.13 ±0.61 | 3.75 ±2.65 | 0.63 ±0.50 |
| **SA/SC/SH** | 6.05 ±1.96 | 1.97 ±1.18 | 9.71 ±4.48 | 7.65 ±2.64 | 13.40 ±6.84 | 12.83 ±2.92 | 4.75 ±1.15 | 2.13 ±0.93 | 18.00 ±15.17 |
| **SA/EE/GH** | 12.55 ±4.93 | 10.86 ±5.26 | 1.41 ±1.02 | 1.78 ±0.74 | 7.13 ±2.85 | 2.86 ±1.59 | 9.00 ±5.34 | 4.88 ±1.81 | 1.50 ±0.46 |
| **SA/EE/SH** | 3.61 ±1.61 | 8.69 ±3.31 | 2.69 ±1.50 | 8.39 ±1.63 | 5.48 ±1.64 | 1.81 ±0.95 | 2.88 ±1.13 | 4.00 ±0.98 | 4.25 ±3.00 |
| **MA/SC/GH** | 9.39 ±4.80 | 3.75 ±1.25 | 2.17 ±0.91 | 4.24 ±1.34 | 8.35 ±4.43 | 11.23 ±3.72 | 4.63 ±1.57 | 2.25 ±0.75 | 3.63 ±1.88 |
| **MA/SC/SH** | 11.29 ±3.37 | 5.24 ±1.68 | 13.53 ±4.30 | 7.39 ±3.51 | 17.41 ±7.35 | 20.34 ±7.11 | 4.13 ±1.33 | 5.75 ±2.02 | 5.75 ±2.20 |
| **MA/EE/GH** | 11.61 ±7.10 | 12.16 ±6.59 | 12.35 ±4.83 | 6.95 ±3.84 | 10.89 ±6.79 | 8.52 ±4.08 | 1.75 ±0.70 | 1.88 ±0.77 | 4.13 ±1.20 |
| **MA/EE/SH** | 10.61 ±4.44 | 14.73 ±7.57 | 10.63 ±5.17 | 5.82 ±2.42 | 6.85 ±3.38 | 11.26 ±6.21 | 6.63 ±0.65 | 4.13 ±1.86 | 3.38 ±1.02 |

***Table 5.c OLT after direct exposure***

*Table shows results from OLT test within animals directly exposed to MA. Parameters presented in this table are: frequency of exploring relocated object during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), latency to original object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), latency to relocated object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| OLT DIRECT | PARAMETERS (means ±SEM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| GROUPS | Frequency-new object 1.min | Frequency-new object 15.min | Frequency-new object 30.min | Latency-old object 1.min | Latency-old object 15.min | Latency-old object 30.min | Latency-new object 1.min | Latency-new object 15.min | Latency-old object 30. min |
| SA/SC/GH | 10.25 ±4.27 | 2.38 ±0.65 | 3.13 ±2.18 | 14.54 ±3.72 | 1.02 ±0.44 | 4.05 ±3.92 | 6.60 ±2.12 | 20.90 ±6.43 | 6.70 ±4.37 |
| SA/SC/SH | 5.00 ±2.19 | 3.88 ±1.68 | 5.50 ±1.78 | 11.42 ±4.99 | 9.10 ±5.99 | 6.32 ±2.97 | 6.10 ±3.24 | 8.16 ±3.15 | 18.34 ±6.82 |
| SA/EE/GH | 1.13 ±0.61 | 2.00 ±0.78 | 1.25 ±0.62 | 2.14 ±0.66 | 7.88 ±2.90 | 9.89 ±6.42 | 3.44 ±1.68 | 8.66 ±4.46 | 2.37 ±1.30 |
| SA/EE/SH | 3.25 ±0.90 | 2.63 ±0.57 | 0.88 ±0.48 | 10.78 ±3.45 | 11.97 ±4.60 | 3.97 ±3.19 | 10.01 ±6.15 | 7.36 ±3.18 | 5.02 ±3.25 |
| MA/SC/GH | 3.75 ±1.69 | 2.88 ±1.06 | 2.88 ±1.36 | 5.26 ±3.06 | 14.74 ±7.39 | 10.19 ±6.27 | 6.99 ±3.44 | 10.44 ±5.01 | 8.63 ±3.96 |
| MA/SC/SH | 3.50 ±1.56 | 3.50 ±2.25 | 2.63 ±1.22 | 13.20 ±6.28 | 4.25 ±2.44 | 7.15 ±5.80 | 7.06 ±3.67 | 5.55 ±3.22 | 10.92 ±4.36 |
| MA/EE/GH | 2.63 ±1.39 | 1.38 ±0.57 | 3.13 ±1.23 | 7.20 ±4.99 | 3.94 ±2.46 | 8.86 ±3.51 | 10.46 ±6.93 | 4.10 ±1.32 | 10.81 ±5.44 |
| MA/EE/SH | 2.50 ±0.76 | 1.25 ±0.45 | 2.38 ±1.03 | 6.18 ±2.39 | 9.69 ±7.00 | 6.23 ±3.14 | 2.53 ±1.62 | 4.11 ±1.93 | 10.83 ±5.09 |

## Indirect exposure

Distance moved was altered by interaction between delay, preweaning and postweaning environment [$F_{(2, 112)} = 3.10$, $p=0.05$]. Velocity was not altered by any factors. Duration around familiar object was altered by interaction between treatment and postweaning environment [$F_{(1, 56)} = 5.67$, $p=0.02$] as well as interaction between delay, treatment and postweaning housing [$F_{(2, 112)} = 3.82$, $p=0.02$]. In general, MA exposed animals in groups spent more time exploring old object than separated as well as SA grouped animals. Duration around familiar object was not significantly altered by any factor, neither recognition index *(Table 6.a, Table 6.b, Table 6.c).*

***Table 6.a OLT after indirect exposure***

*Table shows results from OLT test within animals indirectly exposed to MA. Parameters presented in this table are: distance moved in cm during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), velocity of movement in cm/s during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), recognition index in (s) (RI – ratio between time spent around relocated object in comparison to total time of exploring both objects) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| OLT INDIRECT | PARAMETERS (means ±SEM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **GROUPS** | **Distance 1.min** | **Distance 15.min** | **Distance 30.min** | **Velocity 1.min** | **Velocity 15.min** | **Velocity 30.min** | **IR 1.min** | **IR 15.min** | **IR 30.min** |
| **SA/SC/ GH** | 444.79 ±79.10 | 287.93 ±57.05 | 208.75 ±50.10 | 8.00 ±1.13 | 5.46 ±0.83 | 5.01 ±0.96 | 9.25 ±2.02 | 3.24 ±2.00 | 1.72 ±0.99 |
| **SA/SC/ SH** | 279.83 ±60.85 | 302.75 ±49.20 | 201.74 ±30.77 | 6.95 ±1.14 | 6.57 ±1.18 | 3.64 ±0.57 | 12.16 ±5.83 | 3.26 ±1.89 | 4.67 ±3.75 |
| **SA/EE/ GH** | 311.45 ±51.36 | 265.19 ±65.73 | 198.00 ±39.60 | 7.02 ±1.41 | 7.94 ±2.41 | 5.08 ±1.42 | 3.68 ±1.68 | 1.95 ±0.76 | 2.43 ±1.59 |
| **SA/EE/ SH** | 367.06 ±46.77 | 284.76 ±46.75 | 241.31 ±40.49 | 7.59 ±1.13 | 6.30 ±0.97 | 6.20 ±1.58 | 5.03 ±2.18 | 3.90 ±1.27 | 1.91 ±1.05 |
| **MA/SC/ GH** | 319.10 ±42.66 | 365.95 ±66.89 | 264.31 ±42.68 | 6.09 ±1.16 | 6.34 ±1.75 | 5.00 ±0.85 | 7.05 ±2.27 | 6.74 ±1.40 | 0.54 ±0.27 |
| **MA/SC/ SH** | 199.95 ±70.90 | 226.46 ±75.03 | 223.45 ±59.78 | 9.08 ±2.64 | 9.00 ±2.66 | 7.09 ±2.15 | 3.73 ±2.33 | 0.88 ±0.48 | 1.57 ±0.72 |
| **MA/EE/ GH** | 322.61 ±77.25 | 285.46 ±44.10 | 308.99 ±43.35 | 6.61 ±1.45 | 6.50 ±1.38 | 5.70 ±0.80 | 10.61 ±4.17 | 1.97 ±0.85 | 8.77 ±4.28 |
| **MA/EE/ SH** | 347.18 ±38.43 | 263.21 ±46.16 | 206.56 ±28.05 | 6.64 ±0.87 | 5.29 ±0.90 | 5.65 ±1.58 | 9.3 4±3.41 | 9.98 ±4.84 | 2.47 ±1.10 |

***Table 6.b OLT after indirect exposure***

*Table shows results from OLT test within animals indirectly exposed to MA. Parameters presented in this table are: duration of exploring original (old) object*

*in s during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), duration of exploring relocated object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), frequency of exploring original object during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **OLT INDIRECT** | **PARAMETERS (means ±SEM)** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **GROUPS** | **Duration- old object 1.min** | **Duration- old object 15.min** | **Duration- old object 30.min** | **Duration- new object 1.min** | **Duration- new object 15.min** | **Duration- new object 30.min** | **Frequenc y- old object 1.min** | **Frequenc y- old object 15.min** | **Frequenc y- old object 30.min** |
| **SA/SC/ GH** | 8.37 ±1.94 | 2.52 ±1.94 | 4.27 ±3.01 | 5.76 ±1.44 | 10.42 ±5.55 | 5.22 ±3.76 | 5.75 ±2.08 | 2.13 ±1.11 | 2.25 ±1.58 |
| **SA/SC/ SH** | 21.87 ±7.05 | 6.76 ±3.71 | 11.98 ±7.45 | 2.28 ±0.85 | 10.50 ±4.50 | 1.88 ±1.13 | 6.63 ±1.46 | 25.63 ±22.69 | 1.75 ±0.53 |
| **SA/EE/ GH** | 2.97 ±1.60 | 8.58 ±7.09 | 6.14 ±2.20 | 11.72 ±5.04 | 5.10 ±2.27 | 5.87 ±3.47 | 3.25 ±1.50 | 1.88 ±0.64 | 4.75 ±2.28 |
| **SA/EE/ SH** | 4.72 ±2.02 | 3.15 ±1.16 | 1.29 ±0.96 | 7.77 ±2.55 | 11.78 ±5.54 | 16.81 ±7.34 | 2.75 ±0.86 | 6.75 ±4.93 | 0.88 ±0.48 |
| **MA/SC/ GH** | 6.18 ±2.22 | 5.91 ±1.29 | 21.48 ±9.19 | 13.78 ±6.77 | 9.97 ±6.03 | 2.76 ±2.35 | 5.88 ±2.34 | 6.88 ±1.48 | 5.50 ±3.04 |
| **MA/SC/ SH** | 3.37 ±2.21 | 4.77 ±4.27 | 6.46 ±5.28 | 5.40 ±2.50 | 9.86 ±6.66 | 6.57 ±3.91 | 2.75 ±1.29 | 1.88 ±1.14 | 3.75 ±1.90 |
| **MA/EE/ GH** | 15.99 ±6.23 | 11.28 ±5.99 | 8.62 ±4.08 | 11.27 ±6.14 | 10.01 ±5.52 | 17.95 ±7.08 | 5.38 ±1.73 | 3.88 ±1.72 | 4.50 ±1.65 |
| **MA/EE/ SH** | 11.57 ±3.67 | 9.10 ±4.80 | 1.97 ±0.96 | 7.79 ±2.13 | 12.17 ±5.39 | 12.26 ±5.94 | 6.75 ±2.00 | 3.50 ±1.43 | 1.25 ±0.37 |

***Table 6.c OLT after indirect exposure***

*Table shows results from OLT test within animals indirectly exposed to MA. Parameters presented in this table are: frequency of exploring relocated object*

*during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), latency to original object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay), latency to relocated object in (s) during 1st trial, 2nd trial (15 min. delay) and 3rd trial (30 min. delay). Values are means ± SEM, n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| OLT INDIRECT | PARAMETERS (means ±SEM) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| GROUPS | Frequency-new object 1.min | Frequency-new object 15.min | Frequency-new object 30.min | Latency-old object 1.min | Latency-old object 15.min | Latency-old object 30.min | Latency-new object 1.min | Latency-new object 15.min | Latency-old object 30. min |
| SA/SC/GH | 6.63 ±1.50 | 5.88 ±3.13 | 3.63 ±2.29 | 5.04 ±2.47 | 0.56 ±0.24 | 6.63 ±4.76 | 9.64 ±5.16 | 11.22 ±3.73 | 5.66 ±3.74 |
| SA/SC/SH | 2.38 ±1.10 | 6.13 ±2.13 | 0.88 ±0.44 | 3.94 ±1.87 | 4.88 ±4.63 | 11.04 ±5.14 | 13.32 ±6.40 | 4.23 ±2.02 | 7.60 ±6.32 |
| SA/EE/GH | 4.13 ±1.86 | 2.88 ±1.91 | 2.25 ±1.97 | 2.47 ±1.66 | 10.90 ±6.60 | 3.41 ±2.50 | 16.63 ±6.99 | 5.19 ±2.85 | 13.13 ±7.71 |
| SA/EE/SH | 5.63 ±3.12 | 3.38 ±0.96 | 1.88 ±0.88 | 6.31 ±1.87 | 1.56 ±1.07 | 0.28 ±0.15 | 2.03 ±0.71 | 6.42 ±3.26 | 15.25 ±8.31 |
| MA/SC/GH | 4.75 ±1.97 | 5.50 ±1.52 | 1.00 ±0.63 | 1.88 ±0.71 | 8.33 ±2.83 | 5.68 ±3.46 | 11.71 ±5.67 | 7.53 ±2.27 | 10.26 ±7.15 |
| MA/SC/SH | 2.88 ±1.16 | 2.00 ±0.87 | 2.63 ±1.22 | 4.04 ±2.51 | 0.69 ±0.50 | 6.91 ±6.52 | 4.25 ±2.42 | 2.60 ±1.68 | 8.44 ±4.56 |
| MA/EE/GH | 4.88 ±1.34 | 2.50 ±0.98 | 1.75 ±0.92 | 5.23 ±2.69 | 12.81 ±4.66 | 16.16 ±7.38 | 13.15 ±5.57 | 8.74 ±4.85 | 11.08 ±3.82 |
| MA/EE/SH | 3.63 ±0.94 | 2.13 ±0.67 | 1.13 ±0.48 | 12.65 ±4.71 | 9.92 ±6.46 | 3.36 ±1.86 | 9.95 ±4.99 | 6.42 ±1.85 | 5.73 ±2.41 |

### 4.2.3 Morris Water Maze

**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. (*Fig.* 13 A).

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 (*Fig.* 13 B).

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) (*Fig.* 13 C).

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. (*Fig.* 14 A). 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. (*Fig.* 14 B). 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. (*Fig.* 14 C).

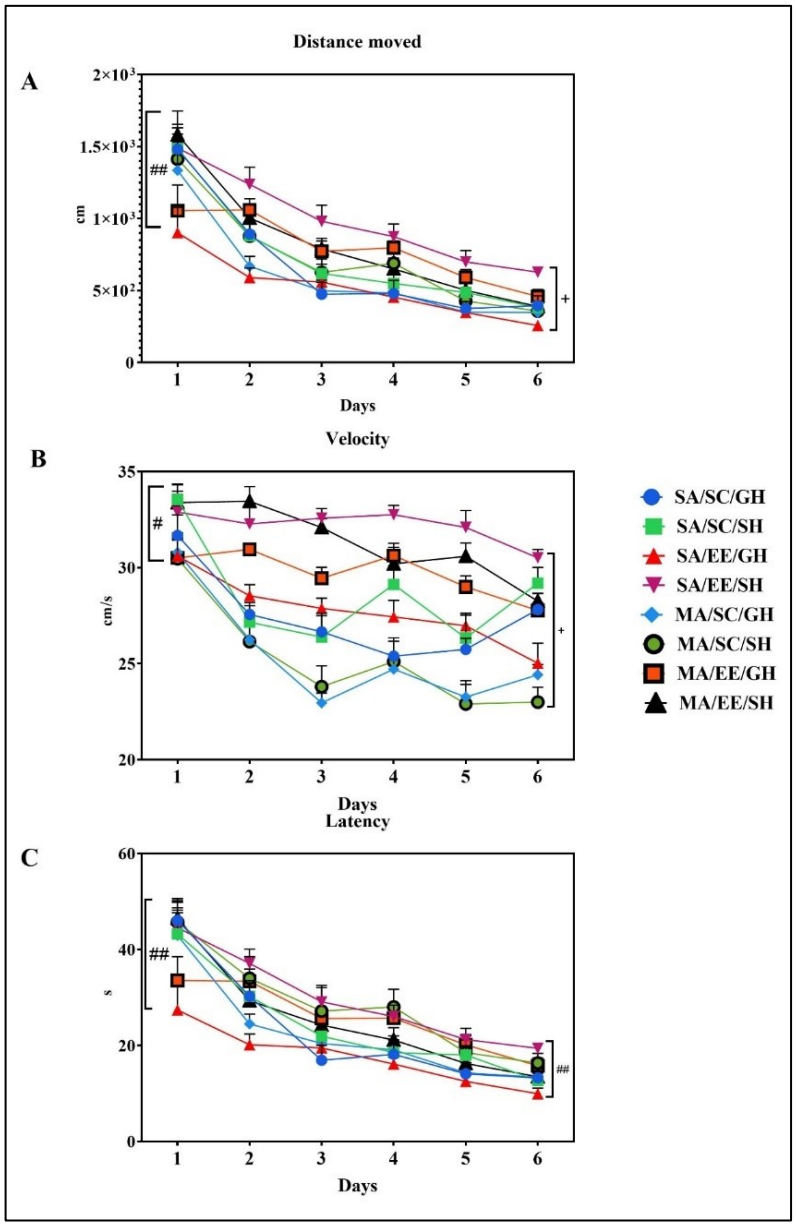


***Figure 13. Performance in learning test after direct exposure.***

*Fig. A shows distance moved of animals. Fig. B shows velocity of swimming and Fig. C shows latency to find a hidden platform. Values are ± SEM. n=8. # ;+ p<0.05, ## p<0. 01. # represents effect of preweaning housing, + represents effect of postweaning housing. Effect of treatment was not present in this case. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

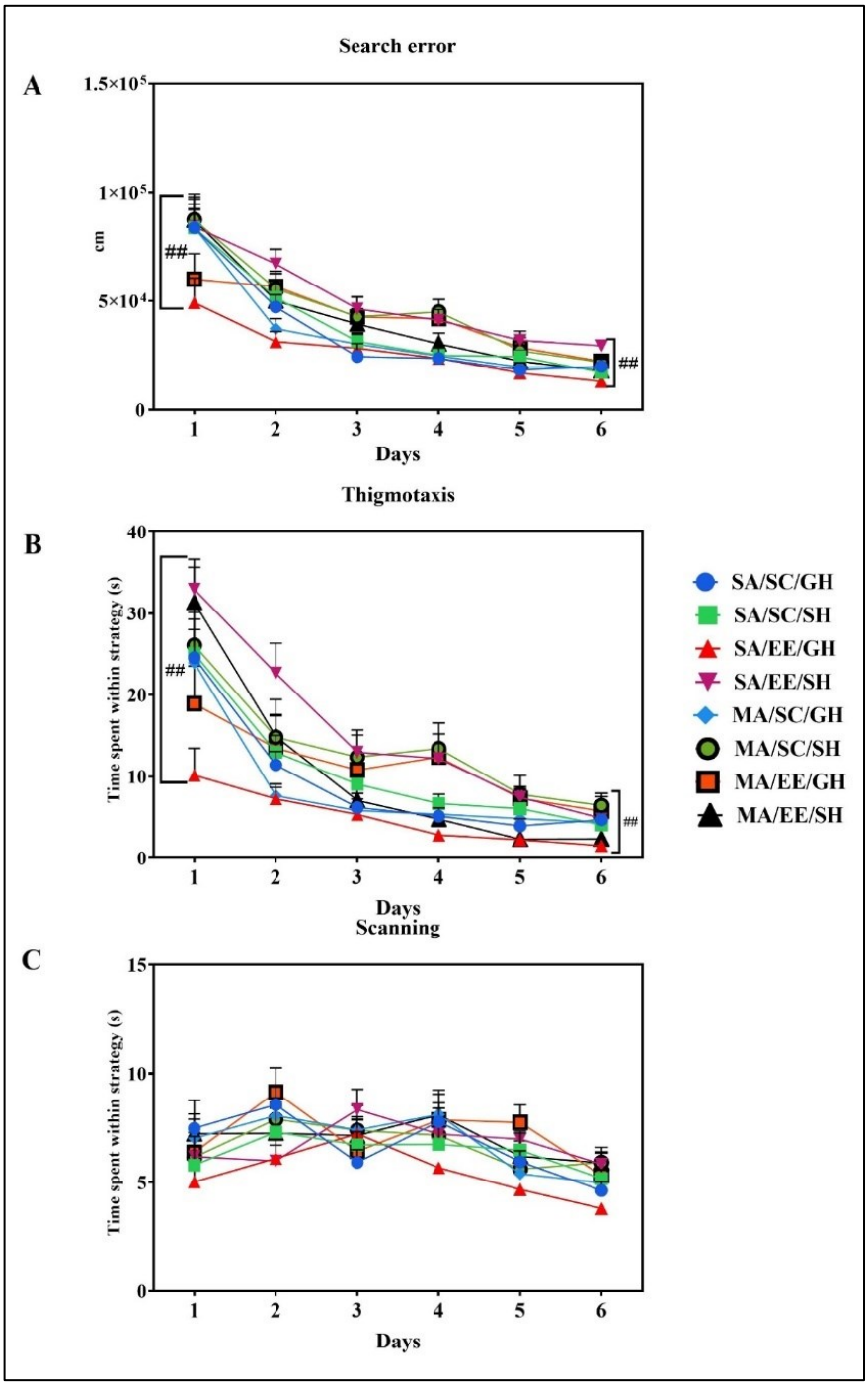


***Figure 14. Performance in learning test after direct exposure.***

*Fig. A shows search error of animals. Fig. B shows time spent within thigmotaxis and Fig. C shows time spent within scanning. Values are ± SEM. n=8. # p<0.05, ## p<0.01. # represents effect of preweaning housing, Effect of preweaning housing, nor effect of treatment were not present in this case. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

**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. *(*Figures *15 A, B, C).* Search error displayed higher tendency of EE exposed animals to have higher search error than standard animals during all days of learning [$F_{(1,56)} = 3.54$, $p=0.004$]. *(Figures 16 A).* This situation was similar in case of thigmotaxis and scanning. *(*Figures *16 B, C).*

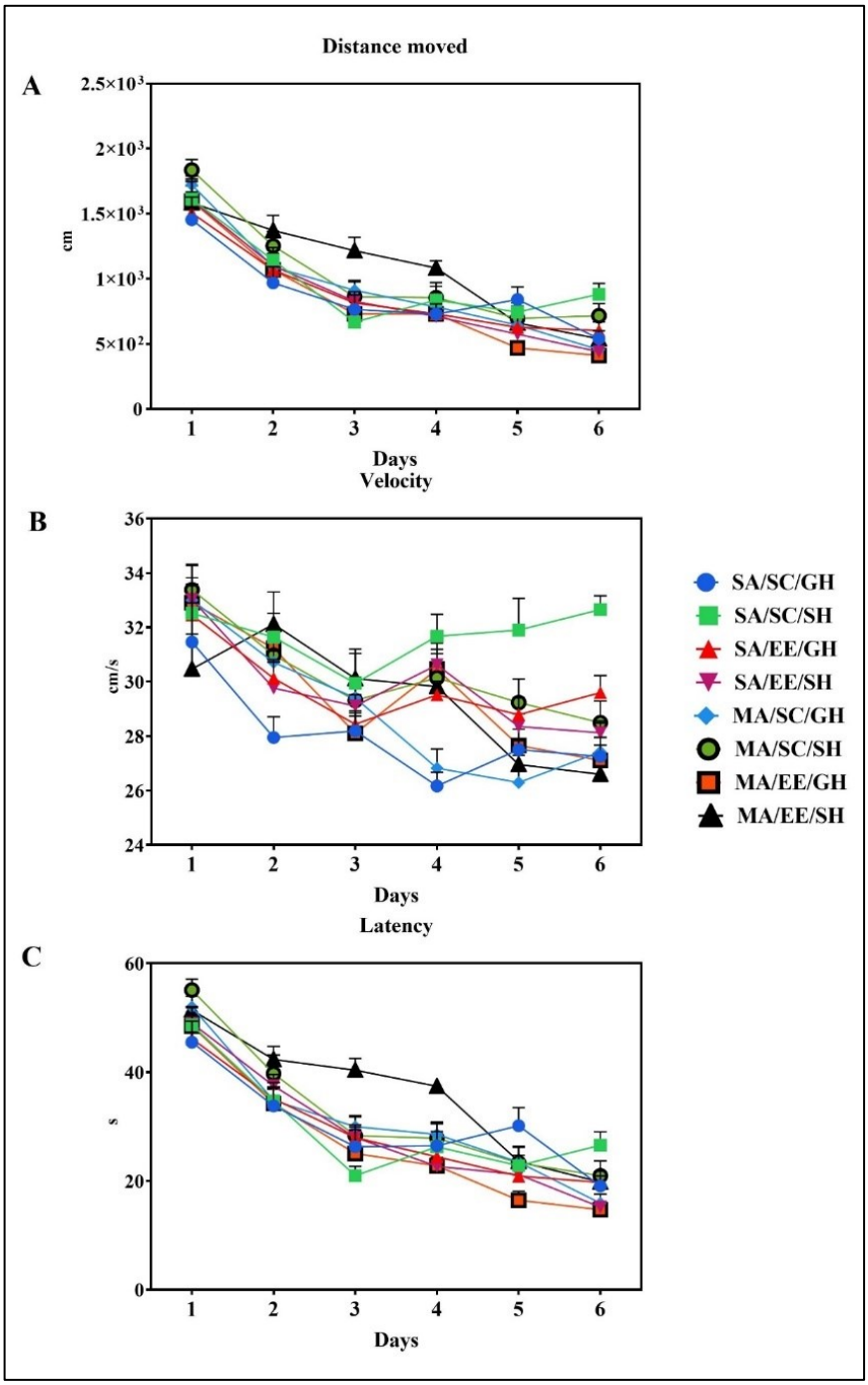


***Figure 15. Performance in learning test after indirect exposure.***
*Fig. A shows distance moved of animals. Fig. B shows velocity of swimming and Fig. C shows latency to find a hidden platform. Values are ± SEM. n=8. No significant impact of treatment, preweaning housing or postweaning housing was present in this case. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

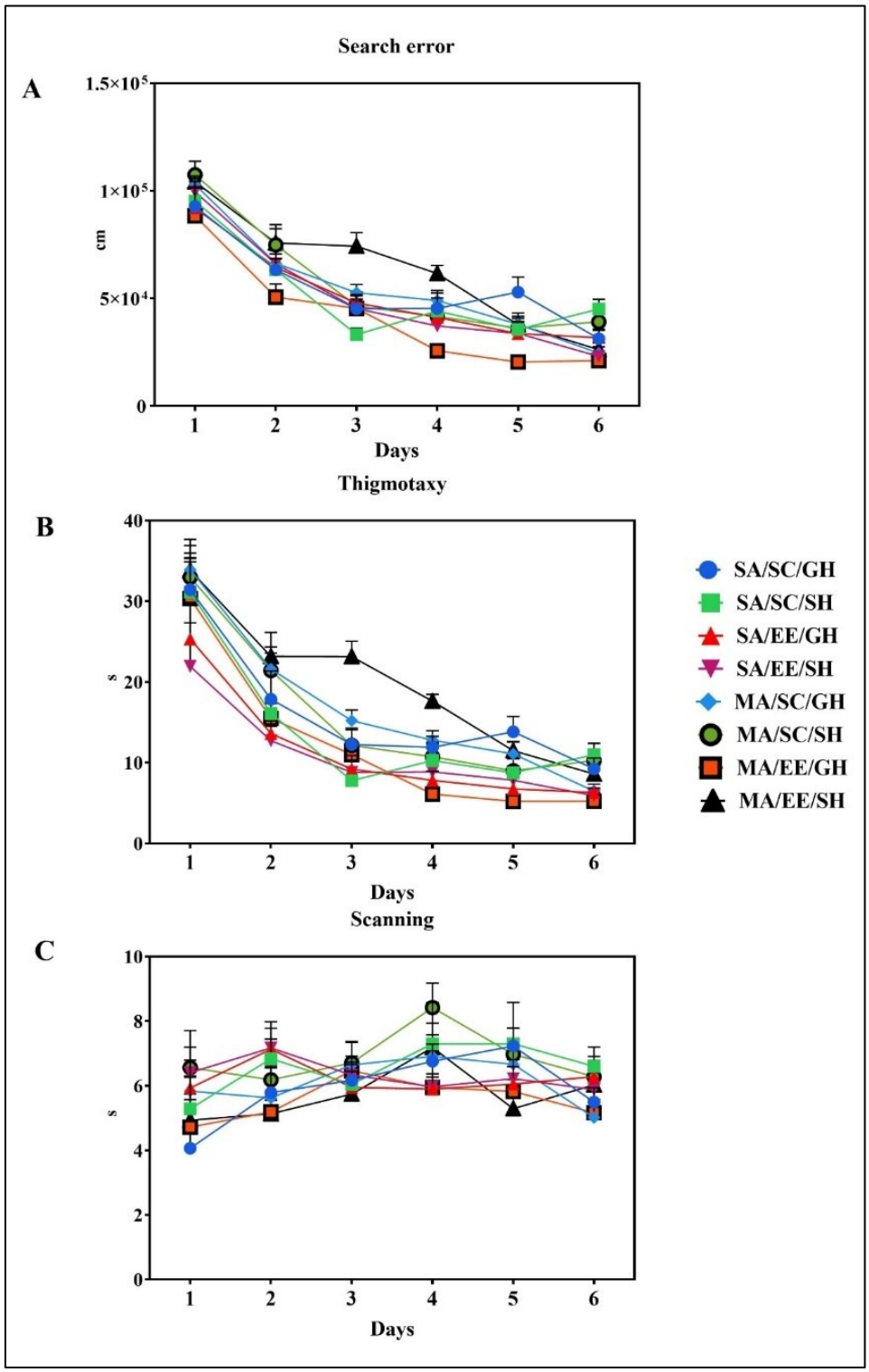


***Figure 16. Performance in learning test after indirect exposure.***

*Fig. A shows search error of animals. Fig. B shows time spent within thigmotaxis and Fig. C shows time spent within scanning. Values are ± SEM. n=8. No significant impact of treatment, preweaning housing or postweaning housing was present in this case. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised*

*in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

**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. (*Fig.* 17 A). 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. (*Fig.* 17 B). Latency was not significantly altered by any factor, (*Fig.* 17 C) neither search error, thigmotaxis, and scanning. (*Fig.* 18 A, B, C).

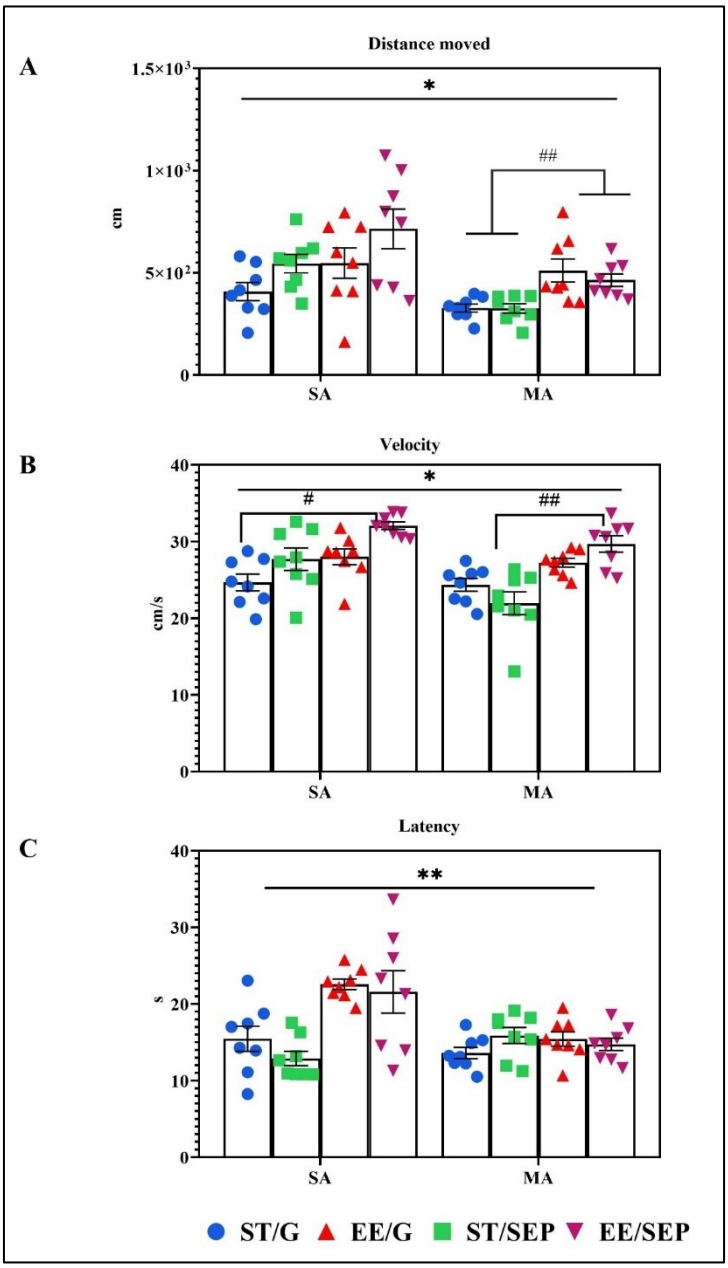


***Figure 17. Performance in memory retention test after direct exposure.***
*Fig. A shows distance moved of animals. Fig. B shows velocity of swimming and Fig. C shows latency to find a hidden platform. Values are ± SEM. n=8. #;∗p<0.05, ##; ∗∗ p<0.01. ∗ represents effect of treatment, # represents effect of preweaning housing. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

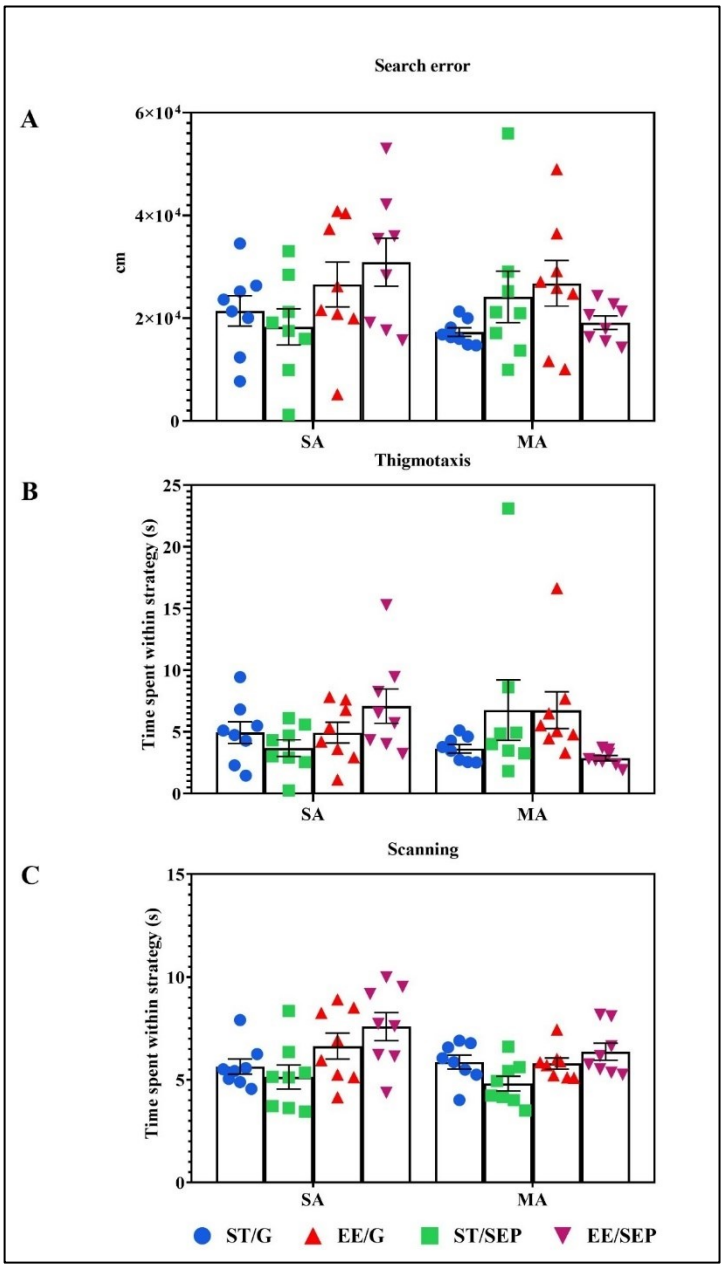


***Figure 18. Performance in memory retention test after direct exposure.***
*Fig. A shows search error of animals. Fig. B shows time spent within thigmotaxis and Fig. C shows time spent within scanning. Values are ± SEM. n=8. No significant impact of treatment, preweaning housing or postweaning housing was present in this case. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

**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. (*Fig.* 19 A, B, C)

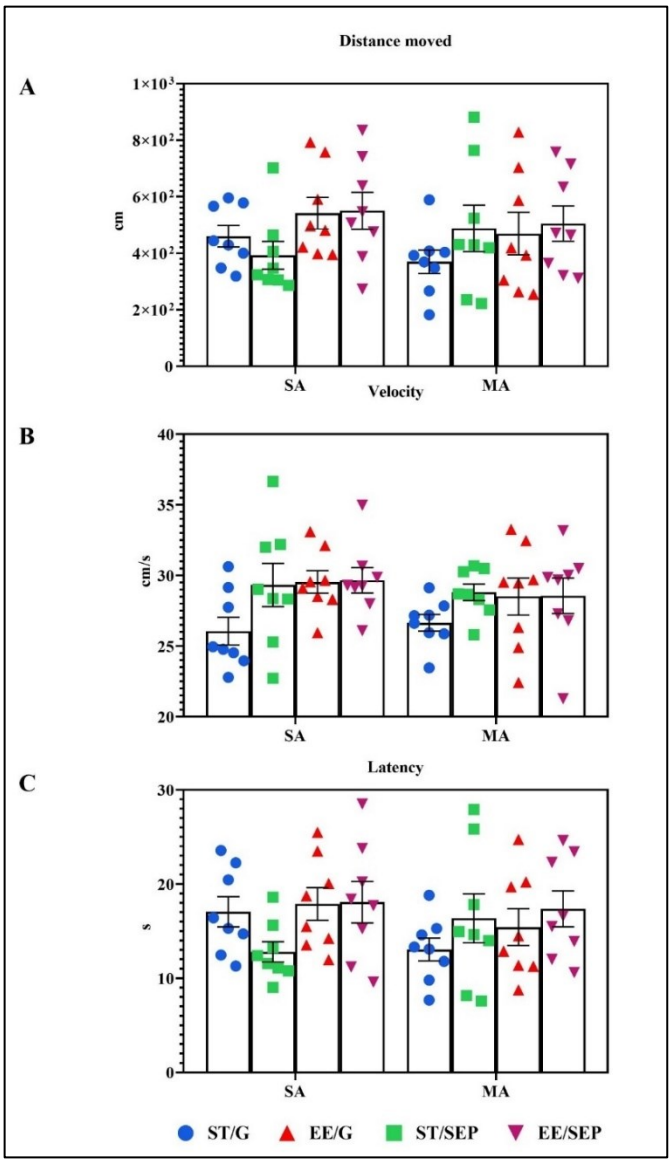


***Figure 19. Performance in memory retention test after indirect exposure.***
*Fig. A shows distance moved of animals. Fig. B shows velocity of swimming and Fig. C shows latency to find a hidden platform. Values are ± SEM. n=8. No*

*significant impact of treatment, preweaning housing or postweaning housing was present in this case. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

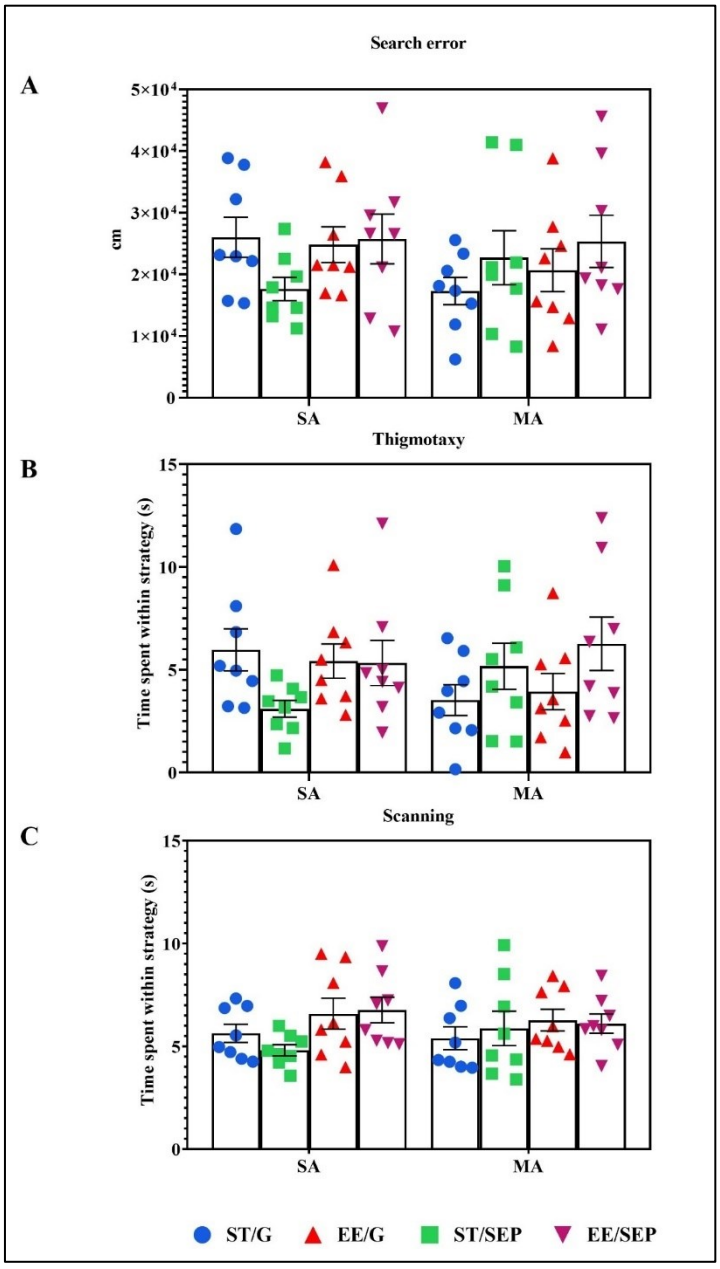


***Figure 20. Performance in memory retention test after indirect exposure.***
*Fig. A shows search error of animals. Fig. B shows time spent within thigmotaxis and Fig. C shows time spent within scanning. Values are ± SEM. n=8. No significant impact of treatment, preweaning housing or postweaning housing was present in*

*this case. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

## 4.3. Immunoanalyses

### 4.3.1 Neurotransmitters

#### 4.3.1.1 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. (*Fig.* 21 A) Differences in NA levels were not statistically significant. (*Fig.* 21 B). 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]. (*Fig.* 24 B).

**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. (*Fig.* 22 A). 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]. (*Fig.* 21 B)

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). (*Fig.* 25 B).

**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. (*Fig.* 23 A) 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). (*Fig.* 23 B)

Within indirectly exposed animals we obtained significant differences in levels of DA and NA as well. (*Fig.* 26 A, B). 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).

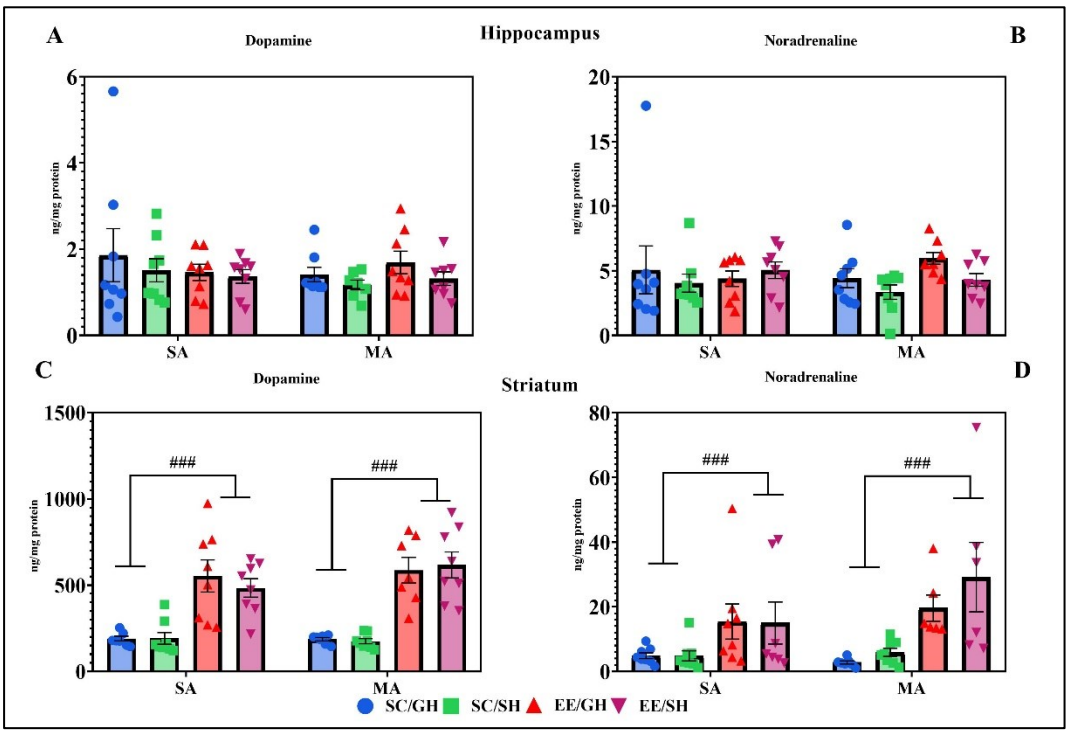

***Figure 21. Levels of catecholamines in hippocampus and striatum on PD 28 after direct exposure.*** *Levels DA or NA are expressed in ng of catecholamine/mg of total protein. Values are ± SEM. n=8 # p<0.05, ## p<0.01, ### p<0.001, # represents effect of preweaning housing.*
*SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

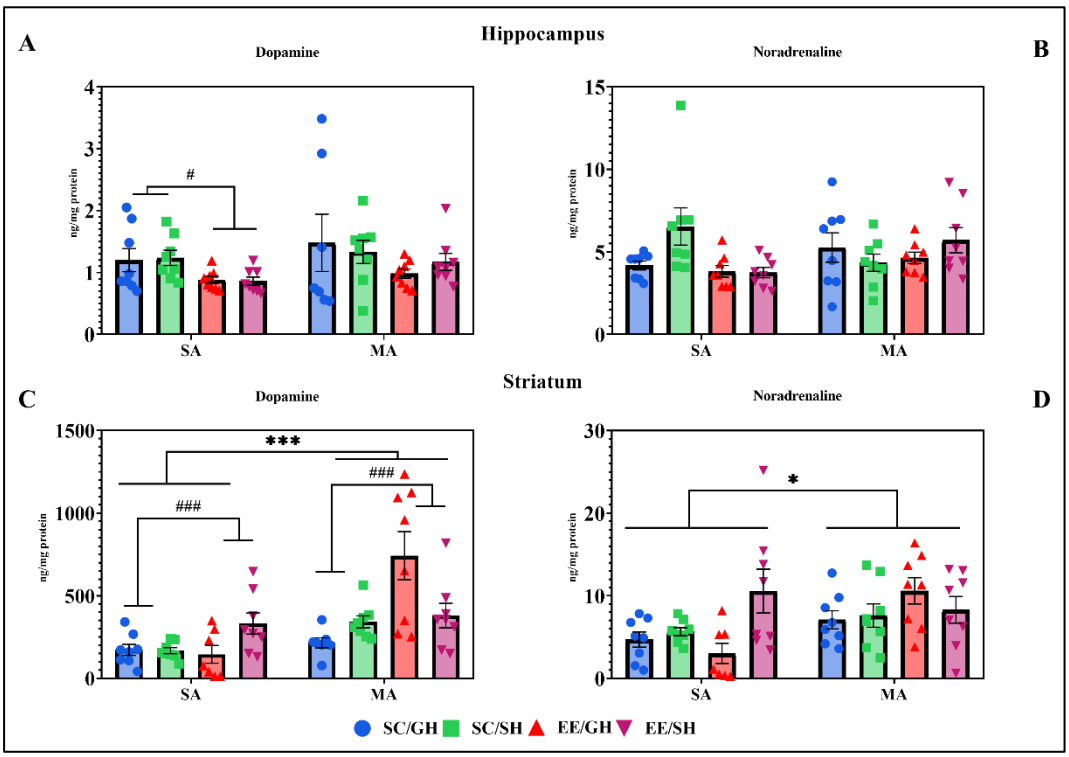


***Figure 22. Levels of catecholamines in hippocampus and striatum on PD 35 after direct exposure.*** *Levels DA or NA are expressed in ng of catecholamine/mg of total protein. Values are ± SEM. n=8. *,# p<0.05, ***, ### p<0.001, ∗ represents effect of treatment, # represents effect of preweaningg housing.*
*SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

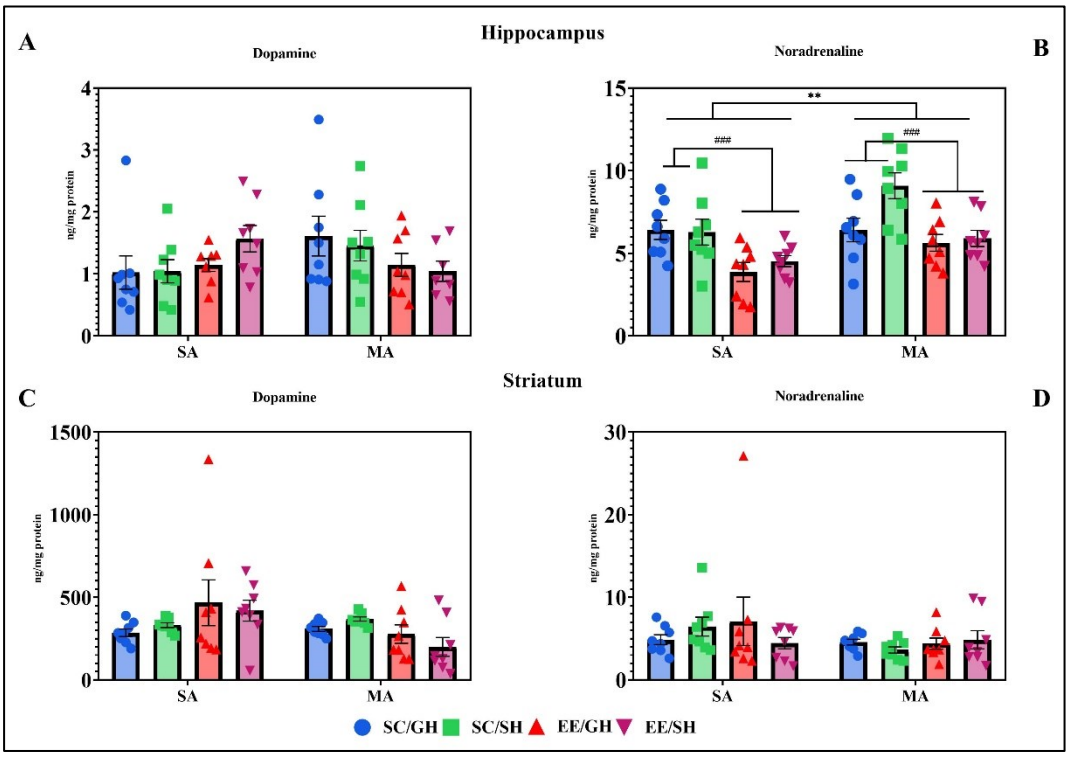


***Figure 23. Levels of catecholamines in hippocampus and striatum on PD 45 after direct exposure.*** *Levels DA or NA are expressed in ng of catecholamine/mg of total protein. Values are ± SEM. n=8. ** p<0.01,### p<0.001, ∗ represents effect of treatment, # represents effect of preweaning housing.*
*SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

## Striatum

### PD 28

Preweaning housing had significant influence on DA levels in striatum [$F_{(1, 54)}$ = 91.05, p=0.0001]. (*Fig.* 21 C). 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). (*Fig.* 21 D).

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). (*Fig.* 21 D).

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). (*Fig.* 24 C,D).

**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). (*Fig.* 22 C).

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). (*Fig.* 22 D).

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). (*Fig.* 25 C, D).

**PD 45**

Within directly as well as indirectly exposed animals, no significant differences were obtained in levels of DA and NA. (*Fig.* 26 C, D).

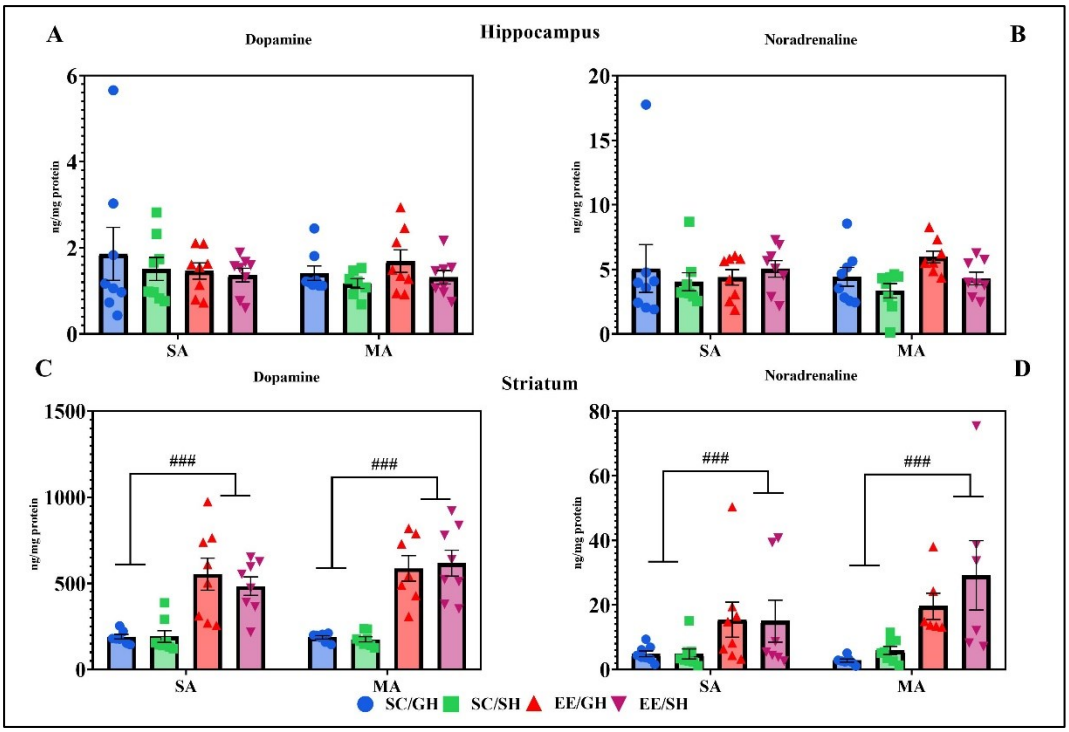


***Figure 24. Levels of catecholamines in hippocampus and striatum on PD 28 after indirect exposure****. Levels DA or NA are expressed in ng of catecholamine/mg of total protein. Values are ± SEM. n=8. ### p<0.001, # represents effect of preweaning housing.*
*SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

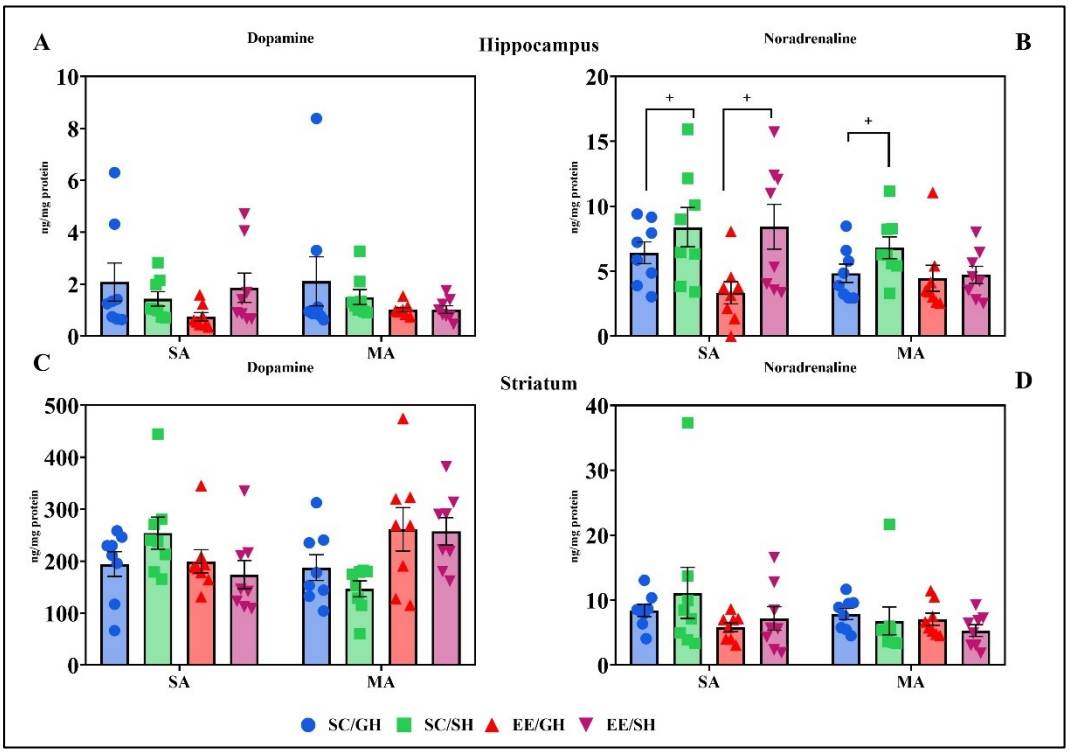


***Figure 25. Levels of catecholamines in hippocampus and striatum on PD 35 after indirect exposure.*** *Levels DA or NA are expressed in ng of catecholamine/mg of total protein. Values are ± SEM. n=8. + p<0.05. + represents effect of postweaning housing.*

*SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

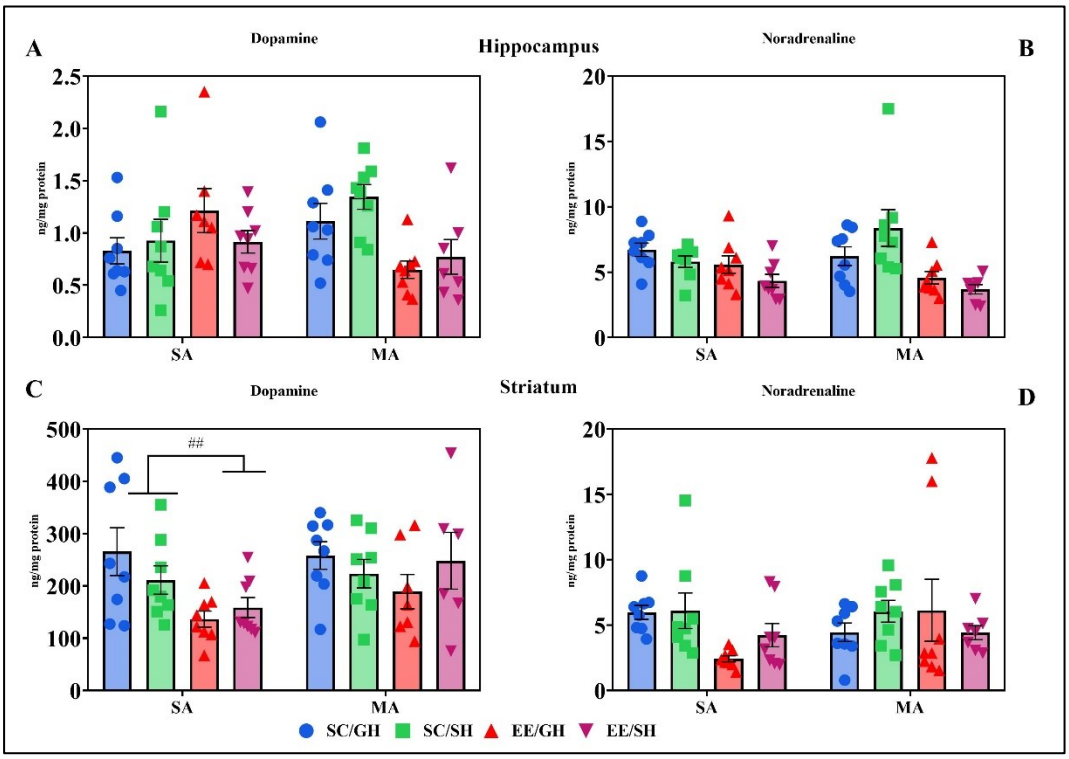


***Figure 26. Levels of catecholamines in hippocampus and striatum on PD 45 after indirect exposure.*** *Levels DA or NA are expressed in ng of catecholamine/mg of total protein. Values are ± SEM. n=8. ##p<0.01. # represents effect of preweaning housing.*
*SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

### 4.3.1.2 Glutamate

**Hippocampus**

**PD 28**

Levels of GLU in HP after direct exposure were significantly influenced by preweaning housing [$F_{(1, 56)} = 8.183$, p=0.0068]. SA/SC/SH animals had significantly higher level of GLU in comparison with SA/EE/GH (p=0.0213) and MA/EE/GH (p=0.0314) *(Table 11).*

There were not any significant differences in GLU levels of indirectly exposed animals on PD 28 *(Table 12).*

**PD 35**

Treatment [$F_{(1, 56)} = 9.328$, p=0.0063] as well as preweaning housing [$F_{(1, 56)} = 8.122$, p=0.0099] had significant impact on levels of GLU in HP. SA/EE/GH had significantly higher levels in comparison with MA/SC/SH (p=0.0041) and MA/EE/GH (p=0.0398) *(Table 13).*

There were not any significant differences in GLU levels of indirectly exposed animals on PD 35 *(Table 14).*

**PD 45**

Treatment significantly impacted levels of GLU in HP on PD 45 [$F_{(1, 56)} = 6.957$, p=0.0139]. MA/SC/SH showed significantly higher levels in comparison with SA/EE/GH (p=0.027) *(Table 15).*

There were not any significant differences in GLU levels of indirectly exposed animals on PD 45 *(Table 16).*

**Striatum**

**PD 28**

Treatment [$F_{(1, 56)} = 4.501$, p=0.0436] and preweaning housing [$F_{(1, 56)} = 4.493$, p=0.0437]. MA/EE/GH animals had significantly higher levels of GLU in comparison with SA/SC/SH (p=0.0349) and MA/SC/SH (p=0.0359) *(Table 11).*

There were not any significant differences in GLU levels of indirectly exposed animals on PD 28 *(Table 12).*

**PD 35**

There were not any significant differences in GLU levels of directly exposed animals on PD 35 *(Table 13).*

In terms of indirect exposure, interaction between treatment and preweaning housing significantly impacted levels of GLU [$F_{(1, 56)} = 3.551$, p=0.0566]. MA/EE/GH animals had significantly higher levels of GLU in comparison with SA/EE/GH (p=0.338) *(Table 14).*

**PD 45**

There were not any significant differences in GLU levels of directly as well as indirectly exposed animals on PD 45 *(Table 15 and 16).*

#### 4.3.1.3 Serotonin

**Hippocampus**

**PD 28**

Regarding 5HT on PD 28 after direct exposure, factors of significance were treatment [$F_{(1, 56)} = 22.48$; $p = 0.0001$], preweaning housing [$F_{(1, 56)} = 9.638$; $p = 0.030$], and interaction between all three factors [$F_{(1, 56)} = 14.34$; $p = 0.0004$]. We observed significantly lower levels of 5HT in the MA/SC compared to the SA/SC ($p = 0.0097$). Exposure to EE alone did not affect basal 5HT levels in SA treated group but enhanced the partially muted 5HT levels in SA treated separated animals ($p = 0.0398$). Low 5HT levels in MA-treated animals were also significantly boosted by the EE ($p = 0.0263$), but 5HT levels in the MA/EE/GH separated rats remained low, without any EE effects. The difference between MA/EE/SH and SA/EE/SH was also significant (0.0037) *(Table 11).*

In terms of indirect exposure, no significant differences were observed *(Table 12).*

**PD 35**

Regarding 5HT levels on PD 35 after direct exposure, the main significant effect was housing [$F_{(1, 56)} = 12.80$; $p = 0.0007$]; there was also interaction between treatment and housing [$F_{(1, 56)} = 18.10$; $p = 0.0001$] and an interaction between all three factors [ $F_{(1, 56)} = 9.167$; $p = 0.0095$]. We observed significantly higher levels of 5HT in the SA/EE/SH compared to the MA/EE/SH ($p = 0.0055$) and in the MA/EE/GH compared to the MA/EE/SH group ($p = 0.0011$) *(Table 13).*

In terms of indirect exposure, the only effect of significance was an interaction between preweaning housing and treatment [$F_{(1, 56)} = 8.180$; $p = 0.0094$]; we observed significantly higher levels of 5HT in the SA/EE/GH compared to the SA SC ($p = 0.014$) *(Table 14).*

**PD 45**

Regarding 5HT levels on PD 45, after direct exposure, factors of significance were preweaning housing [$F_{(1, 56)} = 32.42$; $p = 0.0001$], housing [$F_{(1,56)} = 4.795$; p=0,0327] as well as interaction between preweaning housing and housing [$F_{(1, 56)} = 6,638$; $p = 0.0126$]. We observed significantly higher levels of 5HT in the

SA/SC/SH compared to the SA/EE/SH group (p = 0.0025) and in the MA/SC/SH compared to the MA/EE/SH (p = 0.0059) *(Table 15).*

In terms of indirect exposure, the significant factors of significance were preweaning housing [ $F_{(1, 56)} = 7{,}539$; p = 0.0081] as well as an interaction between preweaning housing and housing [$F_{(1, 56)} = 9{,}056$; p = 0.0039]. We observed a significantly higher level of 5HT in the SA/SC/SH compared to the SA/ EE/SH (p = 0.0082) *(Table 16).*

***Table 11. Levels of glutamate and serotonin on PD 28 after direct exposure***

*The table shows glutamate and serotonin levels in the hippocampus and striatum on PD 28 after direct exposure. Levels are expressed as ng GLU or 5HT/mg protein. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **IMUNOANALYSES OF NEUROTRANSMITTERS** | | | | |
|---|---|---|---|---|
| **Direct** | **Concentrations ng/mg protein (means ± SEM)** | | | |
| **PD 28** | **Hippocampus** | | **Striatum** | |
| **Groups** | **Glutamate** | **Serotonin** | **Glutamate** | **Serotonin** |
| **SA/SC/GH** | 32.02±4.84 | 30.30±7.45 | 29.23±5.29 | 10.34±1.44 |
| **SA/SC/SH** | 64.53±11.93 | 18.18±5.15 | 35.49±6.91 | 3.61±0.80 |
| **SA/EE/GH** | 24.62±5.89 | 27.39±3.20 | 67.92±29.01 | 5.53±0.93 |
| **SA/EE/SH** | 18.27±6.61 | 37.57±6.93 | 109.78±30.44 | 4.32±0.55 |
| **MA/SC/GH** | 51.65. ±7.61 | 8.61±1.36 | 33.81±2.25 | 6.89±0.91 |
| **MA/SC/SH** | 50.26±6.73 | 26.97±4.16 | 37.46±3.77 | 5.81±0.50 |
| **MA/EE/GH** | 24.62±5.89 | 10.06±1.21 | 68.71±12.15 | 6.07±0.66 |
| **MA/EE/SH** | 39.17±19.38 | 12.33±4.22 | 96.37±31.12 | 6.15±0.74 |

***Table 12. Levels of glutamate and serotonin on PD 28 after indirect exposure.*** *The table shows glutamate and serotonin levels in the hippocampus and striatum on PD 28 after indirect exposure. Levels are expressed as ng GLU or 5HT/mg protein. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **Indirect** | **Concentrations ng/mg protein (means ± SEM)** | | | |
|---|---|---|---|---|
| **PD 28** | **Hippocampus** | | **Striatum** | |
| **Groups** | **Glutamate** | **Serotonin** | **Glutamate** | **Serotonin** |
| **SA/SC/GH** | 51.98±4.65 | 12.21±1.30 | 44.18±1.95 | 7.96±0.89 |
| **SA/SC/SH** | 61.65±4.25 | 19.02±2,63 | 47.66±1.86 | 11.55±1.69 |
| **SA/EE/GH** | 48.60±8.91 | 20.35±1.85 | 24.80±5.91 | 14.22±5.01 |
| **SA/EE/SH** | 97.85±41.71 | 13.19±3.44 | 46.68±7.47 | 21.67±5.98 |
| **MA/SC/GH** | 59.79±4.13 | 17.18±5.20 | 48.91±1.90 | 9.74±2.20 |
| **MA/SC/SH** | 39.49±5.89 | 16.90±3.10 | 37.59±3.00 | 9.25±1.41 |
| **MA/EE/GH** | 76.85±33.03 | 18.79±3.91 | 56.23±26.80 | 14.43±2.90 |
| **MA/EE/SH** | 84.03±45.43 | 22.05±3.84 | 46.32±9.16 | 10.39±2.19 |

***Table 13. Levels of glutamate and serotonin on PD 35 after direct exposure.***
*The table shows glutamate and serotonin levels in the hippocampus and striatum on PD 35 after direct exposure. Levels are expressed as ng GLU or 5HT/mg protein. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **IMMUNOANALYSES OF NEUROTRANSMITTERS** | | | | |
|---|---|---|---|---|
| **Direct** | **Concentrations ng/mg protein (means ± SEM)** | | | |
| **PD 35** | **Hippocampus** | | **Striatum** | |
| **Groups** | **Glutamate** | **Serotonin** | **Glutamate** | **Serotonin** |
| **SA/SC/GH** | 47.413±14.24 | 6.07±0.91 | 28.17±2.73 | 12.06±2.80 |
| **SA/SC/SH** | 108.62±26.60 | 4.61±0.91 | 21.67±4.30 | 9.53±0.98 |
| **SA/EE/GH** | 38.94±8.11 | 3.54±0.65 | 20.71±6.94 | 14.29±2.99 |
| **SA/EE/SH** | 67.93±8.08 | 5.81±1.10 | 20.81±4.07 | 9.67±1.70 |
| **MA/SC/GH** | 89.33±19.17 | 6.62±1.05 | 37.02±2.31 | 6.84±0.83 |
| **MA/SC/SH** | 79.97±8.45 | 3.55±0.60 | 19.62±1.50 | 15.09±2.53 |
| **MA/EE/GH** | 67.93±8.08 | 6.87±0.92 | 30.47±6.67 | 18.26±4.83 |
| **MA/EE/SH** | 90.13±21.05 | 1.61±0.22 | 27.06±4.60 | 13.62±2.41 |

***Table 14. Levels of glutamate and serotonin on PD 35 after indirect exposure.*** *The table shows glutamate and serotonin levels in the hippocampus and striatum on PD 35 after indirect exposure. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **Indirect** | **Concentrations ng/mg protein (means ± SEM)** | | | |
|---|---|---|---|---|
| **PD 35** | **Hippocampus** | | **Striatum** | |
| **Groups** | **Glutamate** | **Serotonin** | **Glutamate** | **Serotonin** |
| **SA/SC/GH** | 64.14±22.76 | 3.59±0.56 | 40.70±2.22 | 10.08±1.92 |
| **SA/SC/SH** | 59.96±24.77 | 5.69±0.84 | 36.96±2.56 | 9.76±1.29 |
| **SA/EE/GH** | 71.85±13.94 | 7.54±0.40 | 34.35±1.86 | 6.94±0.79 |
| **SA/EE/SH** | 127.87±32.81 | 6.16±1.24 | 36.35±2.68 | 7.06±0.95 |
| **MA/SC/GH** | 53.54±28.97 | 5.98±0.82 | 27.13±6.27 | 9.70±0.96 |
| **MA/SC/SH** | 44.73±22.59 | 6.62±0.55 | 36.96±2.56 | 8.32±1.45 |
| **MA/EE/GH** | 107.88±16.32 | 5.75±1.05 | 29.28±3.47 | 4.67±1.25 |
| **MA/EE/SH** | 163.07±57.35 | 5.08±1.03 | 28.26±5.29 | 13.33±3.63 |

***Table 15. Levels of glutamate and serotonin on PD 45 after direct exposure.***
*The table shows glutamate and serotonin levels in the hippocampus and striatum on PD 45 after direct exposure. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **IMMUNOANALYSES OF NEUROTRANSMITTERS** | | | | |
|---|---|---|---|---|
| **Direct** | **Concentrations ng/mg protein (means ± SEM)** | | | |
| **PD 45** | **Hippocampus** | | **Striatum** | |
| **Groups** | **Glutamate** | **Serotonin** | **Glutamate** | **Serotonin** |
| **SA/SC/GH** | 76.45±19.91 | 14.30±2.46 | 42.76±2.76 | 3.65±1.69 |
| **SA/SC/SH** | 61.31±26.50 | 19.30±2.99 | 41.25±1.78 | 3.10±0.90 |
| **SA/EE/GH** | 65.23±8.21 | 7.11±1.02 | 40.95±6.27 | 3.25±0.49 |
| **SA/EE/SH** | 89.94±18.80 | 9.15±0.97 | 56.06±9.94 | 2.41±0.23 |
| **MA/SC/GH** | 70.59±12.12 | 10.48±1.46 | 37.93±6.09 | 3.92±0.97 |
| **MA/SC/SH** | 55.50±3.66 | 16.59±1.85 | 43.19±3.24 | 2.64±0.51 |
| **MA/EE/GH** | 42.68±2.80 | 10.36±0.98 | 44.86±8.19 | 1.85±0.80 |
| **MA/EE/SH** | 71.01±8.35 | 9.15±0.97 | 31.66±5.38 | 1.99±0.18 |

***Table 16. Levels of glutamate and serotonin on PD 45 after indirect exposure.*** *The table shows glutamate and serotonin levels in the hippocampus and striatum on PD 45 after indirect exposure. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **Indirect** | **Concentrations ng/mg protein (means ± SEM)** | | | |
|---|---|---|---|---|
| **PD 45** | **Hippocampus** | | **Striatum** | |
| **Groups** | **Glutamate** | **Serotonin** | **Glutamate** | **Serotonin** |
| **SA/SC/GH** | 7.98±4.34 | 11.46±1.32 | 47.51±2.16 | 1.08±0.35 |
| **SA/SC/SH** | 38.71±10.06 | 17.71±2.29 | 44.62±4.35 | 3.22±0.80 |
| **SA/EE/GH** | 27.71±5.87 | 12.49±1.11 | 42.01±5.75 | 3.54±0.52 |
| **SA/EE/SH** | 19.96±4.24 | 10.14±0.84 | 38.88±8.23 | 4.48±0.86 |
| **MA/SC/GH** | 45.62±3.45 | 12.95±2.14 | 46.91±3.73 | 5.58±0.90 |
| **MA/SC/SH** | 52.56±4.81 | 12.68±1.21 | 44.32±4.67 | 5.26±0.83 |
| **MA/EE/GH** | 61.60±10.78 | 12.39±0.48 | 46.94±9.66 | 8.02±0.41 |
| **MA/EE/SH** | 19.96±4.24 | 15.42±1.29 | 22.89±5.86 | 6.56±0.61 |

### 4.3.1.4 Neurotrophins

**BDNF**

**Direct exposure**

On PD 28 there were no significant differences between groups *(Fig. 27 A).* 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) *(Fig. 27 B)***.** 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) *(Fig. 27 C)*.

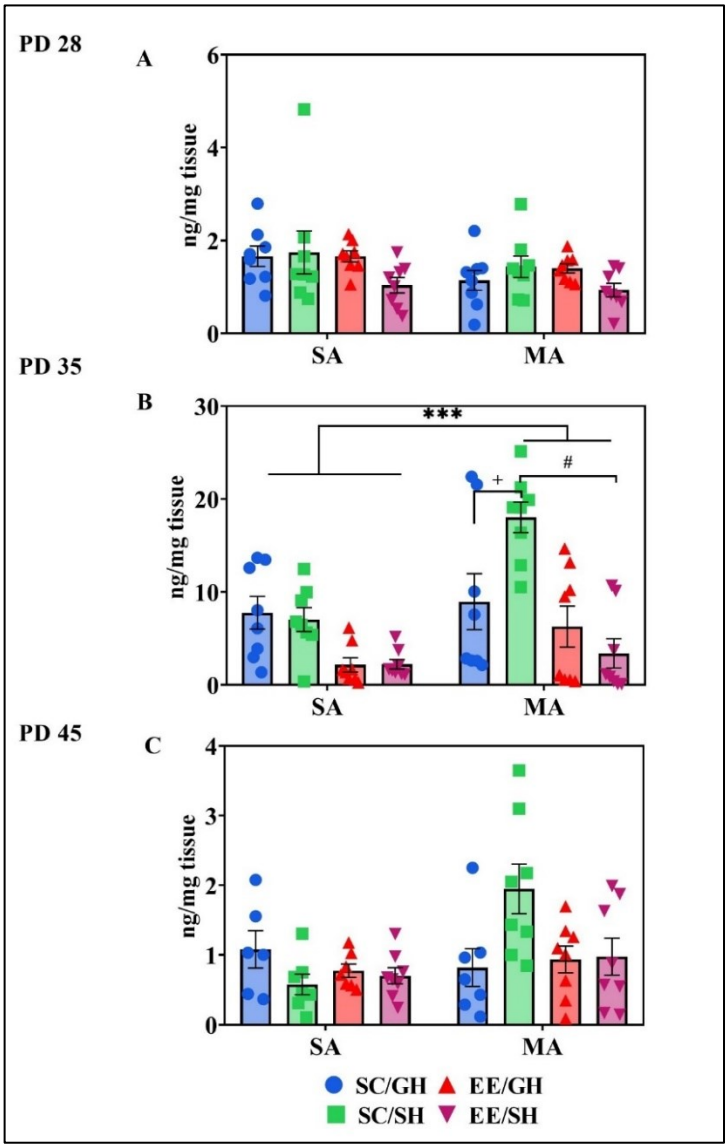


***Figure 27. Levels of BDNF in hippocampus after direct exposure.*** *Levels BDNF are expressed in ng BDNF /mg tissue. Values are ± SEM. n=8. #, + p<0.05, *** p<0.001. + represents effect of preweaning housing , * represents effect of treatment, # represents effect of postweaning housing.*
*SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

**Indirect exposure**

According to statistical analyses, our results shows, that indirect exposure of MA did not eminently altered BDNF levels and we did not obtain significant differences *(Fig. 28).*

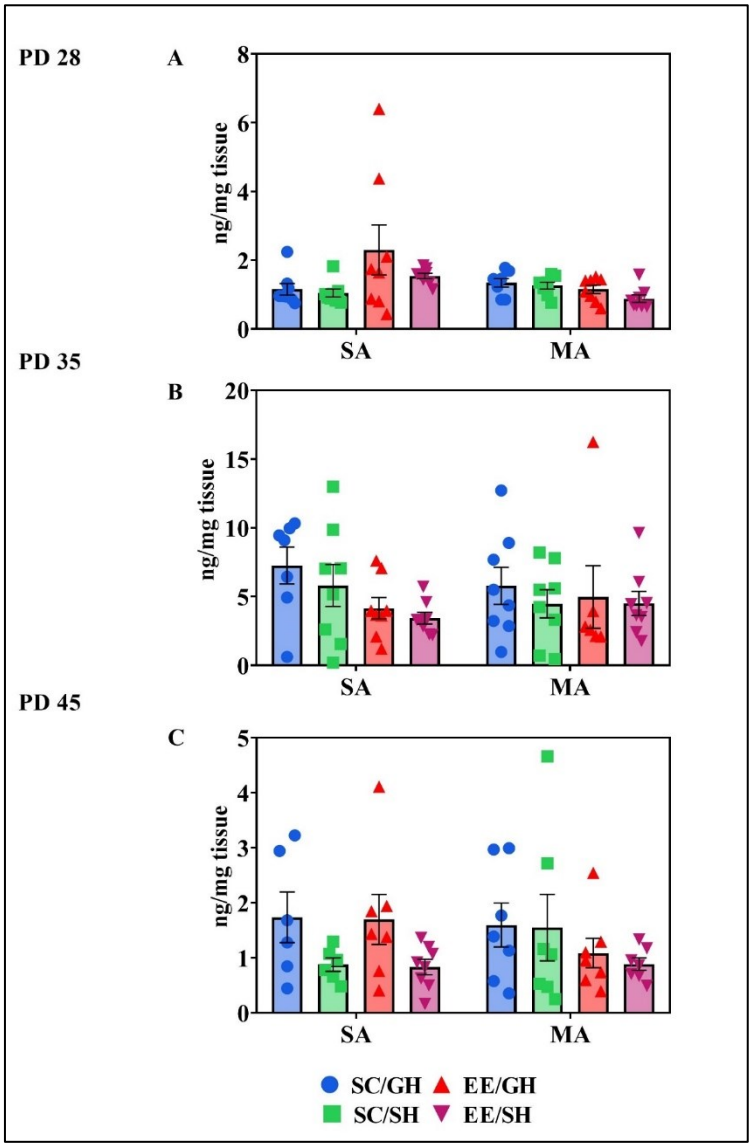


***Figure 28. Levels of BDNF in hippocampus after indirect exposure.*** *Levels BDNF are expressed in ng BDNF /mg tissue Values are ± SEM. n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

## NGF

### Direct exposure

On PD 28 after direct exposure, there were no significant differences between groups *(Fig. 20 B)*. 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 *(Fig. 20 D)*. 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) *(Fig. 20 F)*.

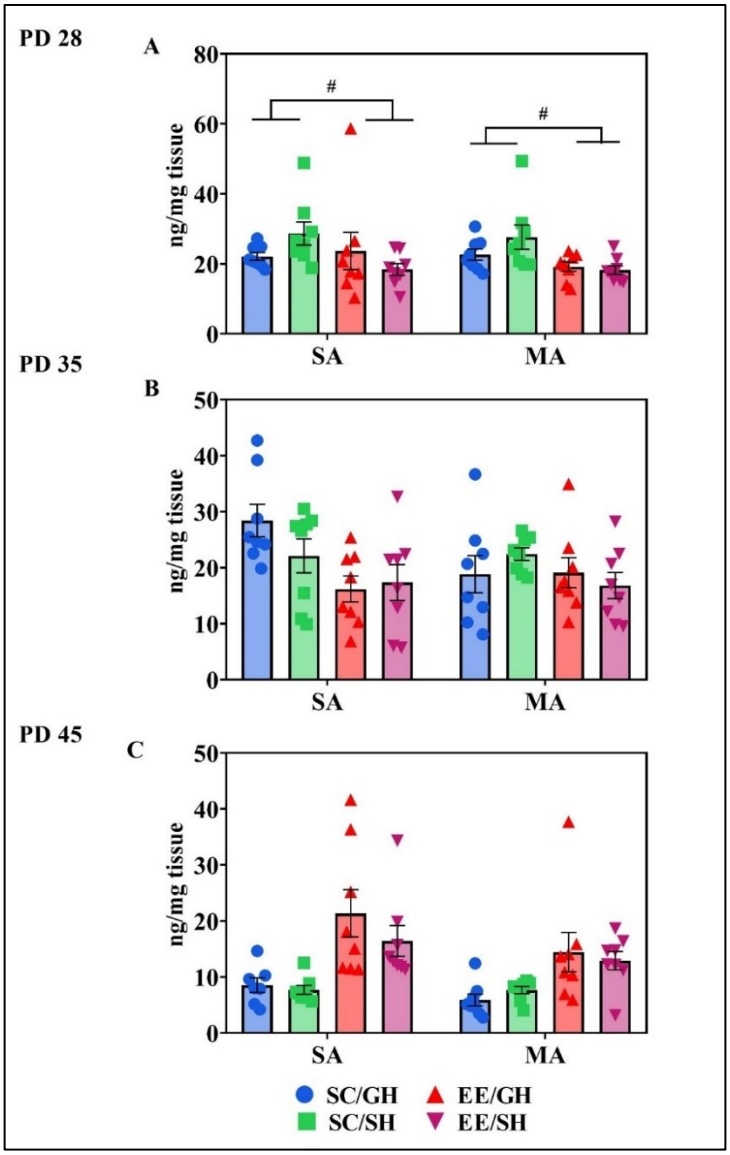


***Figure 29. Levels of NGF in hippocampus after direct exposure.***
*Levels NGF are expressed in ng NGF /mg tissue. Values are ± SEM. n=8–10. # p<0.05, # represents effect of preweaning housing.*

*SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

**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) *(Fig. 21 B).* 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) *(Fig. 21 D).* On PD 45, there were none significant differences *(Fig. 21 F).*

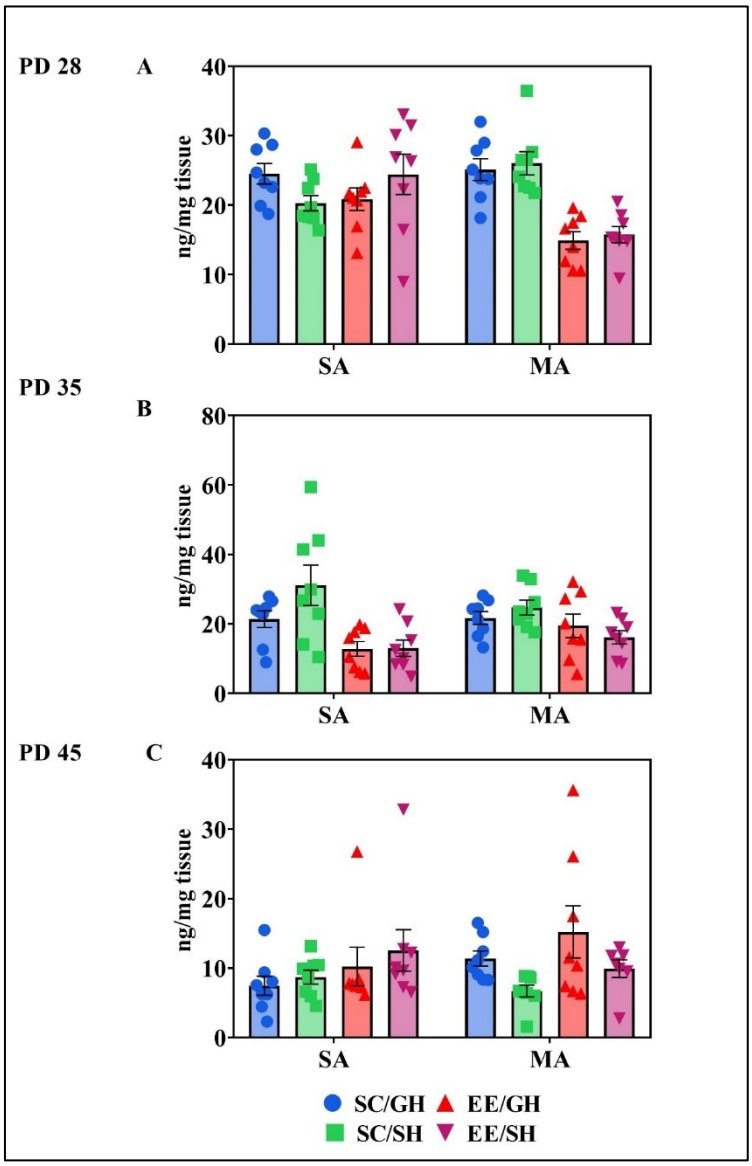


***Figure 30. Levels of NGF in hippocampus after indirect exposure.*** *Levels NGF are expressed in ng NGF /mg tissue. Values are ± SEM. n=8. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

### 4.3.2 c-fos in hippocampus

**PD 28**

In terms of direct exposure, factor of significance was treatment [$F_{(1, 56)} = 5.459$, p=0.0281] and activation of c- fos was higher in SA/SC/GH than MA/SC/GH (p=0.0060). In terms of indirect exposure, factor of significance was preweaning

housing [$F_{(1, 56)} = 4.510$, p=0.0442] and activation of c-fos was lower in MA/SC/SH than MA/EE/SH (p=0.0270). (Table 17).

**PD 35**

Within direct exposure as well as indirect exposure there were not any significant differences between groups. (Table 17).

**PD 45**

Within direct exposure, interaction between all factor was significant [$F_{(1, 56)} = 8.441$, p=0.0082] and activation of c-fos was higher in SA/SC/SH than MA/SC/SH (p=0.0097) and SA/SC/GH (p=0.0275). Also, this activation was lower in SA/SC/GH than SA/EE/GH (p=0.0075) and in MA/SC/SH than MA/EE/SH (p=0.0407). In terms of indirect exposure, treatment [$F_{(1, 56)} = 5.735$, p=0.0248] and interaction between preweaning and postweaning housing were significant [$F_{(1, 56)} = 6.016$, p=0.0218]. Activation of c-fos was lower in SA/SC/GH than MA/SC/GH (p=0.0402). (Table 17).

***Table 17. c-FOS***

*SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

| **c-FOS in hippocampus OD (means ± SEM)** | | | | | | |
|---|---|---|---|---|---|---|
| | **PD 28** | | **PD 35** | | **PD 45** | |
| **GROUPS** | **Direct** | **Indirect** | **Direct** | **Indirect** | **Direct** | **Indirect** |
| **SA/SC/GH** | 0,2±0,03** | 0,09±0,01 | 0,17±0,03 | 0,09±0,01 | 0,09±0,01** | 0,11±0,01* |
| **SA/SC/SH** | 0,20±0,01 | 0,09±0,05 | 0,20±0,05 | 0,11±0,01 | 0,23±0,02* | 0,09±0,00 |
| **SA/EE/GH** | 0,17±0,03 | 0,09±0,03 | 0,11±0,01 | 0,12±0,01 | 0,26±0,09* | 0,12±0,01 |
| **SA/EE/SH** | 0,19±0,02 | 0,10±0,01 | 0,13±0,01 | 0,14±0,01 | 0,14±0,03 | 0,16±0,01 |
| **MA/SC/GH** | 0,12±0,02** | 0,09±0,04 | 0,09±0,01 | 0,12±0,02 | 0,12±0,02** | 0,22±0,06* |
| **MA/SC/SH** | 0,15±0,01 | 0,08±0,01* | 0,18±0,05 | 0,11±0,01 | 0,06±0,01 | 0,13±0,01 |
| **MA/EE/GH** | 0,17±0,02 | 0,12±0,02 | 0,15±0,02 | 0,15±0,03 | 0,14±0,01 | 0,14±0,01 |
| **MA/EE/SH** | 0,17±0,03 | 0,12±0,01* | 0,15±0,01 | 0,15±0,01 | 0,19±0,04 * | 0,16±0,01 |

### 4.3.3 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. *(Fig. 31 A, B)*

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. *(Fig. 32 A, B)*

**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). *(Fig. 31 C, D)*

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). *(Fig. 32 C, D)*

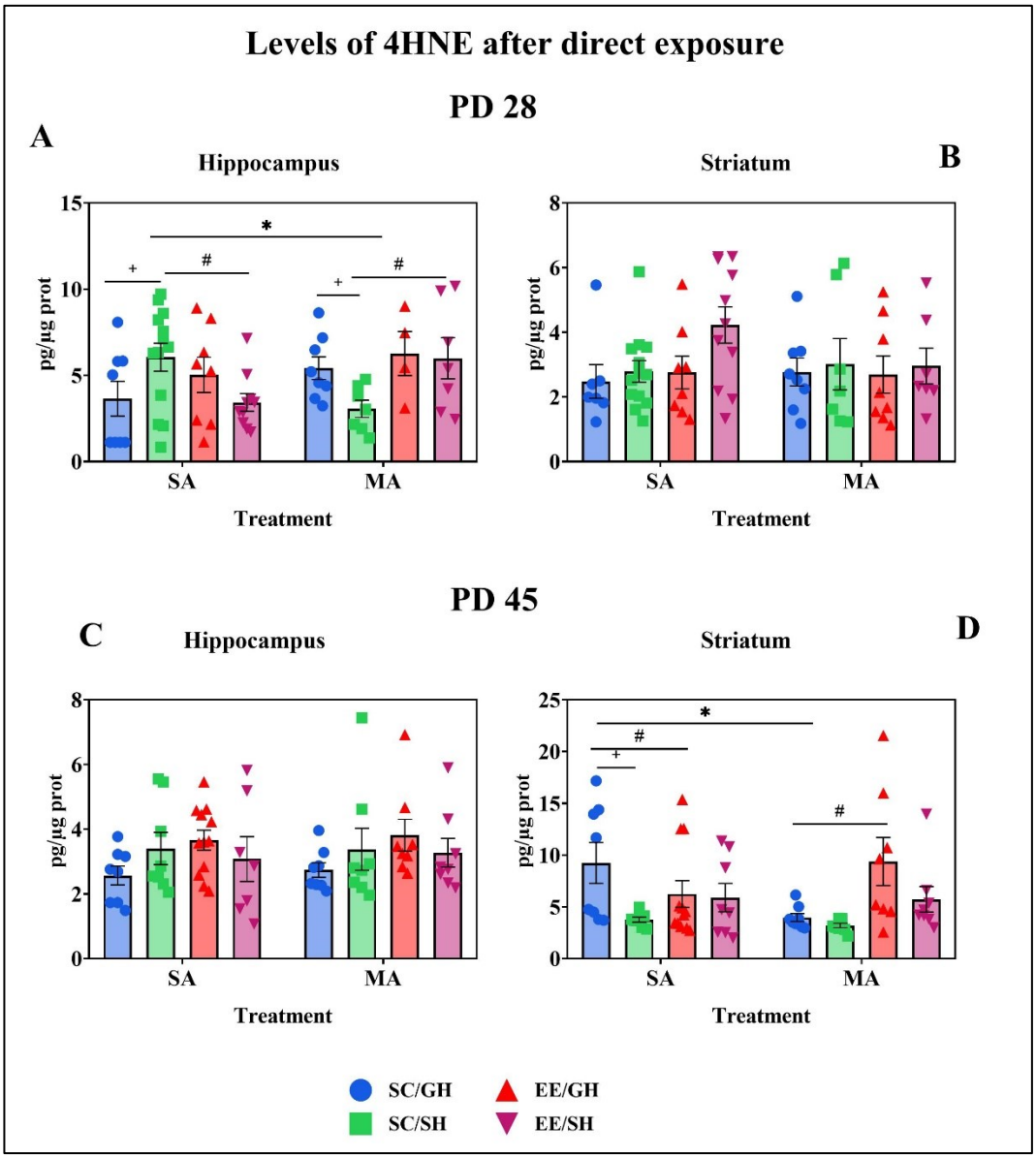


***Figure 31. Levels of 4-HNE after direct exposure.*** *Levels of 4-HNE are expressed in pg of 4HNE/µg of total protein. Values are ± SEM. n=8. *,#p<0.05, ,∗represents effect of treatment, # represents effect of postweaning housing. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

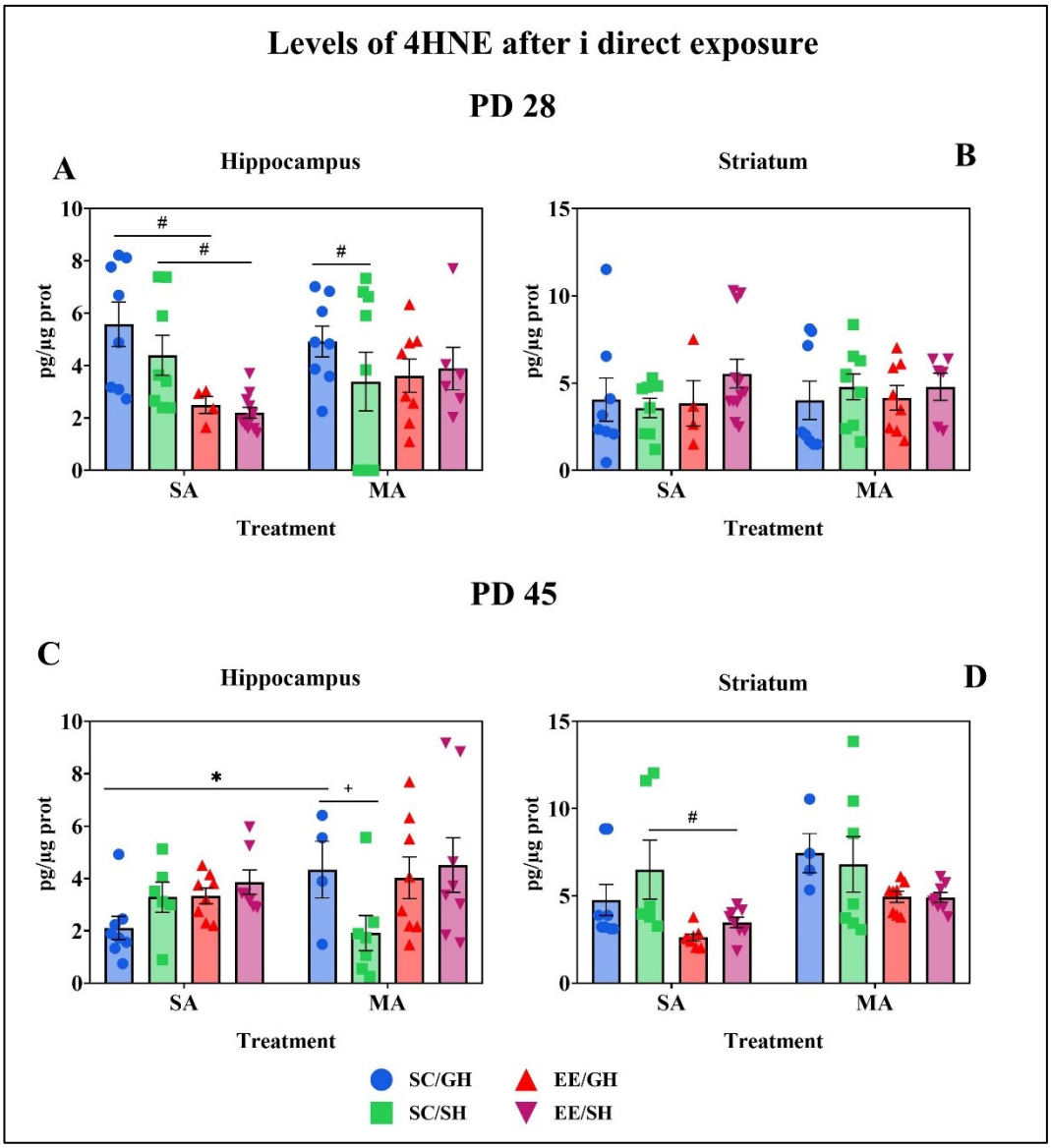


***Figure 32. Levels of 4-HNE after indirect exposure.***
*Levels of 4-HNE are expressed in pg of 4HNE/µg of total protein. Values are ± SEM. n=8. *,#p<0.05, ∗represents effect of treatment, #represents effect of postweaning housing. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

**Levels of TBARS**

**PD 28**

In terms of direct exposure, there were not any significant differences between groups in HP neither STR (*Fig. 33 A, B*). 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) *(Fig. 34 A, B).*

**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).*(Fig. 33 C,D).*
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). *(Fig. 34 C, D).*

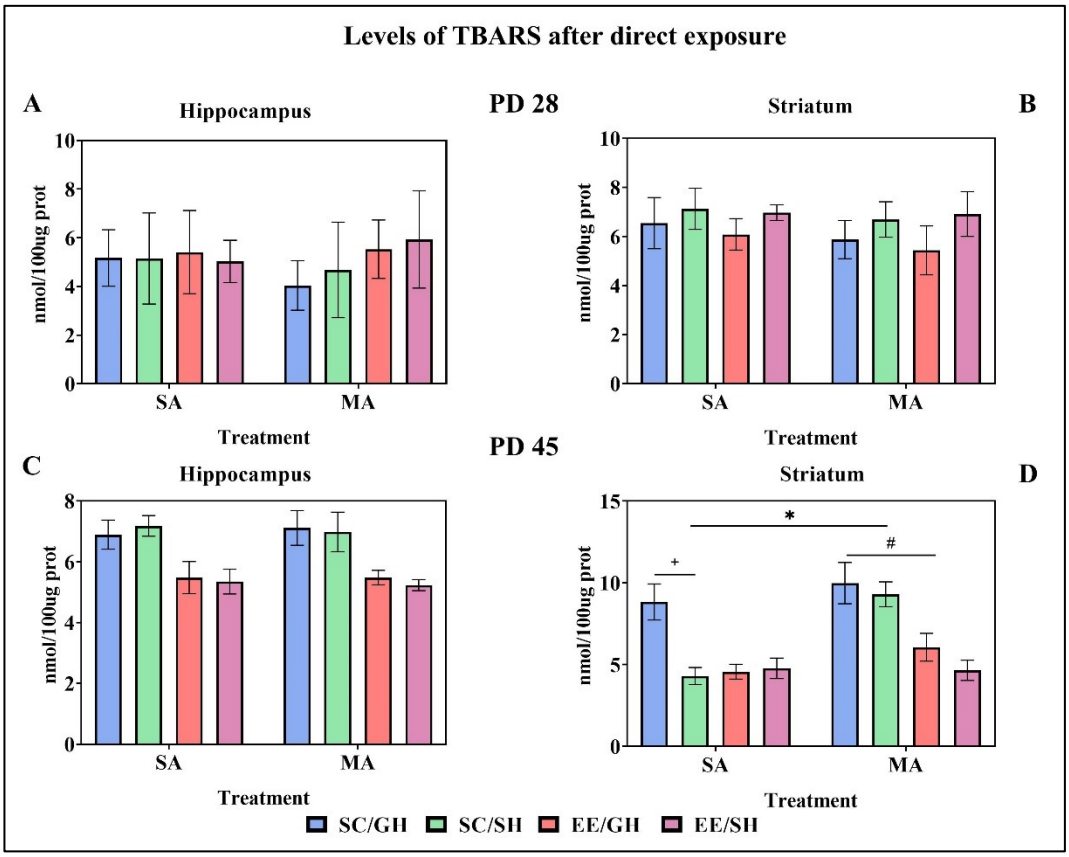


***Figure 33. Levels of TBARS after direct exposure***.

*Levels of TBARS are expressed in nmol of TBARS/100µg of total protein. Values are ± SEM. n=8. * p<0.05, ** p<0.01, ***,### p<0.001, ∗ represents effect of treatment, # represents effect of postweaning housing. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

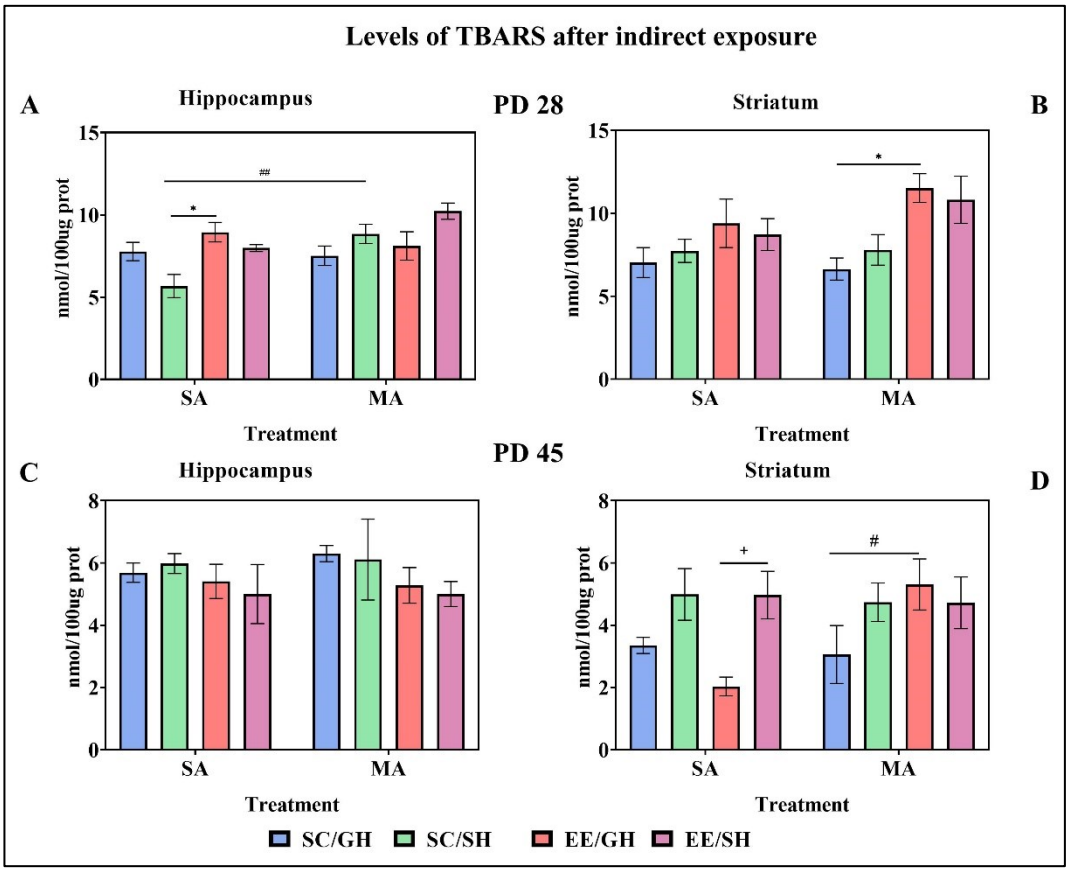


***Figure 34. Levels of TBARS after indirect exposure.***
*Levels of TBARS are expressed in nmol of TBARS/100µg of total protein. Values are ± SEM. n=8. * p<0.05, **,## p<0.01, ∗ represents effect of treatment, # represents effect of postweaning housing. SA/SC/GH – SA-treated animals raised in standard cages and postweaning grouped housing, SA/SC/SH- SA-treated animals raised in standard cages and postweaning separated housing, SA/EE/GH – SA-treated animals raised in EE cages and postweaning grouped housing (stayed in EE after weaning), SA/EE/SH- SA-treated animals raised in EE cages and postweaning separated housing (without EE after weaning).*

# 5. DISCUSSION

In the current study, we monitored the influence of early postnatal administration of MA on various behavioral and molecular parameters, but also the role of the individual's environment during development on these parameters. We will discuss these results in the context of observed factors - MA administration (direct vs indirect), preweaning housing (EE vs SC) and postweaning housing (GH vs SH). We worked with two hypotheses, which are going to be discussed in this chapter:
The long-term effects of early postnatal MA exposure are influenced by pre-weaning and post-weaning 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.

For the sake of the understanding this complex study, we may consider indirectly exposed animals with standard preweaning housing and postweaning grouped housing as form of absolute control, since these animals were not exposed to drug, injection neither stress nor enrichment.

## 5.1 Levels of neurotransmitters on PD 12

We discovered significantly decreased levels of DA in HP and STR of MA-treated pups on PD 12. This discovery directly proves, that subcutaneous MA exposure during this period significantly alters DA neurotransmission. Other neurotransmitters such as NA, GLU and 5HT were not significantly altered, however in terms of 5HT, these levels were decreased in STR and increased in HP after MA exposure. This is in contrast with study by Jablonski et al. (2017), which reported that neonatally administered MA decreased 5HT levels in the HP and PFC [112]. They also found a decrease in NA levels by MA in HP. It is also important to note, that our animals were not exposed to any environmental alterations during

MA exposure, while Jablonski et al. studies combination of stress and MA. MA and stress presented by barren cages decreased hippocampal DA levels. Results from this experiment lead us to aforementioned hypotheses.

### 5.2. Behavioral testing outcomes

These hypotheses were teste on behavioral and molecular level. We used 4 types of behavioral test - habituation, object recognition and object location tests, Morris Water Maze learning and memory tests. We chose these tests on the basis that our previous data as well as data of others have shown that that MA affects learning and memory abilities [113, 114]. Studies by Vorhees, whose team published several studies related to postnatal MA administration, showed that rats exposed to MA in first 10 PDs and later PD 11–20 showed reduced locomotor activity [113, 114]. The effect was most powerful at PD 30 (adolescence) and was smaller at PD 45 and PD 60 (late adolescence and adulthood). This study suggests that the effects of MA on behavior are long lasting and stage dependent [113, 114].

Therefore, we performed these behavioral tests on animals during adolescence till adulthood. It has also been reported that social isolation causes several behavioral and molecular impairments, while EE, on the other hand, supports the neurogenesis of HP, on which the memory consolidation process depends [115]. Studies have shown that EE can lead to improved cognitive function, reduced anxiety levels, increased exploratory behavior, and enhanced neural development in rats [116]. Specifically, exposure to EE has been linked to changes in emotional behavior, spatial memory, and neural activation in the HP of rats [117]. Learning and memory are dependent on proper function of HP. However, our results did not show such effects.

#### 5.2.1 Habituation (PD 28-31)

The novel setting is often an open area, which might be either relatively empty or full of stimuli. A rodent begins to construct an internal representation of the surrounding spatial information when it is placed in a new environment, according to the "cognitive map" notion[104]. After the completion of this hippocampus "map," the rodent stops exploring its surroundings and is said to have acclimated to

its new surroundings. Behavioral habituation to a novel environment, thus, is a widely used paradigm for studying no associative learning and memory processes in rats and mice. Furthermore, habituation is widely employed to evaluate the positive or negative effects of drugs on memory and learning [103].

**Direct exposure to MA**

In our experiment, directly exposed animals displayed differences which suggest that MA has impact on ability of creating map in HP. This is evident from the fact that MA/SC/GH animals spent less time exploring the arena on the last day of testing than SA/SC/GH, indicating a positive influence of MA on the creation of aforementioned map.

A reduction in exploratory behavior following prolonged or repeated exposure to a new environment is one of the most prevalent types of habituations observed in rodents [104]. However, on the first day when the animals were exposed to the new environment, MA/SC/GH animals showed a lower rate of exploration than SA/SC/GH. This phenomenon may indicate other mechanisms associated with the effect of MA on animal behavior, such as fear of novelty. We did not observe any effect of EE during this test.

**Indirect exposure to MA**

The situation was different among indirectly exposed animals. While on the first day the MA/SC/SH animals, which were exposed to postweaning stress and MA through milk, explored the arena the most in comparison to SA/SC/GH animals that were not exposed to injection or stress explored the least. Exploration on the last day of the test showed that MA/SC/SH explored the arena the most of all groups, together with animals from the MA/EE/SH group. This result suggests that MA exposure through breastfeeding together with post-weaning stress impairs map formation in HP. Animals from the SA/EE/SH group showed the least exploration on the last day, which indicates that this effect is apparently present only in combination with MA.

In conclusion of the results from habituation test we can state that animals directly exposed to MA had better habituation results than SA-treated animals, which proves impact of MA, however, not impact of preweaning environment, neither postweaning housing. Indirectly exposed animals displayed worse habituation rate

when were exposed to MA as well as postweaning stress. Preweaning exposure to EE did not suppress this effect, however it did suppress this effect in SA-treated animals.

It is important to note, that there is lack of studies observing MA exposure during early postnatal development and most of studies are performed on animals exposed during adolescence or adulthood. Several studies reported consequences of substance exposure on habituation of rodents. In study by Lloyd et al. (2014), nicotine was found to decrease habituation rate as well as MA. These findings show that stimulant drugs may disrupt normally occurring habituation of reinforcer effectiveness by increasing DA neurotransmission[118].

This effect was not present in our study. Study by Seyedhosseini Tamijani et al. (2018) reported that MA administration decreased rates of spontaneous exploration in Y-maze and increased anxiety-like response [119]. Similarly in study by Struntz et al. (2018), MA-exposed mice showed increased locomotor activity and anxiety-like behavior in the open field test compared with SA controls, regardless of age [120]. This kind of results were also obtained by Rud et al. (2016), which reports that MA-exposed adolescent mice showed increased locomotor activity and anxiety-like behavior in the open field compared with SA controls [121]. Thanos et al. (2017) reported that chronic MA treatment disorganizes open field activity, impairs alert exploratory behavior and anxiety-like state, and downregulates DA transporter in the STR [122].

In context of our study, Siegel et al. (2011) reported that anxiety-like behavior, sensorimotor gating, and contextual and cued fear conditioning were not affected by MA exposure. Thus, neonatal MA exposure affects cognition in adolescence and unlike in adulthood equally affects male and female mice [123]. In conclusion, as in several mentioned studies, in our study MA had rather anxiety-like effect in the beginning of this test, however, did apparently improved habituation rate in rats, which is not in agreement with other studies.

Role of environmental condition and stress exposure as well as in context of drugs exposure was studied by several authors. Study by Brenes et al. (2009) reported that alteration of an environment impacted on locomotor habituation and on sensory-motor exploration at the first minute and during the 10-min session without

modifying the plus-maze behavior as well as concentration of NA, 5HT, and its turnover in HP [124]. Overall, EE accelerated open-field habituation and led to behavioral and neurochemical antidepressant-like effects. In contrast, isolation rearing strongly impaired habituation and simple information processing, but showed marginal effects on depressive-like behavior and on HP neurochemistry [124].

Study by Neugebauer et al. (2004) investigating role of EE after prenatal cocaine exposure on open field activity, social interaction and DA transporter function in PFC of rats [125]. Rats prenatally exposed to cocaine without EE displayed divergent social interaction and altered function of DA transporter in PFC, while animals with EE did not different from prenatally SA-treated groups, suggesting that EE attenuates the behavioral and neurochemical effects of prenatal cocaine [125]. Effect of EE was not present in our study during habituation test.

In terms of stress, Carneiro de Oliviera et al. (2022) found that repeated restraint stress increased anxiety-like behavior in the open field test and induced locomotor cross-sensitization in the stressed mice and their cage mates [126]. In our study the effect of stress was visible only in combination with MA. Study by Ahmadalipour et al. (2015) reported that EE exposure during adolescence alleviates behavioral deficits induced by prenatal morphine exposure and up-regulates decreased levels of BDNF [127]. Prenatal morphine exposure caused a reduction in time spent in the elevated plus maze open arms, decreased step-through latency and increased time spent in the dark side of passive avoidance task [127]. Postnatal rearing in an EE counteracted with behavioral deficits in the elevated plus maze and passive avoidance task, which suggests that exposure to an EE during adolescence period alters anxiety profile in a task-specific manner [127].

Study by Brancato et. al (2015) observed effect of EE during adolescence on alcohol exposed animals during perinatal period. Constant perinatal alcohol drinking decreased locomotor activity, exploratory behavior, and declarative memory [128] but in term of our study is very important to mention that alcohol is rather depressant in contrast to MA which is stimulant. Continuously perinatal drinking pups exposed to EE showed ameliorated declarative memory while it mitigated spatial learning and reference memory impairment in intermittent alcohol drinking

rats. These data suggest that even though maternal alcohol consumption produces drinking pattern-related long-term consequences on cognition and vulnerability to alcohol in the offspring, increased positive environmental stimuli during adolescence may curtail the detrimental effects of developmental alcohol exposure [128].

In study by Rodríguez - Ortega et al. (2018) EE-housed adult animals with long-term exposure to ethanol binge-drinking showed lower anxiety-like, compulsive-like, and novelty-seeking behaviors than standard housing exposed mice, irrespective of the specific housing conditions during adolescence [129]. In conclusion, within habituation test, we did not observe results as mentioned studies, since effect of EE on habituation was not visible and effect of stress was displayed only in combination with MA.

### 5.2.2 ORT and OLT (PD 31-32)

During the ORT task, memory is consolidated, and spatial or contextual characteristics of objects could be relocated in different parts of the brain [130]. However, when a given memory is recovered in the presence of novelty, it is set into a labile phase and requires stabilization to persist. This processing memory is called reconsolidation, and it is involved in reorganization of the already formed memories, allowing incorporation of new information [131]. It is known that rate of neurogenesis in HP is linked with spatial memory consolidation[132].

Object recognition memory is HP-dependent, and if there are lesions on this structure, moderate and reliable anterograde memory impairment will occur [133].

**ORT**

**Direct exposure to MA**

As previously mentioned, when animals are exposed to a familiar and a novel object, they approach frequently and spend more time exploring the novel than the familiar one. We were observing these changes in three trials delayed 15 minutes from each other.

MA/EE/SH as well SA/EE/SH animals spent more time near the familiar object in all trials, suggesting tendency of alteration in memory consolidation. SA/SC/GH

spent at least time around this object suggesting better memory consolidation processes. SA/SC/SH and SA/EE/SH showed the highest frequency with which the animals approached the familiar object. SA/SC/SH spent most time around new object suggesting proper memory consolidation and recognition index (time spent exploring novel object relative to total duration of exploring) was the highest in in SC/SH animals within both treatment in comparison to grouped housing during the first trial. These results indicate an improvement in short-term memory after exposure to stress but not in context to MA. Positive effect of EE was not present in this test.

**Indirect exposure to MA**

Animals from MA/SC/GH showed increasing trend of the recognition index. On the other hand, this index showed declining trend in SA/EE/GH, however also in MA/SC/SH. This result suggests positive impact of indirect MA exposure on the short-term memory, however EE had tendency to worsen short-term memory as well as stress in combination with MA.

**OLT**

**Direct exposure to MA**

SA/SC/GH animals showed the highest recognition index MA/SC/SH in all trials. This result indicate that MA impair memory consolidation processes and EE does not have significant impact on these processes.

**Indirect exposure to MA**

In conclusion we may speculate that MA exposure cause negative impairment on process of memory consolidation, however, is not clear what role the environment plays in this case.

Firstly, is important to mention study by Schindler et al. (2010) which report that animals which were exposed to cocaine in prenatal period displayed a preference for the novel object when tested after 20 min, but no preference for the novel object after either 1 or 24 h indicating a deficit in short-term memory in the task caused by stimulant drug [134]. These results differ with our findings, since in our case novel object was preferred by SA- treated animals, or stressed animals regardless to treatment. Herring et al. (2008) described the role of MA which decreased the novelty index in adult rats [135]. Novel object recognition has been also impaired

by psychostimulant drugs in study by Schröder et al. (2003)[136].These studies suggest similar effect of MA that is present in our study, however in our case, we may conclude stress as more eminent factor that alter short-term memory.

Botton et al. (2010) used this object recognition test to evaluate learning and memory after caffeine administration and reported that caffeine had a positive effect on cognition [137]. This study also observed dose and schedule of its administration impact on the memory recognition. They concluded that the acute treatment with caffeine followed by its withdrawal may be effective against cholinergic-induced disruption of memory and could prevent cognitive decline [137]. Goulart et al. (2010), used novel object recognition test to evaluate the effects of ketamine on consolidation phase of memory, when it was administrated systemically and acutely. They showed that after training, the impaired effect of this drug on long-term retention of memory in animals was dose dependent. The consolidation phase of long-term recognition memory was impaired by ketamine, probably by preventing learning-induced increase in BDNF levels in the HP [138].

Study by Seyedhosseini Tamijani et al., (2018) reported that short-term recognition memory was unchanged in MA-treated animals, while long-term memory was impaired in these animals, however only in higher doses. Though MA deleterious effect especially in recognition memory is somehow dose dependent [119]. Findings from this study are strongly in an agreement with our presented results, since we used MA dose of 5 mg/kg. In several different analyses, it seems as not sufficient dose to promote several behavioral and molecular consequences. EE reversed anxiety-like behaviors induced by sleep deprivation and impaired cognitive ability tested by open field, novel object recognition memory, and passive avoidance memory test in study by Ghaheri et al. (2022). On the other hand, social separation in rats has been shown to have a significant impact on their behavior [139]. Studies have demonstrated that maternal and social separation during early life stages can lead to hyperactivity, anxiolytic behavior, and changes in neurobiological responses in rats [140]. Specifically, research has highlighted that adolescent rats subjected to social separation exhibit increased hyperactivity and anxiety-like behaviors, as observed in tests like the open field test, elevated plus-maze test, and forced-swim test [141].

### 5.2.3 Morris Water Maze

Study by Williams et al. (2003) have confirmed that the early postnatal period is more susceptible to alterations caused by MA, [142] as well as study conducted by Hrebíčková et al. (2016) which shown that the administration of MA at a dosage of 5 mg/ml/kg from postnatal days 1-11 had a significant impact on the cognitive abilities of adult male rats. The study examined the impact of MA exposure during several periods of rat brain development, specifically the first and second half of gestation, as well as the early neonatal stage [57]. The findings of that study indicate that the primary consequence of exposure to MA is a significant impairment in spatial learning that relies on the HP. This impairment is specifically linked to the injection of MA during the neonatal period according this study [57].

**Learning**

**Direct exposure to MA**

On the first day of learning, MA/EE/SH swam the greatest distance, in contrast to MA/EE/GH. The last day of learning showed that SA/EE/SH greatest distance and SA/EE/GH the least. These results again indicate the effect of EE on the learning of animals, and negative impact of combination of MA with stress. Highest velocity of swimming on the first day was visible in MA/EE/SH and SA/SC/SH (suggesting the effect of separation) and the slowest swimmers were SA/EE/GH, MA/SC/GH and SA/EE/SH (suggesting the effect of EE in controls and MA). The last day of learning SA/EE/SH and were the fastest and SA/EE/GH were the slowest. Speed of swimming can be used as a measure of motivation to find the hidden platform [143]. Motivation is assumed to be mediated by the DA system[144], which mature, as does the HP, in the end of the third postnatal week [38, 50]. The fact, that in this test, the highest velocity of swimming was displayed in separated animals regardless off treatment, may be explained by higher motivation of this animals due to long-term separated housing. On the other hand, we speculate that animals exposed to EE were less motivated due to many different distractions and toys placed in their cages. On the first day of learning, the latency to the platform was the highest for MA/EE/SH and the lowest for SA/EE/GH (stress effect), and on the

last day SA/EE/SH and lowest at SA/EE/GH. Again, we can conclude that separation has the greatest influence on the ability to learn and EE does not have any beneficial effects, since separated animals were exposed to EE only during postweaning period in contrast to grouped animals

Because previous studies showed that animals may have similar escape latencies or length of trajectories during the trials, while having markedly different performances, [93, 145] we chose to observe two strategies during this test – thigmotaxis and scanning. These two strategies were chosen according to previous study from our laboratory by Hrubá et al. (2010), which revealed that these strategies are most used by tested animals [93]. Also, in study by Petríková et al. (2021), female rats which were directly exposed to MA during PD 1-11 more frequently used thigmotaxis during the learning period compared to females who were indirectly exposed to MA. Specifically, females with direct neonatal MA exposure spent more time using thigmotaxis and scanning and indirect neonatal MA exposure spent more time using scanning instead of using a direct trajectory to the hidden platform during advanced phases of MWM.

In present study, the time spent in thigmotaxis was the highest in SA/EE/SH and MA/EE/SH (separation effect) and the least in SA/EE/GH and MA/EE/GH on the first day of learning. On the last day of learning, SA/EE/GH used this strategy the least and MA/SC/SH the most. In this case is very important to note the contrast of environments between these two groups. This result clearly suggest that animals exposed to EE, but not MA and stress have better ability to learn, while MA and stress apparently disrupt this process. 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. In this case, the effect of separation is also evident. Search error was the same for all animals on the first day, however on the last day, SA/EE/GH showed the smallest learning error and SA/EE/SH the highest. We can conclude that separated may have higher motivation however in the end of the test, their ability to learn was poorest among all animals. This interesting result may be explained by anxiety-driven motivation among separated animals, while animals housing in EE cages in groups were exposed to several social and sensory stimulants, which may have caused their lack of motivation.

**Indirect exposure to MA**

During the learning test, results indicating impairment of learning processes were recorded in the control separated animals. While these animals performed best in almost all parameters, they had the worst results at the end of the test. It is interesting, however, that during the first four days, animals exposed to EE in combination with MA learned the worst, but at the end of the testing, this trend changed positively. Within the distance swam parameter, animals from SA/SC/SH swam the most on the first day, while on the last day, MA/EE/GH, together with MA/SC/GH and SA/EE/SH showed the least swimming distance, while SA/SC/SH showed the highest.

These results indicate the influence of the drug but also the environment and the negative influence of separation on the ability to learn. Conversely, MA together with EE or group housing obviously improves this ability. The swimming speed was relatively similar in all groups on the first day of learning, but on last day of learning, SA/SC/GH showed the lowest speed, this speed was also similar for MA/SC/GH, MA/EE/GH and MA/EE/SH, while SA/SC/SH swam the fastest. Latency was similar in all groups on the first day, and at the end of learning, SA/SC/SH animals had the highest latency to the platform, and SA/EE/SH, MA/SC/GH, and MA/EE/GH the lowest.

It seems that separation stress in this case caused a reduced learning ability. The time spent in the thigmotaxis strategy was comparable in all groups on the first day of learning, but with a decreasing tendency in SA/EE/GH and SA/EE/SH, indicating a positive influence EE on control animals. However, on the last day of learning, this strategy was used the least by MA/EE/GH and the most by SA/SC/SH and MA/SC/SH, which indicates a negative effect of separation on learning but regardless of the drug. Differences in scanning strategy were visible only on the last day and was used mostly by SA/SC/SH and MA/SC/SH, which again points to the impact of separation on learning ability.

The highest search error on the first day was visible for MA/SC/SH and the lowest for MA/EE/GH, on the 1st day of learning showed the highest search error for SA/SC/SH, and the lowest for MA/EE/GH, MA/SC/GH, SA/EE/SH. These results

show that MA exposed through breast milk does not affect the ability to learn, but the change in the environment turns out to be very important.

In conclusion we may state that social separation as form of postweaning stress significantly impaired learning ability, while role of MA in this case is disputed. Effect of EE did not show to be beneficial.

**Memory recall**

**Direct exposure to MA**

In the case of animals directly exposed to MA, we noted the influence of the environment in all parameters. In terms of distance swum, animals exposed to EE generally swam a greater distance than animals in standard cages. This phenomenon points to the apparently negative influence of EE on memory formation. Swimming speed was the fastest in separated animals exposed to EE in both treatments. Latency to the platform was again affected by EE in controls, where EE animals searched for the platform significantly longer, but in MA this phenomenon was not as intense. In the case of search error in the controls, the same situation was repeated, and the EE animals showed a higher error, while the MA-exposed animals showed the highest error if they were exposed to EE but not to separations. 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. In the end, it should be added that animals exposed to MA showed better memory abilities than controls regardless of the environment.

**Indirect exposure to MA**

Our data partly point to the importance of combining the type of exposed substance with the type of environment. While in control animals the combination of SA and EE caused a tendency to impair recall, social separation stress in combination with MA improved these processes. EE in combination with MA did not show any differences, however within the animals exposed to MA that were after weaning in groups we can see a slight improvement. These suggestions are represented by

longer distance swam in EE exposed animals with indirect SA treatment in comparison with standard housing. Similar tendencies are visible among separated MA treated animals in comparison with grouped animals. Interestingly, velocity of animals was almost identical among all experimental groups suggesting that drug exposure, neither change in environment do not have impact on velocity of animals. Latency of animals was also lower among SA treated separated animals with standard housing. This was also apparent in combination of MA and separation. Again, EE among SA treated animals had tendency to increase latency of animals which suggest poorer memory consolidation; however, it seems to have minimal effect in combination with MA.

Strategies and search error were also apparently impaired by EE, since all these parameters are increased in control animals but not in MA exposed animals. Increased time spent in thigmotaxic and scanning strategy was also apparent in MA exposed separated animals, while in grouped animals, these strategies were apparently less used. Very important is to note that stress from separation also improved performance of control animals, since these all parameters have decreasing tendency in comparison with grouped controls.

Other studies have documented the negative effects of MA treatment during specific developmental periods, such as PD 6–15 and PD 11–20. These studies 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 by MA administration [50, 146-149]. Vorhees et al. (1994) demonstrated that the administration of MA at a dosage of 30 mg/kg/day during PD 1-10 only affected the locomotor activity of adult rats tested in a water maze. However, when administered at a dosage of 40 mg/kg divided into 4 doses/day during PD 11-20, it resulted in a decline in memory performance during probe trials [113, 114]. A further research shown that when MA was administered to neonates at dosages of 5, 10, or 15 mg/kg four times a day from postnatal day 11 to 20, it resulted in deficits in spatial learning and memory [142].

Based on our findings and the findings of other researchers, it seems that exposing rats to MA during the early postnatal period has a detrimental effect on their cognitive abilities. Our study is in contrast with these studies. It is probably based by the fact that we did use smaller dose of the drug in comparison with other studies.

Also, we exposed animals only once a day to MA, while other studies often use multiple administrations during the day.

Study by Madhavadas et al. (2017) revealed, that EE exposure to obese rats (induced by monosodium glutamate) with cognitive deficits had significantly increased the volume of HP along with increase in neuron number in the CA1 subfield of the HP [150]. These effects were present in rats exposed to EE from PD 45 as well as PD 75 for 15 days. EE completely recover cognitive functions suppressed by obesity only in 2-month-old rats but not in 3-month-old rats [150]. In our study the effect of EE was rather opposite. Therefore, it seems that correct determination of critical window period for EE interventions in restoring the cognitive functions is crucial [150].

Study by Saadati et al. (2023) observed effects of EE during adolescence on serotonergic system following postnatal 5HT depletion, which is also often caused by MA [151]. This work obtained results which indicated that adolescent EE exposure alleviated memory impairment, decreased BDNF levels, and anxiety-like behavior induced by experimental depletion of 5HT [151]. Leggio et al. (2005) reported that EE exposure during adolescence in rats promoted high performance levels, by exploiting procedural competencies and working memory abilities [152]. In the MWM, EE animals more quickly acquired tuned navigational strategies and they also reported increased density of dendritic spines of EE animals [152].

In experiment by Pautassi et al. (2017), adolescent mice exposed to EE, exhibited ethanol induced conditioned place preference. Standard environment exposed adolescents, but not EE adolescents had BDNF levels were significantly lower in those treated with ethanol than in that given vehicle, but not in adults [153]. In experiment by Dandi et al. (2022), chronic unpredictable stress decreased learning impairments which were limited to males while depressive-like behavior to females. EE exposure protected against stress related behavioral deficits and body weight loss [154]. Study by Fan et al. (2021), EE normalized reduced locomotor activity, increased anxiety-like behaviors, enhanced contextual fear memory and elevated basal plasma ACTH levels, caused by early adolescent stress exposure [155].

Study by Gill et al. (2013), EE exposed animals showed decreased locomotor activity and anxiety-like behavior in comparison with early adolescent isolated animals [156]. The results of study by Bator et al. (2018) showed that exposure to EE prevented the development of adult behavioral deficits induced by prenatal methylazoxymethanol administration, that is used as model of schizophrenia [157]. Study by Korkhin et al. (2020) reported that EE exposure during juvenile period improved selective attention, increased foraging-like behavior, and reduced anxiety levels as reflected in the open field as well as in low corticosterone levels [158]. Results of this study suggest mid-adolescents as the sensitive time that induces the most beneficial and long-term effects of EE on attention [158].

Postweaning EE reversed maternal separation- lowered grooming behavior in the open field in study by Vivinetti et al. (2013). Inhibitory avoidance but not object recognition memory was impaired in maternally separated animals, suggesting that early maternal separation alters learning and memory in a task-specific manner. EE reversed the effects of maternal separation on the inhibitory avoidance task [159]. Stressful experience early in life such as maternal separation can persist until adulthood, some of them can be compensated by early favorable environments, possibly through nervous system plasticity [159]. EE during adolescence improves passive avoidance memory and increases nociceptive response against thermal stimulus in both sexes in study by Sadegzadeh et al. (2020). EE also increased BDNF level in the PFC of female rats [160]

In study by Kentrop (2018) male rats deprived from mothers showed a longer latency to play and a decreased total amount of social play behavior, after a 24 h isolation period. In adulthood, social discrimination was impaired in deprived male and female rats in the three-chamber social approach task. However, after 24 h of isolation, these animals showed shorter latencies to engage in social play behavior [161]. Study by Yazdanfar et al. (2021) showed that maternal morphine exposure and postweaning social isolation could dramatically impair memory in offspring, while EE could reverse these adverse outcomes [162]. All above mentioned studies reported positive effect of EE however in our study this effect was not Shown as positive, since these animals displayed greater distance swam, greater search error

and so on. However, in contrast with learning task, in this case EE exposed animals displayed greater velocity of swimming.

## 5.3 Molecular analyses

### 5.3.1 Neurotransmitters

#### Catecholamines

#### Dopamine

DA levels in HP within PD 28 in directly exposed animals were not significantly different between groups, but in STR these values were significantly increased in EE animals in case of both treatments. This indicates an obvious effect of EE on DA levels in this age category. Within the indirectly exposed animals, DA levels were increased in HP on PD 28 in SA/SC/SH and MA/EE/SH, other values were similar, while in STR the same situation as in directly exposed animals was repeated, because these values were increased in EE animals in both treatments. This result indicates that both direct and indirect exposure to MA still has some effect in this PD and this effect is the same for both forms of administration. However, in PD 35 these levels were different. There were no significant changes in HP, but there was an increasing tendency in animals with standard environment compared to EE. In STR, the situation was different in control animals, where we recorded an increase in DA in SA/EE/SH, but in MA these values were significantly increased in MA/EE/GH.

In indirectly exposed animals in PD 35 there were very variable differences in DA levels in HP. Animals in a standard environment housed in groups had the same DA levels in both treatments as in separated animals with a standard environment, but these values were lower than in the groups. EE animals in controls showed lower DA in group housing, while in MA the values in EE animals were comparable. DA levels in STR were the highest among SA/SC/SH in controls, while in MA, as in the previous day, the highest values were in EE animals compared to standard environment. Here we already see the different influence of environment and MA in this age group, but the form of administration has similar effects. On PD 45 in HP, in directly exposed controls, levels were slightly increased in SA/EE/SH

animals, but in MA, the situation was the same as the previous day, and these values had an increased trend in animals with standard environment.

In STR, the ratios of levels in controls were increased in EE animals, but in MA, these values were decreased in EE animals. In indirectly exposed animals in HP, DA levels were highest in EE/GH, while in MA, values were highest in SC/SH. In STR, DA levels were significantly higher in animals with a standard environment compared to EE in both treatments, but in MA, EE/SH levels were increased.

Several studies observed different protocols of MA exposure and its effect on DA neurotransmission. In terms of subcutaneous administration, study by Chu et al. (2008) revealed decreased DA content in STR and nucleus accumbens after MA exposure in dose of 7.5 mg/kg 4-times a day in 2-hour intervals [163]. There was also significantly decreased number of transporters of DA in both structures but also in frontal cortex [163].

Study by Zaczek et al. (1989) showed decreased DA content in STR after MA administration in dose of 15 mg/kg 2 times a day for 4 days [164]. Also, DA transporters were decreased in STR as well. These data correspond with our observations, however only on PD 28. Therefore, we may speculate that negative effect of MA on DA transmission in STR is temporary since this effect was visible further, but also that development and environment may reverse potential harmful effect of this drug. Intraperitoneal administration of MA caused decreased content of DA as well as DA transporters in STR after 3-times in one day in 3-hour intervals in study by Granado et al. (2011) [165]. Similar results were reported in studies by Keller et al (2011) and Krasnova et al. (2011) [166, 167]}.

These reports clearly show that also different form of exposure is effective. Kokoshka et al. (1998,2000) reported that MA effect are strongly age dependent as well as reversible since MA exposure 4-times a day in dose of 10mg/kg cause significant decrease of striatal activity of DA transporters, however much severely in adult animals [168, 169]. Also, content of MA in brain of adolescent animals were two times lower than adult animals, suggesting difference of MA metabolism during development. There is also important to note, that there are developmental differences in function of DAT [168, 169].

**Noradrenaline**

NA 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. This result means that EE can influence higher alertness. In indirectly exposed animals, however, this situation was different, because the levels of NA in the HP were increased in EE animals within MA but partly also in SA. In STR, these levels were similarly distributed, but in MA, the values of EE/GH were reduced.

At PD 35 in directly exposed animals, NA levels in HP were significantly increased in SA/SC/SH but this situation did not occur 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. In indirectly exposed animals in HP, the levels in SA/EE/GH were significantly lower compared to the other groups, but this difference was not visible in MA. In STR, the values were increased in SA/SC/SH, but the other groups did not show big differences among themselves, even within MA.

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 other cases, the difference was not very visible. In indirectly exposed animals, NA levels in EE had an increasing trend, but these differences were not very significant in controls. In MA, these differences were significant. The situation was similar in STR controls, but in MA animals, the levels were reduced in MA/SC/GH and MA/EE/SH, which are groups that differ in all observed factors.

Seiden et al. (1976) then reported, that long-term administration of high doses of MA to rhesus monkeys depleted NA in the frontal cortex and midbrain and DA in the caudate nucleus [170]. These depletions have been speculated to be most likely irreversible since they are found to remain as long as twelve months past the repeated injection period. However, in our research, we clearly showed, that this is rather not so accurate. Ellison et al. (1978) reported that continuous exposure to high doses of amphetamine results in neurotoxic effects manifested by enlarged and swollen fluorescent axons in the STR [171].

Wayment et al. (1998) performed an in vitro experiment on reserpine pretreated rats to study the amphetamine binding site in the striatal DA transporters [172]. Results show that amphetamine and DA compete at the same binding site and are transported. Once amphetamine is transported, the preloaded intercellular DA is released. Rutledge and his team performed different in vitro experiments to study the effects of amphetamine on NA in the cerebral cortex as well as DA in the STR. Their results showed that the amphetamines increase the release and inhibit the uptake of NA and DA [173-177].

In terms of environmental changes and their effect on DA, study by Yazdanfar et al. (2021) maternal morphine exposure and social isolation reduced DA levels and altered expressions of D1R, D2R, and DA transporter in STR. However, postweaning EE partially buffered these changes. These findings provided evidence on reversibility of these alterations following EE [178]. In schizophrenia model (induced by methylazoxymethanol acetate) by Zhu et al. (2022), prepubertal EE has been shown to prevent methylazoxymethanol acetate-induced increased DA neuron population activity measured in adults, while 10-day prepubertal EE, regardless of age of exposure, was shown to prevent adult DA-related pathophysiology [179].

In our study, animals exposed to postweaning EE had significantly higher levels of DA in STR, however on PD 45 this was not displayed anymore. Since we did not investigate DA receptors distribution in HP and STR, we do not exactly know what role EE play in terms of DA neurotransmission.

Study by Gabriel et al (2020) revealed that EE exposure causes decreased D2 receptor expression in HP [180]. Similar results were observed in study by Ko et al. (2019) since treadmill exercise enhanced thyroxin hydrolase expression and suppressed D2 dopamine receptor expression in rats with traumatic brain injury in which these receptor expressions were increased [181].

**Serotonin**

The results of observing 5 HT levels in the context of the presented protocol were published in the article by Čechová et al. (2024) [44]. Here we shortly discuss findings of this study. On PD 28, EE significantly increased the muted 5HT in SA pups after separation and restored the pronounced inhibition of 5HT by MA. No

beneficial effect of EE was present in pups exposed to combination of MA and separation. 5HT development declined over time; EE, MA and separation had different effects on 5HT relative to adolescence stage. In STR, levels of 5HT were not impacted by MA exposure in terms of direct exposure, however on PD 45, indirectly exposed animals have shown increased levels among MA animals with standard environment as well as EE in comparison to SA/SC/GH and SA/EE/GH, which is opposite results than expected. Based on these results, MA along with preweaning and postweaning environment affect 5HT levels, depending on both the age and the method of application (direct or indirect).

Studies by Schaefer et al. (2008, 2010) reported that the effect of regular neonatal MA treatment on the activity of 5HT during the neonatal period can be detected very soon after treatment, and serotonergic neurotransmission declines during ontogenesis [147, 182]. The authors reported an immediate 5HT decline in the HP 1 day after a 5-day (PD 11–15) or 10-day (PD 11–20) MA administration dosing schedule of 10 mg/kg; 4×/day. On day 30, the effect was no longer visible, suggesting that the MA effect was only transient. It seems that the timing and method of MA administration are crucial due to the different susceptibility of 5HT neurons during postnatal development. It must be emphasized that the effect was manifested only after direct administration.

Interestingly, according to the study by Fosnocht et al (2019), social isolation during adolescence led to vulnerability to cocaine seeking behavior and alter behavioral responses to cocaine later in adulthood [183]. It also altered the sensibility of reward circuit in the brain. Conversely, EE has been shown to produce an opposite effect. It was reported that EE can stimulate various biochemical, and functional changes in the HP, especially the network connectivity, as well as the developing of new neurons in the dentate gyrus of mice exposed to an EE compared with standard housing environment [183].

A study by Sbrini et al. (2020) reported that during 1 month of EE exposure the anhedonia and anxiety-like phenotype characteristics were normalized in animal model of depression [184]. Similar study as ours by Gutierrez et al. (2017) studied the effect of stress in adult animals on HP-dependent learning, memory performance and several neurotransmitter levels [185]. Stress was demonstrated by

barren cages housing in comparison to standard cages. Their research reported that 5HT levels in HP were decreased by MA in comparison to SA, but stressor environment did not seem to have eminent effect on these levels in comparison to standard environment [185]. Although it is very important to highlight the fact, that all mentioned studied were performed mostly on adult animals with different doses of MA, type of injection and different type and duration of stress exposure [44].

**Glutamate**

GLU levels has been impacted by social isolation in case of indirect MA administration on PD 35. Levels of GLU were higher within MA administered grouped animals in comparison to separated animals, both exposed to EE. Opposite situation appeared within control animals with standard environment where levels of GLU were higher in separated animals in comparison to grouped animals. In terms of direct exposure, we obtained significantly higher GLU levels on PD 45 within MA administered grouped animals in comparison to separated animals. Both groups were exposed to EE. It has been reported that EE in adulthood also induced a set of modifications in the expression of proteins related to glutamatergic neurotransmission. To this date, the effect of EE on neurotransmitters levels has not been sufficiently documented, especially not in connection with MA and adolescent brain. GLU levels was also impacted by EE. On PD 28 within direct exposure, we obtained higher levels of this neurotransmitter in control separated animals exposed to standard environment in comparison to EE. On PD 35 with direct exposure, we acquired same situation with GLU levels in control as well as MA exposed animals. GLU levels were higher in EE grouped animals in comparison to standard environment exposed animals. Within indirect exposure on PD 35, GLU level were higher in MA exposed separated animals also exposed to standard environment in comparison to EE. Finally, levels we acquired higher levels of GLU within control grouped animals exposed to standard environment in comparison to EE on PD 45. Significantly higher levels were obtained in MA exposed animals separated with standard environment in comparison to EE.

Kokoshka et al. (1998) investigated effect of MA induced oxidative stress reaction on GLU receptors and acquired interesting results [169]. The MA induced decrease

in transport activity completely recovered by 24 h but was decreased again 1 week later. In contrast, GLU transport was essentially unchanged after MA treatment. These findings indicate that MA causes a rapid and reversible decrease in 5HT transporter activity and GLU transporters are less susceptible than 5HT transporters to effects of MA treatment [169].

Isolation during adolescence, study by Deutschmann et al. (2022) demonstrated several conclusions. Mentioned study concludes that adolescent social isolation leads to decrease of GLU presynaptic neurotransmission, in ventral HP and nucleus accumbens, and that adolescence is critical developmental period for the development of reward circuit, which is also highly altered by MA administration [186]. Deutschmann and his team demonstrated that social isolation leads to vulnerability to cocaine reinstatement in compared to animals isolated in adulthood [186].

### 5.3.2 Neurotrophins

The results of the observation of neurotrophins levels in the context of the presented protocol were published in the article by Čechová et al. (2023) [187]. Here we shortly discuss findings of this study.

The results of our study indicate that exposure to EE did not lead to an increase in BDNF levels in either the control group or the group exposed to MA. Furthermore, this lack of increase was observed in both direct and indirect exposure scenarios. However, social separation following weaning did decrease BDNF levels compared to animals in conventional housing. Nevertheless, this impact was counteracted by direct exposure to MA. During late adolescence, the levels of NGF in the EE environment increased only in indirectly exposed controls and MA animals. Conversely, social isolation led to an increase in NGF levels in most animals. Unlike our findings, a study conducted by Grace et al. (2008) found that there was a rise in BDNF levels in the hippocampus after repeated injection of MA during postnatal days 11-15 [96].

The authors propose that the impact of MA on BDNF is only evident after several days of exposure or is shielded from oscillations by an unknown mechanism, as no alterations in these levels were observed on PD 11. It is worth noting that the dosage

utilized in this research was twice as high as the dosage used in our present investigation. Additionally, the levels were assessed after exposure, which is different from our study. Furthermore, research has demonstrated that exposure to EE resulted in a more robust dorsal hippocampal BDNF response and elevated serum BDNF levels. Animals that were exposed to EE had a greater brain weight in comparison to rats that were kept in isolation. The enhanced BDNF profile observed in enriched animals may indicate the neurobiological manifestation of a resilient phenotype in response to a stressful scenario [188].

Another study has discovered that exposure to EE leads to greater levels of NGF in the HP) of rats, compared to those housed in ordinary conditions [189]. As in terms of most of our investigated parameters, all mentioned studies by other authors were performed mostly on adult animals with different doses of MA, type of injection and different type and duration of stress exposure, so it is very difficult to compare these results [187].

### 5.3.3 Oxidative stress

The role of oxidative stress in the neurotoxicity caused by MA has been recognized from the initial research conducted by De Vito in 1989. De Vito revealed that the neurotoxic effects of MA can be reduced by pre-treating with antioxidants. Since then, these systems have undergone thorough investigation, revealing diverse outcomes with numerous techniques and species. Various doses ranging from 0.25 to 10 mg/kg have been administered, falling into three categories: pharmacological (0.2 - 2 mg/kg), toxic (2 - 4 mg/kg), and neurotoxic (> 5 mg/kg). These doses closely resemble the ones that are routinely abused by people. Nevertheless, in experimental research, adult rats are commonly administered doses of 10 mg/kg and above [for further details, refer to [7]. The presence of a significant amount of lipids in the brain makes it possible to evaluate the levels of lipid peroxidation products, such as MDA and HNE. This assessment can provide valuable information about oxidative damage in the brain.

Studies have demonstrated that giving adult male rats repeated doses of MA (5 mg/kg, 15 days) increased levels of MDA, which is measured as thiobarbituric reactive species (TBARS). Additionally, it also enhanced the activity of the

antioxidant defense enzyme superoxide dismutase (SOD), which catalyzes the breakdown of superoxide anion radicals into oxygen (O2) and hydrogen peroxide [190]. The authors also demonstrated that administering a single dose of 15 mg/kg increased TBARS levels in the striatum and prefrontal brain [190]. Similarly, in adult male Wistar rats, both short-term and long-term administration of MA (2 mg/kg) resulted in increased levels of TBARS in the striatum, amygdala, and cortex [191]. Additionally, multiple one-day administrations of MA (4 x 10 mg/kg) led to an increase in MDA levels one week later in the nigrostriatal system, as well as in the cortex and hippocampus of adult male rats [192]. The effect of MA exposure of rats in preweaning period (PD 1 - 20) approximately equals to that of developing human fetus during the second half of gestation. Brain structures that form higher cognitive functions develop at that period and are susceptible to the damaging effects of drugs. Even in this developmental window there are differences in the neurochemical and neurobiological responses between the intervals of MA administration at PD 1 - 10 and 11 - 20, for Review see Jablonski 2017 [112].

We tried to ascertain the impact of repeated neonatal MA administration on striatal and hippocampal monoamines and peroxidation of lipids at the threshold of adolescence, and in addition, in combination with emotional stress applied after weaning, till various stages of adolescence. Unlike the long-term striatal DA depletion along with the reduction of all dopaminergic markers observe after 10 mg/kg of MA treatment at PD 10-21 [193] in our conditions the depleted DA in STR and HP was no more present on PD 28. This fact indicates that MA administration in developmental window PD 1-12 causes only temporary DA depletion. On the threshold of adolescence, at PD28, the prolonged effect of MA became visible only in the reduction of body weight gain. At this postweaning period no differences in monoamines were observed in response to MA given either directly to the pups, or indirectly via mother´s milk, which speaks for the recovery of the juvenile brain from the early MA insult. These two ways of administration have their specific features: The direct injection resembles more human abuse, where the injected drug directly penetrated from mother´s circulation through placenta to fetus. However, these pups were daily stressed by handling and injecting. In the indirect administration, the pups remained intact, and the drug was

given to them with each suckling, even though the amount was not precise. The early stress models in rodents focus mainly on preweaning period, where stressful challenges activate corticosterone release with its detrimental effects on immature hippocampus (for review see [194].

Neonatal MA exposure (PD 11-15) alone was shown to trigger corticosterone release, [182, 195] which exceeded the effect of various stressors such as forced swim or isolation [96] during preweaning period. Recently Jablonski et al. [112] studied the combination of chronic stressor of barren cage with MA in two intervals of pre-weaned rats, namely PD 11 - 15 and 11- 20. These authors found NA increase in neostriatum on PD15 in response to 5 mg/ml of MA and to the same extent after stress exposure but no additive effect of these two stimuli.

We have focused on the next phase in the rat ontogeny – juvenility - the earlier phase of adolescence from post-weaning to pre-pubertal period, i.e. day 21 - 28. This developmental stage corresponds approximately with early (10–13 years), human adolescent [196].

Manipulation of the social experience during this specific period in rats has been shown to impair cognitive, behavioral, learning and emotional balance later in adulthood [197]. Because of the importance of social learning in adolescence, isolation has the most potent negative effect during the phase between weaning to early adulthood. The adolescent brain is also more responsive than the adult brain when confronted to a stressor. Daily isolation during this period resulted in a robust corticosterone release which persisted after the termination of stress exposure.

In our series, although brain monoamines were not affected by MA, 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. In the directly treated pups, the daily repeated stress of manipulation may account for the unresponsiveness to additional effects of separation. This hypothesis is supported by our repeated observations that injections of rat alone prevented subsequent interventions [198]. Since neonatal pre-treatment of the rats with MA did not potentiate the stress effect it speaks for the recovery of the juvenile brain from neonatal MA insult.

When analyzing lipid peroxidation, surprisingly none of the intervention, MA treatment stress or combination of both did affect TBARS (MDA) or 4HNE in striatum after direct or indirect exposure. The only enhanced activation was found in TBARS (MDA) after the combination of stress and MA. Similar trend was followed in 4-HNE. It is difficult to explain, but again, the enhanced sensitivity was observed in indirectly exposed pups. The MA activation of MDA in immature rats is not unequivocal. A chemical congener of amphetamine, mephedrone, when given in high doses to periadolescent rats caused enhanced lipid peroxidation measured as MDA in frontal cortex but not in striatum or hippocampus [199]. The same drug administered chronically during adolescent period resulted in enhanced DA turnover rate in striatum and more importantly in oxidative DNA damage in cortex later in adulthood [200]. Our results clearly demonstrated 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 periadolescent period, however, there are no conclusive data in the literature yet, showing at which level MA produces oxidative stress in immature brain. The immediate effect of early MA intervention with a long-lasting consequence has to be studied in more details a perhaps at the level of more subtle mechanisms, such as (im)balance between pro- and antioxidant enzymes or DNA impairment.

### 5.3.4 Activation of c-fos

In our study, activation of c-fos in HP was lowered by direct MA exposure on PD 28 and eminently by separation within indirectly exposed animals. On PD 35 this protein remained without significant changes. Activation of c-fos on PD 45 showed significant decrease in MA exposed animals again, as well as preweaning housing, however indirectly exposed animals displayed opposite situation. Our results are in contrary to several publications by other authors, but it firstly must be emphasized that our experiment significantly differs in methodology from others. Study by Tomite et al. revealed that high doses of MA (20mg/kg) increased c-fos expression of nine brain regions in mice while not in HP [201].

This study apparently contradicts our results; however, it is important to note, that our dose of MA was 4-times lower and animals in their experiment were adults. Another study by Deng et al. reported that c-fos knock-out mice showed more DNA fragmentation after MA treatment than wild-type, however wild-type mice treated with MA demonstrated a greater number of glial fibrillary acidic protein–positive cells than did c-fos knock-out mice. These data suggest that c-fos induction in response to toxic doses of MA might be involved in protective mechanisms against this drug-induced neurotoxicity [25].

Study by Umino et al. showed that intraperitoneal injection of MA (1.6–4.8 mg/kg) induced a widespread nuclear c-Fos-like immunoreactivity in the brain regions such as amygdala, hypothalamus, thalamus, nucleus accumbens and STR in rats. This study used lower dose of MA, however there is not any evidence of c-fos activity in HP in this study [202].

Results of the study by Cornish et al. showed that acute MA caused increased expression of c-fos in cortical and extended amygdala regions [203]. Interestingly, study by Cabrerizo et al. showed that MA administration during PD 54-57 induced enduring HP cell damage observed on PD 91. This was proven by decreasing cell survival, BDNF content, as well as c-Fos protein content, which is more in agreement with our study however animals in their study were adults [204].

Schmauss et al. (2002) reported that a single dose of MA (5 mg/kg) enhances c-fos expression levels in preadolescent wild-type mice that normally express low c-fos mRNA in response to D1 agonist stimulation, which is again different situation and results in comparison to our study [205].

# 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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Čechová B., Jurčovičová J., Vaculín Š., Šandera Š., Šlamberová R.

**Czech Neuroscience Society Meeting 2021**

Early postnatal methamphetamine administration and changes in environment alter levels of neurotransmitters and oxidative stress in different stages of adolescence in rats.
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**FENS forum 2022**

Does early postnatal methamphetamine administration along with altered environment affect neurotransmitter and oxidative stress levels in adolescence of laboratory rat?
Čechová B., Jurčovičová J., Vaculín Š., Šandera Š., Šlamberová R.

**97. Fyziologické dny**

Early methamphetamine exposure along with altered environment affects neurotransmitters and oxidative stress levels in brain of laboratory rat

Čechová B., Jurčovičová J., Vaculín Š., Šandera Š., Šlamberová R.

**65. česko-slovenská psychofarmakologická konference**

How does early postnatal methamphetamine exposure along with altered environment impact levels of some regulatory proteins in adolescent rat hippocampus?

Čechová B., Jurčovičová J., Vaculín Š., Šandera Š., Šlamberová R.

**98. Fyziologické dny**

Vliv prostředí na hipokampální regulační proteiny dospívajících potkanů neonatálně vystavených metamfetaminu.

Čechová B., Jurčovičová J., Vaculín Š., Šandera Š., Šlamberová R.

**Studentská vědecká konference 2023**

Role of environment in neurotrophin levels in hippocampus of adolescent rat after early postnatal methamphetamine exposure.

Čechová B, Šlamberová R

**11th IBRO World Congress of Neuroscience 2023**

Neurochemical and behavioral consequences of methamphetamine exposure during early and late rat adolescence.

Čechová B, Vaculín Š, Panušková K, Šlamberová R

**The 14th Conference of the Czech Neuroscience Society 2023**

Methamphetamine exposure during adolescence causes molecular and behavioral alterations.

Čechová B, Vaculín Š, Panušková K, Šlamberová R

**66. česko-slovenská psychofarmakologická konference**

Molecular and behavioral alterations caused by methamphetamine exposure to adolescent laboratory rat.

Čechová B, Vaculín Š, Panušková K, Šlamberová R

**FENS forum 2024**

Does methamphetamine exposure alter hippocampal neurogenesis during preweaning period of laboratory rat?

Barbora Čechová, Kristýna Patková, Ivana Fišerová, Šimon Vaculín,Romana Šlamberová

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