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Paternal methamphetamine exposure - effect on the development of offspring

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DECLARATION OF AUTHENTICITY

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CONTENT

ABBREVIATIONS

ANOVA- analysis of variance

CNS- central nervous system

DA- dopamine

DHT- dihydrotestosterone

EMCDDA- European Monitoring Centre for Drugs and Drug Addiction

GnRH- gonadotropin-releasing hormone

i.p.- intraperitoneal application

LH- luteinizing hormone

MA- methamphetamine

MDMA- 3,4-Methylenedioxymethamphetamine

NA- noradrenalin

OPIAD- opioid-induced androgen deficiency

PD- postnatal day

s.c.- subcutaneous application

SA- saline

SERT-serotonin

SHAM- single injection of an inactive substance

T- testosterone

THC- tetrahydrocannabinol

ABSTRACT

Introduction: Drug addiction and its effect on the behavior and development of children has become a serious problem in our society. Methamphetamine (MA) is one of the most abused psychostimulants in the Czech Republic, and its abuse is rising worldwide. Previous studies have demonstrated the adverse long-term effects of maternal drug abuse on rat offspring. However, the father's contribution as a parent and donor of half the genetic information is unclear.

Aim: First, the present study aimed to examine the effect of MA administration on male sexual behavior, locomotor activity, spermatogenesis, and testosterone level. Second, the impact of paternal MA exposure on behavioral development, locomotor activity, and social interaction in rat offspring was examined.

Methods: MA was administrated for 30 days at a dose of 5 mg/kg s.c. to adult male rats (PD 90). The control group was exposed to saline (SA). During the experiments, 6–8 individuals from each group were tested. The sensorimotor development of rat pups was examined during PD 1–23. The Social play experiment was conducted with juvenile rats (PD 30). The sexual behavior, spermatogenesis, and locomotor activity of fathers and offspring were tested in adulthood. Prior to testing, adult offspring were exposed to an acute challenge dose of MA (1 mg/kg) to examine the possible sensitizing effect of the paternal treatment.

Results: Our results demonstrated that MA exposure did not affect the sexual behavior of male rats. Moreover, MA administration did not influence testosterone levels or spermatogenesis in adult males compared to the control group. The data from the Laboras test showed that chronic MA administration impairs locomotor activity in fathers. Further, our results demonstrated a significant increase in locomotor activity on the Laboras test after acute MA application.

Regarding the paternal administration effect on offspring, there were no significant differences in behavioral development or locomotor activity in adulthood. Our data showed that paternal and acute MA administration significantly impaired the social interaction of adolescent offspring. Paternal MA exposure significantly decreases the frequency of pouncing, increases the duration of rearing in males and decreases the duration of mutual sniffing in both genders. When comparing sex differences, males were more active during development, whereas females showed more activity in adolescence and in adulthood than males.

Conclusion: In conclusion, our results demonstrate that chronic MA abuse does not impair the sexual behavior and reproductive functions of adult male rats. Additionally, paternal MA administration does not affect offspring's behavior during development and adulthood, as it was seen after maternal MA administration. However, MA exposure significantly impairs the social behavior of progeny. These results suggest that drug addiction in fathers may not have the same serious consequences for their offspring as drug addiction in mothers. Our study is critical because it is the first to assess the effect of MA on the male's role as a parent and donor of half the genetic information of their offspring.

ABSTRAKT

Úvod do problematiky: Drogová závislost a její vliv na chování a vývoj potomstva se v naší společnosti stává závažným problémem. Metamfetamin (MA) je považován za jednu z nejčastěji zneužívaných drog v České republice, jehož zneužívání roste i celosvětově. Předchozí studie prokázaly negativní účinky zneužívání drog matkami, a především jejich nepříznivý vliv na jejich potomky. Ovšem podíl otce jako rodiče a dárce poloviny genetické informace zůstává dosud neobjasněný.

Cíle práce: Prvním cílem této studie je prozkoumat vliv dlouhodobého podávání MA na sexuální chování samců, jejich lokomoční aktivitu, spermatogenezi a hladinu testosteronu. Druhým cílem je prozkoumat vliv paternitní expozice MA na vývoj, chování, lokomoční aktivitu a sociální interakci u potkaních potomků.

Metodika: MA byl podáván dospělým samcům denně po dobu 30 dní v dávce 5mg/kg subkutánně. Kontrolní skupině byl aplikován fyziologický roztok (SA) ve stejným objemu a ve stejný čas. Během pokusů bylo testováno 6-8 jedinců z každé skupiny. Potkaní mláďata byla testována na senzomotorický vývoj v průběhu postnatálních dnů (PD) 1-23. Adolescentní mláďata (PD 30) byla dále testována v testu sociální hry. V dospělosti byl testován vliv MA na hladinu testosteronu, spermatogenezu a sexuální chování samců. Lokomoční aktivita (Laboras test) byla testována u potkaních otců i jejich potomků. Před testováním lokomoční aktivity byli dospělí potomci vystaveni akutní dávce MA (1 mg/kg) na možný senzitizační účinek paternitní aplikace drogy.

Výsledky: Naše výsledky ukázaly, že expozice MA neovlivnila sexuální chování potkaních samců. Podávání MA navíc neovlivnilo hladinu testosteronu ani spermatogenezi u dospělých samců ve srovnání s kontrolní skupinou. Údaje z testu Laboras ukázaly, že chronické i akutní podávání MA významně ovlivňuje lokomoční aktivitu dospělých samců. Pokud jde o vliv

paternitní expozice MA na potomstvo, nebyly zjištěny žádné významné rozdíly ve vývoji chování nebo lokomoční aktivitě v dospělosti. Naše výsledky ukázaly, že paternitní a akutní podání MA významně snížilo sociální hru u dospívajících potomků. Výsledky dále prokázaly významné zvýšení lokomoční aktivity v testu Laboras po akutní aplikaci MA. Při porovnání rozdílů mezi pohlavími byli samci aktivnější během vývoje, zatímco samice vykazovaly větší aktivitu v adolescenci a dospělosti než samci.

Závěr: Závěrem lze říci, že naše výsledky ukazují, že chronické zneužívání MA nenarušuje sexuální chování a reprodukční funkci dospělých potkaních samců. Navíc paternitní aplikace MA nemá vliv na chování potomků během vývoje a v dospělosti v porovnání s vlivem mateřské aplikace MA. Expozice MA však významně zhoršuje sociální chování potomků. Tyto výsledky naznačují, že drogová závislost u otců nemusí mít pro jejich potomky stejně závažné důsledky jako drogová závislost matek. Naše studie je unikátní, jelikož jako první hodnotí vliv podávání MA z pohledu muže jako rodiče.

INTRODUCTION

Methamphetamine as a psychostimulant drug

Statistical surveys of methamphetamine abuse

Methamphetamine (MA) is a psychotropic stimulant that affects the body on a biological, behavioral, and psychological level. In many countries, it is one of the most widely used illicit drugs. Psychostimulant drugs such as MA activate the dopaminergic and serotoninergic pathways of the central nervous system (CNS), which are mainly associated with reward circuits, affective states, sexual behavior, and also in control of motor function and cognition (Frost and Cadet, 2000). In humans, MA induces feelings of happiness and pleasure, suppresses anxiety and depression, increases concentration, reduces appetite, and promotes weight loss (Logan et al., 2002). Controlling MA abuse has been a significant change over the decades. It is known to be a powerfully addictive drug, and due to its low price and relatively simple production, it has become increasingly popular in our society. The compound was first synthesized from ephedrine in 1893 by the Japanese scientist Nagai Nagayoshi, six years after the discovery of amphetamine. The stable crystallized form of the drug was first synthesized in Japan in 1919 by Ogata, which provides the basis for producing the drug on a larger scale (Panenka et al., 2013). MA was patented in 1920 and later licensed to Burroughs Wellcome, who marketed it as the anorectic Methedrine® (Logan et al., 2002). However, worldwide use did not occur until World War II. During World War II, it was administered to Japanese soldiers on suicide air missions. In the 1950s, MA was legally administered to treat depression, and athletes used MA as a permitted stimulant. After its massive expansion in the 1970s and recognition of severe side effects, its production, and use were declared illegal (EMCDDA and addiction 2009, Mravcik et al., 2007).

In the Czech Republic (previously Czechoslovakia), MA has been illicitly produced since the mid-1970s. Its popularity increased significantly in the 1990s. Recently, it was estimated that there are about three MA abusers per thousand inhabitants in the Czech Republic (EMCDDA and addiction 2009).

In 2017, statistical surveys by the EMCDDA reported 34,700 active MA users in the Czech Republic. The annual report of the EMCDDA in 2019 considered MA the 4th most abused illegal drug in the Czech Republic after cannabinoids, ecstasy, and hallucinogenic fungus (psilocybin). Approximately 66% of male and female drug addicts consider MA their drug of choice (Vavřínková et al., 2001). Currently, most MA users are reproductive-age men between 15 and 34 years.

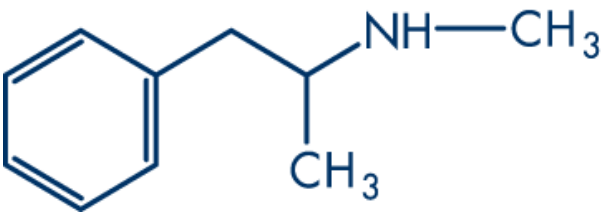
The Czech Statistical Office estimates that about 4.5 tons of MA are consumed annually in the Czech Republic. However, world statistics give far more alarming estimates of MA consumption. A 2018 report by the United Nations Office on Drugs and Crime reported a significant rise in global consumption of amphetamine-type stimulants from 205 tons to 247 tons in one year (data collected in 2015–2016). The same tendency is seen in MA abusers, where global consumption in 2016 increased by about 12% and stabilized at 158 tons. Worldwide, the largest group of abusers is concentrated in East and Southeast Asia, where most of the global MA production is located.

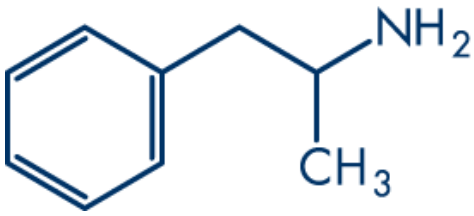
Chemical properties of the methamphetamine molecule

Methamphetamine ((S)-N-methyl-1-phenylpropan-2-amine) is a synthetic drug formed by reductive amination of pseudoephedrine. Methamphetamine hydrochloride is characterized by its manageable volatility and advantageous chemical properties. Due to its characteristics and chemical stability, MA can be administered via various routes, and users typically progress from oral or nasal insufflation and smoking to administration by injection. The effects of MA depend on the route of administration. When the drug is administered orally, the drug's gradual absorption from the gastrointestinal tract leads to a slow release of dopamine in the brain.

In contrast, smoking or intravenous administration of MA causes a rapid increase in neurotransmitter levels (Davidson et al., 2001). About 80% of MA users prefer injection (Mravcik et al., 2007) because when MA is injected subcutaneously, the drug enters the bloodstream faster, and its effect occurs more quickly, frequently causing "cravings" as well as acute health issues associated with overdose.

MA is a lipophilic molecule that rapidly penetrates membrane structures (Dattel, 1990). After MA injection, the drug rapidly distributes in the bloodstream and penetrates the brain. In addition, MA has been shown to cross the placenta and enter the breast milk of mothers, which may affect the prenatal development of the fetus or postnatal development of the newborn during lactation, respectively (Smith et al., 2001). Unlike other addictive drugs, the biological elimination time of MA is very long, reported to be between 12 and 32 hours (Kalina, 2003). This corresponds to the relatively long activity period, roughly 4 to 8 hours (at a dose of 5–50 mg). N-demethylation metabolizes MA to amphetamine via the cytochrome P4502D6 isoenzyme system. Amphetamine is metabolized to various metabolites such as norephedrine and p-hydroxy-amphetamine, both of which are pharmacologically active (Logan et al., 2002).





Methamphetamine Amphetamine

(https://www.unodc.org/wdr2018/prelaunch/WDR18_Booklet_3_DRUG_MARKETS.pdf)

Methamphetamine mechanism of action

Effect of psychostimulants on neurotransmitters

MA is a psychostimulant drug that is similar in molecular structure to amphetamine. Amphetamine and its derivates have a molecular structure similar to catecholamines and, therefore, can either directly increase the concentration of monoamines (dopamine, serotonin, and noradrenaline) or indirectly act as a reuptake inhibitor of the relevant neurotransmitters (Iversen et al., 2013).

Repeated administration of amphetamine induces sensitization that results from increased dopamine release in the mesoaccumbens and nigrostriatal dopaminergic nerve endings (Suzuki et al., 2002). Elevated concentrations of neurotransmitters at inter-neuronal synapses are manifested by prolonged and increased activity at the postsynaptic membrane, which enhances the psychostimulatory effect of the drug (Rothman et al., 2001). Administration of higher doses of MA stimulates the axonal endings of these structures, increasing monoamine reuptake (Weissman and Caldecott-Hazard, 1995). Enhanced release of noradrenaline appears to be responsible for the anorectic effects of amphetamines and, together with dopamine release from dopaminergic nerve terminals, for the locomotor stimulating effects.

The typical stereotyped repetitive behavior characterized by higher doses of amphetamines is also caused by dopamine release, particularly in the neostriatum. Enhanced release of 5-hydroxytryptamine (serotonin) may be responsible for both disturbances of perception and psychotic behavior (Logan et al., 2002, Ellinwood and Kilbey, 1980). It seems that the effect of cocaine (another psychostimulant drug) is mediated through dopaminergic, serotoninergic, and adrenergic neurotransmission. In contrast, the effects of amphetamine are mediated mainly through dopamine and noradrenaline (Ševčíková et al., 2020, Achterberg et al., 2016, Trezza et al., 2010, Vanderschuren et al., 2008).

Effect of MA exposure on neurotransmission

The effects of MA exposure result mainly from an acute increase in serotonin and dopamine levels. MA abuse causes neuronal damage (apoptosis in the striatum and prefrontal cortex, long-term damage to dopaminergic and serotonergic axon terminals in the hippocampus, striatum, and prefrontal cortex) (Zhu et al., 2006, Wagner et al., 1980, Ochozková et al., 2021). Consequences of MA exposure include a reduction in the volume of several brain structures, such as the hippocampus, dentate gyrus, and striatum (Bubeníková-Valešová et al., 2009, Chang et al., 2004, Šlamberová et al., 2006), followed by long-term cognitive deficits, e.g., impairment in spatial learning and memory (Chang et al., 2004, Hrebíčková et al., 2014). Cognitive functions are altered quantitatively and manifest as increased concentration and attention with a reduced ability to effectively select appropriate stimuli (Silber et al., 2006). The increase in noradrenaline levels after MA exposure results in an overall stimulation of the sympathetic nervous system, leading to a release of energy resources and an increase in locomotor activity. Elevated MA levels are associated with increased motor activity and a reduced sense of fatigue. The characteristic “fight or flight” effects of MA are mediated mainly through the sympathetic α-receptors (Logan et al., 2002).

Effect of MA abuse on the human body

As a highly potent stimulant, MA increases blood pressure, heart rate, and body temperature and causes bronchodilation and an overall elevation in respiratory activity. A typical symptom is dilated pupils (mydriasis). The addicted person may have a dry mouth, dizziness, and shivering. MA also reduces appetite and has anorectic effects. The cardiac effects of MA may be indirectly connected to the release of adrenaline into the circulation (Perez-Reyes et al., 1991) and may lead to changes in heart muscle following chronic use.

Chronic MA use is associated with malnourishment (Werb et al., 2010). Oral administration can result in "meth mouth" (Rhodus and Little, 2008), a dental condition associated with severe decay and loss of teeth. Skin lesions are commonly observed in MA abusers due to the compulsive scratching that accompanies drug use (Kerr et al., 2005, Panenka et al., 2013). MA abusers experience euphoria, joy, well-being, and a sense of self-confidence (Iversen et al., 2013, Mravčík et al., 2007), which often involves the loss of social restraints. MA also has an anxiogenic effect, which can lead to anxiety, fear, panic, paranoia, and hallucinations. When higher doses of MA are administered frequently over a short period, forms of stereotypic behavior (Robinson and Becker, 1986) or a state of acute psychosis (Bell, 1973) have been reported. Cessation of high doses of MA in humans and animals induces psychological effects opposite to the acute effects of the drug (Koob and Volkow, 2010). Thus, the positive effects of energy, confidence, and elevated mood are replaced by fatigue, anxiety, depression, inability to concentrate, and even suicidality (Panenka et al., 2013). Acute MA overdose is associated with elevated blood pressure, tachycardia, chest pain, dyspnea, shivering, a very high fever, as well as mental changes such as psychosis, agitation, and suicidal tendencies. The most common cause of overdose death is cardiac collapse or stroke (Iversen et al., 2013).

Gender differences in MA abuse

MA releases dopamine and serotonin equally, while amphetamine is more specific to dopamine (Sabol et al., 1995). This difference in the release of serotonin could impact the behavioral differences between genders related to the known differences in the serotonergic system between males and females (Biver et al., 1996, Rubinow et al., 1998, Zhang et al., 1999).

Gender differences are also reflected by how MA is metabolized and eliminated from the body. Studies have shown that drug elimination is much slower in females due to the activity of degradative enzymes, especially cytochrome P450 (Roth and Carrol, 2004). In addition, females are more sensitive to drug exposure due to their body composition, lower body weight, and higher proportion of adipose tissue, resulting in higher plasma and brain concentrations when administered the same MA dose as males (Rambousek et al., 2014).

When considering the mechanisms of action relative to sex differences in drug abuse, it is important to consider gonadal hormones. These hormones include testosterone in males and estrogen and progesterone in females. Adult women secrete small amounts of testosterone compared to adult men, who have plasma concentrations of testosterone 7–8 times higher because men utilize more testosterone. Daily testosterone production in males is about 20 times greater than in women (Southren, 1965, 1967). A study by Forgie and Stewart (1993) indicated that neonatal exposure to testosterone suppresses responsiveness to amphetamine (a psychostimulant drug) in adult males. However, females were more responsive to amphetamine regardless of testosterone exposure due to lifelong exposure to estradiol, which alters responsiveness to the drug (Forgie and Stewart, 1993). Previous literature showed that ovarian hormones are related to the sex differences observed in drug abuse (Roth and Carrol, 2004).

The phase of the reproductive cycle and the concentration of female hormones are essential aspects that influence the effect of MA. Estrogens have been experimentally shown to increase the body's sensitivity to MA. Previous studies report that females are more sensitive to the acute and chronic effects of methamphetamine on locomotor activity (Mattei and Carlini, 1996). Females have also been shown to be more sensitive to the behavioral effects of other psychomotor stimulants (Becker, 1999, Robinson et al., 1982, Stohr et al., 1998). The association between the effect of MA and a woman's reproductive cycle has also been investigated (White et al., 2002). These studies showed that the effect of MA was more potent during the follicular phase (compared to the luteal phase), where the level of estrogens was significantly increased. A study by Shen et al. (2014) demonstrated that long-term use of MA disrupts menstrual cycles and leads to dysfunction of the hypothalamic-pituitary-gonadal axis in women. Despite of negative impact of MA exposure on the menstrual cycle, previous studies by Dluzen et al. (2000) indicate that estrogens can act as a neuroprotectant against nigrostriatal dopaminergic neurotoxicity after drug exposure. However, the same effect was not observed in the case of testosterone. Women tend to become drug addicted more quickly; however, they are less likely to relapse during withdrawal than men (White et al., 2002).

Maternal psychostimulant exposure

Psychostimulants affect the behavior of addicts, increase aggression and disrupt social and maternal behavior (Šlamberová et al., 2013, Holubová et al., 2019). These facts highlight the very serious worldwide problem of women abusing MA during pregnancy (Marwick, 2000). While MA abuse has apparent advantages for drug-addicted pregnant women compared to other drugs, such as helping to maintain lower body weight, reducing appetite, and increasing energy, prenatal MA exposure causes many negative impacts. Since MA crosses the placenta, maternal MA exposure may cause prenatal hypoxia and fetal malnutrition, resulting in irreversible fetal changes. Maternal MA exposure has also been shown to cause morphological changes in the brain and altered brain metabolism (Abar et al., 2014, Acuff-Smith et al., 1996). Previous studies showed that MA administration to pregnant rats impairs postnatal sensorimotor development of pups during the pre-weaning period (Hrubá et al., 2009, Šlamberová et al., 2006, Ševčíková et al., 2020), affects learning abilities (Macúchová et al., 2013), and leads to a higher susceptibility to drug sensitization in adulthood (Šlamberová et al., 2009).

Furthermore, prenatal exposure to MA impairs cognition (Hrebíčková et al., 2017, Macúchová et al., 2017, Šlamberová et al., 2014), evokes anxiety-like behavior in offspring (Schutová et al., 2009, Macúchová et al., 2016) and affects pain sensitivity later in life (Yamamotová et al., 2011). Prenatal MA exposure is also associated with increased physiological stress, decreased arousal, and poor quality of movement during the first five days of life (LaGasse et al., 2011, Smith et al., 2008). Previous studies showed that low doses of MA (1–2 mg/kg) cause increased dopaminergic activity in the nucleus accumbens (Kelly et al., 1975) that manifested as increased locomotor activity and exploration (Schutová et al., 2010; Schutová et al., 2009, Hrubá et al., 2012). This effect of MA exposure on behaviors in adulthood was even higher in animals prenatally exposed to maternal MA during gestation and corresponded with increased dopamine

levels (Bubeníková-Valešová et al., 2009). Maternal MA abuse has also been shown to affect stress responses in adult animals (Holubová et al., 2016). Alterations in behavioral patterns manifested in social behavior during adolescence (Ševčíková et al., 2020) and in aggressive and sexual behavior during adulthood (Hol et al., 1999). Previous studies also demonstrated that the administration of MA during pregnancy attenuates the maternal behavior of rat mothers (Šlamberová et al., 2005, Šlamberová et al., 2012).

In the general population, however, MA abuse is frequent in both female and male populations, primarily because of its stimulant effects and easy availability. While the effect of MA on mothers and offspring has been the subject of detailed research over the past few decades, the effect of MA on fathers and offspring has received far less attention. Therefore, our study aimed to investigate the impact of paternal MA abuse on the behavior of addicted male rats and their offspring.

Effect of psychostimulants on the social behavior of offspring

An important part of the development, which occurs in all mammalian species, involves characteristic patterns of social play behavior (Panksepp et al.,1984, Ševčíková et al., 2020). Social play is modulated by neurotransmitters involved in reward and motivation circuits, which makes social play a natural reinforcer (Vanderschuren et al., 1997, Douglas et al., 2004, Siviy and Panksepp, 2011, Ševčíková et al., 2020). Recent literature provides evidence that social-cognitive functions are significantly affected by chronic MA abuse and causes social isolation, depression, and aggressiveness in addicts (Homer et al., 2008). Previous studies demonstrated that neurotoxic effects of prenatal MA exposure on dopaminergic and serotoninergic neurons might cause neurochemical changes in the CNS associated with alterations in social interactions of rats (Homer at al., 2008, Schutová et al., 2013, Šlamberová et al., 2015, Hrebíčková et al., 2017). MA administration impairs social interaction in a dose-

and stress-dependent manner (Šlamberová et al., 2010). Additionally, the unknown environment of the open field test arena led to increased exploratory behavior (locomotion and rearing) in all MA-treated groups.

A study by Arakawa (1994) showed that MA and methylphenidate (indirect dopamine agonists) administration induce less interaction and decreases social behavior in rats. An earlier study by Syme (1974) correlates with recent study outcomes. The study showed that MA significantly reduced social contact more in the unfamiliar group than in the familiar group of rats. An acute MA exposure significantly decreased all social interaction patterns and increased non-social activities compared to the acute saline group (Hrebíčková et al., 2017). However, prenatal/early postnatal MA exposure decreased only specific patterns of social interaction, such as the time spent in genital investigation or following; it also decreased non-social activity in rats. A study by Šlamberová et al. (2015) confirmed that psychostimulants (e.g., amphetamine, cocaine, and 3,4-Methylenedioxymethamphetamine (MDMA)) suppress social interaction but also indicate differences in the effects of psychostimulants on specific patterns of social interaction. Amphetamine exposure decreased sniffing, allogrooming, and following behaviors. Higher doses of cocaine decreased social interaction but did not affect locomotor activity. Administration of MDMA decreased mutual sniffing and climbing over each other; at higher doses, MDMA decreased allogrooming and following behaviors. Moreover, higher doses of amphetamine and all the doses of MDMA increased locomotion and rearing.

Paternal drug exposure

Effect of paternal drug exposure on offspring

There are few studies examining the impact of paternal drug exposure on rat pup development and behavior in adulthood. A study by Abel et al. (1989) showed that paternal cocaine administration leads to increased hyperactivity and behavioral changes in rat pups. Other studies report that paternal cocaine exposure affects the birth weight of offspring, with birth weights being decreased, increased, or unchanged (Abel et al., 1989, Fischer et al., 2017, George et al., 1996, He et al., 2006, Killinger et al., 2012, White et al., 2016). Relative to neurocognitive outcomes, a study by Le et al. (2017) showed that the offspring of addicted male rats had increased cocaine self-administration. Studies by Bielawski et al. (1997, 2002) found that paternal alcohol abuse results in offspring malformations and reduced fetal weight. Another study by Dalterio et al. (1984) showed that paternal THC (delta-9-tetrahydrocannabinol) exposure significantly impairs the development of rat pups. On the other hand, a study by Levin et al. (2019) demonstrated that paternal THC exposure does not significantly impact the clinical health of the offspring, including litter size, sex ratio, pup birth weight, survival, and growth. However, it results in neurocognitive alterations with increased habituations of locomotor activity and decreased attentional function of offspring in adulthood (Levin et al., 2019).

The above few studies present very conflicting findings about the effect of drugs administered to male rats and the effect on their offspring. There are no published studies examining the effects of paternal MA on the postnatal development of rat offspring and persistence into adulthood. Our study attempts to fill a gap in second-generation drug addiction research.

Effect of methamphetamine exposure on fathers

Effect of drug exposure on spermatogenesis

Rats were considered pubertal until 50 days of age, when spermatozoa were first found in the tail of the epididymis. Sperm production increases up to age 75 days, and testicular weight increases until 100 days of age. Sperm reserves in the tail of the epididymis were not maximal until 100 days of age. Therefore, Wistar rats should not be considered sexually mature until 100 days (Robb et al., 1978). Sexually mature rats had testes weighing 3–7g, and the average sperm concentration ranged from 152.5–230.0 $\times 10^7$ spermatozoa/ml (Kempinas and Carvalho, 1988).

Drugs such as cocaine, amphetamines, and cannabinoids may adversely affect the quality and quantity of sperm and result in infertility of drug users (Verstegen et al., 2020). Previous experiments have demonstrated the adverse effect of cocaine abuse on reproduction and spermatogenesis in males (George et al., 1996). A study by George et al. also found that the significant toxic effect of cocaine on spermatogenesis is attributed to the ischemic effect of cocaine. Cocaine, which enhances noradrenaline and adrenaline release, induces intense vasoconstriction. Moreover, it has been demonstrated that chronic cocaine administration increases germ cell apoptosis (Li et al., 1999). Other studies have shown the negative impact of cocaine and its metabolites on Sertoli cell function (Zhang and Loughlin, 1996). Cocaine-induced apoptosis in sperm cells is significantly increased 15 days after the first administration of the drug (Li et al., 1999), reaches a maximum 30 days after application, and persists until day 90. It has been known since the 1980s that ethanol and acetaldehyde (the first ethanol metabolite) are potent Leydig cell toxins (Van Thiel et al., 1983). Moreover, it has been experimentally determined that alcohol abuse reduces cytosine methyltransferase mRNA levels in sperm cells of addicted males (fathers), leading to spermatogenesis malfunctions (Bielawski

et al., 1997). Opioids are also known to impair spermatogenesis. Rats chronically treated with tramadol (a commonly prescribed effective opiate painkiller) exhibited degenerative histological changes in the seminiferous tubules, the Sertoli cells, and the Leydig cells (Abdellatief et al., 2015). We know that human spermatozoa express μ-, δ-, and κ- opioid receptors located on the head, middle region, and tail of sperm (Agirregoitia et al., 2006).

Other substances such as nicotine, cannabis, and amphetamines also alter spermatogenesis by inducing oxidative stress and subsequent apoptosis in testicular tissue. The study by Condorelli et al. (2013) showed that nicotine is responsible for all the negative impacts of cigarette smoke on sperm. Chronic, intensive marijuana usage has been associated with oligospermia in 35% of men who provided semen samples (Kolodny et al., 1974). Human spermatozoa express cannabinoid receptor 1 (CB1) receptors, and in vitro studies exposing human spermatozoa to marijuana extracts have demonstrated decreased sperm motility, viability, and function (Schuel et al., 2002, Rossato et al., 2005, Whan et al., 2006). Daily administration of synthetic THC derivates leads to a significant reduction in sperm count and daily sperm production and a reduction in the number of Sertoli cells (Lewis et al., 2012).

A previous study also demonstrated that MA administration significantly decreases cell proliferation and increases apoptosis in rat spermatogonia and primary spermatocytes (Alavi et al., 2008). A study by Saberi et al. (2017) demonstrated the adverse effects of MA exposure (after 7 and 14 days) on rat testes structure and spermatogenesis. A study by Montagnini et al. (2014) demonstrated that repeated administration of methylphenidate (Ritalin) during childhood to early adulthood interfered with testicular functions in adult rats. Moreover, a study by Gonzalez et al. (2015) showed the potential role of the local dopaminergic system in psychostimulant-induced testicular pathology. The above studies show that drug exposure may induce reduced sperm cell production and leads to changes in sexual behavior.

Effect of drugs on the sexual behavior of rats

The mechanism of action for MA is based on the release of neurotransmitters (dopamine (DA), serotonin (SERT), and noradrenaline/norepinephrine (NA/NE)) by the central nervous system (CNS) and by blockade of their reuptake from synapses and into synaptic vessels (Rothman et al., 2001, Kish et al., 2009). Dopamine in the nigrostriatal tract influences motor activity, in the mesolimbic tract it activates behaviors including copulation, and in the medial preoptic area, DA controls genital reflexes, copulatory patterns, and sexual motivation. In addition, SERT positively or negatively affects copulatory patterns such as erection and ejaculation by targeting a specific subtype of the 5-HT/serotonin receptors (Hull et al., 2004). According to these studies, DA and SERT strongly influence sexual behavior. Therefore, there is a possibility that MA could mediate sexual activities in drug-exposed males. Some MA users report experiencing enhanced sexual pleasure, sexual confidence, and sexual performance compared to when they were not using MA (Semple et al., 2005). A previous study by Winland et al. (2011) showed that MA enhances sexual motivation in female rats, i.e., MA-treated female rats were less discriminating about how and with whom they mated and were more interested in sex than SA-treated female rats. A study by Frohmader et al. (2010) demonstrated that MA administration in male rats activates neurons in the brain's mesolimbic system, which regulates sexual behavior. As for amphetamines, experimental animal models have demonstrated that repeated exposure to amphetamine stimulates sexual behavior in naïve male rats regardless of the environment in which the experiment was conducted (Fiorino and Phillips, 1999). Prenatal cocaine and morphine exposure also affect the development of spinal sexual reflexes in males (Vathy et al.,1998).

Effect of drug exposure on testosterone levels in males

Testosterone physiology

Testosterone is the primary androgenic steroid hormone. Testosterone is secreted mainly by the Leydig cells of the testes of males and the ovaries of females. In both sexes, testosterone is synthesized in the adrenal gland cortex (Burger, 2002, Dohle et al., 2003, Celec et al., 2015). The hypothalamic-pituitary-gonadal axis regulates sexual behavior in male rats. Gonadotropin-releasing hormone (GnRH) released from the hypothalamus initiates the release of luteinizing hormone (LH), which in turn stimulates the release of testosterone from the testes (Shulman and Spritzer, 2014, Neave, 2007, Nyby, 2008). Male rats reflexively release testosterone when they smell or mate (ejaculatory release) with a novel receptive female. LH is elevated 10 min after exposure to a female and is followed by a release of testosterone about 30 min after exposure (Shulman and Spritzer, 2014, Nyby, 2008, Pfaus et al., 2001, Kamel et al., 1978). GnRH secretion in adulthood is pulsatile, with the highest testosterone peaks during the early morning hours (Lord et al., 2014). Although, the circulating serum testosterone concentration decreases with age, mainly due to the attrition of Leydig cells and declined secretion of hypothalamic GnRH (Basaria, 2013, Celec et al., 2015).

As a steroid hormone, testosterone crosses cellular membranes and binds to testosterone receptors before attaching to DNA and facilitating RNA and protein synthesis (Handelsman, 2010). Testosterone and its metabolites initiate male sexual behavior by acting on key brain regions. Aromatization of testosterone to estradiol in the medial preoptic area of the hypothalamus is essential for initiating copulation, while dihydrotestosterone, another testosterone metabolite, is crucial for controlling genital reflexes (Shulman and Spritzer, 2014, Hull et al., 2007).

Testosterone is the principle androgen responsible for sexual development and maintaining male secondary sexual characteristics (Johnson et al., 2013). Testosterone is the only steroid hormone essential to maintain spermatogenesis (Smith and Walker, 2014). The primary cellular target and translator of testosterone signals are the Sertoli cells. In the Sertoli cells, testosterone signals can be translated directly to changes in gene expression (the classical pathway), or testosterone can activate kinases that regulate processes required to maintain spermatogenesis (Walker, 2011). Without testosterone or functional androgen receptors, males are infertile because spermatogenesis rarely progresses beyond meiosis (De Gendt et al., 2004).

Testosterone (T) and its metabolic products, dihydrotestosterone (DHT) and estradiol, directly lead to sexual maturation, indicated by increased production of steroids during puberty (Jameson and Finlayson, 2010). Testosterone and its derivatives are responsible for the increases in muscle mass, bone growth and mass, and body hair growth and distribution; additionally, T is responsible for the growth of the penis and the scrotum and the development of male secondary sexual characteristics. T also exerts anabolic effects in both sexes and influences behavior (Jameson and Finlayson, 2010). T has been associated with aggression, violence, and sexually motivated behavior (Dabbs et al., 1987, Isidori et al., 2005, Montoya et al., 2012).

Testosterone levels of males who self-reported to be in excellent health correlated with physiological levels of T. Daily average testosterone levels in men range from 300–1000 ng/dL (Johnson et al., 2013). Male testosterone levels below the reference range indicate partial or complete hypogonadism caused by testicular failure. Low testosterone levels are encountered in male patients with the following diseases: primary hypogonadism (e.g., Klinefelter's syndrome), testicular feminization, enzymatic defects, anorexia, liver cirrhosis, drug abuse, or intake of anabolic steroids.

Much (40–50%) of the total testosterone in the blood is bound with high affinity to sex hormone-binding globulin (SHBG), while 40–50% is bound with low affinity to albumin, and only 1–2% exists as unbound or free testosterone. Only free testosterone and albumin-bound testosterone are considered bioactive (Cumming and Wali, 1985).

Testosterone levels in rats

The testosterone levels in adult male rats have been shown to be significantly higher than in females. Plasma testosterone concentrations in adult male rats and female rats during estrus and proestrus were determined to be 5.71 ± 0.84, 1.24 ± 0.29, and 0.80 ± 0.36 ng/ml, respectively (Falvo et al., 1972). The interval between birth and sexual maturity in rats is approximately 50 days (Lee et al., 1975). A study by Lee et al. found a rise in testosterone levels from PD 25 that persisted until sexual maturity. Moreover, a stepwise rise occurred to reach levels in excess of 230 ng/100 ml between PD 70–80. Another study (Heywood, 1980) found that plasma levels varied significantly during the day, with the acrophase occurring between 9.00–13.00 hours.

Moreover, the study showed that testosterone levels varied significantly during the day, not only in peripheral plasma but also in testicular venous plasma and testis of adult male rats (Heywood, 1980). The plasma levels of testosterone are elevated during sexual intercourse. However, a study by Shulman and Spritzer (2014) concluded that when male rats have daily sexual interactions, sexual behavior tends to show cyclic changes, and testosterone levels are significantly elevated only on the first day of sexual interaction.

Effect of drug exposure on testosterone levels

Alcohol, opioids, and anabolic steroids can reduce testosterone production in males, thus interfering with testicular and/or hypothalamic-pituitary function (Duca et al., 2019). Van Thiel et al. (1983) showed that alcohol-abusing rats had testosterone levels reduced by 50% compared to the control group. Moreover, in vitro studies demonstrated that rat testes perfused with ethanol and acetaldehyde showed reduced production and secretion of testosterone. Decreased testosterone level in heavy drinkers is linked to reduced Leydig cell production and increased androgen metabolism. Alcohol also induces the aromatase enzyme that catalyzes the conversion of testosterone in estradiol (Gordon et al., 1979). Opioid-induced androgen deficiency (OPIAD) is a well-known syndrome characterized by decreased testosterone levels, reduced libido and muscle mass, fatigue, and osteopenia (Hsieh et al., 2018). However, the inhibitory effects of opioid drugs on the hypothalamic–pituitary–testicular axis and, thus, on testosterone production, have been described for over 40 years (Froczak et al., 2012, Duca et al., 2019). Anabolic-androgenic steroids are drugs frequently used by athletes, amateur athletes, and bodybuilders to improve sports performance (Duca et al., 2019). Anabolic-androgenic steroids suppress gonadotropin release from the pituitary gland by a negative feedback mechanism that results in down-regulation of gonadotropins and decreased secretion of testosterone (La Vignera et al., 2018).

It has also been demonstrated that nicotine and its metabolites inhibit multiple steps in testosterone biosynthesis (Duca et al., 2019). Chronic administration of nicotine in male rats leads to a reduction in testosterone levels (Kavitharaj and Vijayammal, 1999). However, the decreased testosterone levels seem to return to physiological levels after nicotine cessation, indicating the potentially reversible effects of nicotine on Leydig cell function (Oyeyipo et al.,

2013). Chronic, intensive marijuana usage has been associated with decreased serum testosterone levels in a dose-dependent manner (Kolodny et al., 1974).

However, a recent population study on 1,500 men indicates no differences in serum testosterone levels among marijuana users compared to non-users (Thistle et al., 2017). Surprisingly, the chronic administration of cocaine to rats (15 mg/kg for 100 days) did not induce changes in testosterone levels (George et al., 1996). However, in another study, male Wistar rats receiving low doses, i.p., injections of cocaine, showed an increase in testosterone concentration, but the same effect was not demonstrated with high doses of cocaine (Rodriguez et al., 1992).

In vivo and *in vitro* studies have demonstrated that amphetamine exposure decreases testosterone concentrations (Tsai et al., 1996). However, a single intraperitoneal administration of methamphetamine showed a biphasic effect on testosterone production in mice: serum testosterone concentrations, which initially decreased and then increased (Yamamoto et al., 1999). Rats chronically treated with high doses of MA have lower testosterone levels than controls (Lin et al., 2014).

HYPOTHESIS

Previous studies focused mainly on the adverse impact of MA on the development and behavior of offspring from the perspective of mothers. Surprisingly, few investigated the effect of paternal MA exposure on offspring. Therefore, the aim of my thesis is:

1) To investigate the effects of chronic 30-day MA administration on reproductive toxicity and sexual behavior of adult male rats.
2) To investigate the impact of paternal MA administration on the development, social behavior, and locomotor activity of offspring, evaluated using behavioral methodologies.

Based on current findings regarding MA abuse and its consequences, we hypothesize that:

1) The long-term application of MA (30 days) to adult male rats should induce changes in their reproductive system. Hypothetically, we expect increased sexual and locomotor activity after MA application. However, it is questionable whether long-term MA administration will induce stereotypic behavior and rejection of a sexual partner. Another unanswered question is whether the MA application will affect spermatogenesis and testosterone levels in males. Since this effect has been demonstrated in other psychostimulants, we expect reduced sperm production and a negative impact on reproductive functions after long-term MA exposure.
2) Paternal MA administration (30 days) could also lead to changes in rat pup functional development, social behavior, and locomotor activity, as occurs with maternal MA administration.

Based on our hypotheses, we set the following aims for the dissertation:

1. Investigate the impact of MA administration on **male sexual behavior**.
2. Investigate the effect of MA exposure on **male spermatogenesis** and analyze testosterone concentrations.
3. Investigate the impact of paternal MA exposure on the functional and **morphological development of rat pups**.
4. Investigate the effect of paternal MA exposure on the **social behavior of adolescent progeny** and the **locomotor activity of offspring in adulthood**.

My research is related to previous and current projects in our department that have focused mainly on the impact of prenatal maternal MA exposure and its influence on maternal behavior and offspring development. According to previous results, we have reason to believe that behavioral changes may occur in the offspring of MA-exposed fathers, and we anticipate functional changes in the reproductive system of exposed male rats. At the same time, I hope my work will help clarify the mechanism of paternal drug addiction and its negative consequences and provide new insights into the field of drug addiction.

METHODS

Laboratory animal breeding

Adult male and female Albino Wistar rats were purchased from Velaz (Prague, Czech Republic) raised by Charles River Laboratories International, Inc. Males (300-350 g) were housed 2 per cage and females (250- 300 g) were housed 3 per cage. All animals were left undisturbed for a week in a temperature- controlled (22-24 °C) colony room with free access to food and water on a 12 hours light: 12 hours dark cycle with lights on from 6:00 a.m. After one week of acclimatization, adult males were randomly assigned to methamphetamine-treated (MA) group and saline-treated (SA) group. D-methamphetamine hydrochloride was administrated subcutaneously (s.c.) in a dose of 5 mg/ml/kg for 30 days. This administration period was chosen in accordance to adverse impact of cocaine as also a psychostimulant on sexual behavior and spermatogenesis in male rats (Li et al.,1999). Moreover, this dose of MA induces similar behavioral changes that correspond to those found in humans and which is standardly use in all our experiments (Šlamberová et al., 2005). Control group was exposed to saline s.c. injection (1 mg/kg) at the same time in the same volume as MA group.

All experimental models used in our laboratory at the Department of Physiology of Third Faculty of Medicine are using approved methods for the study of drug addiction and long-term effects of MA exposure on morphological and functional development of the laboratory rat. The aim of our research is to investigate the influence of methamphetamine (Pervitin) as one of the most commonly abused "hard" drugs in the Czech Republic.

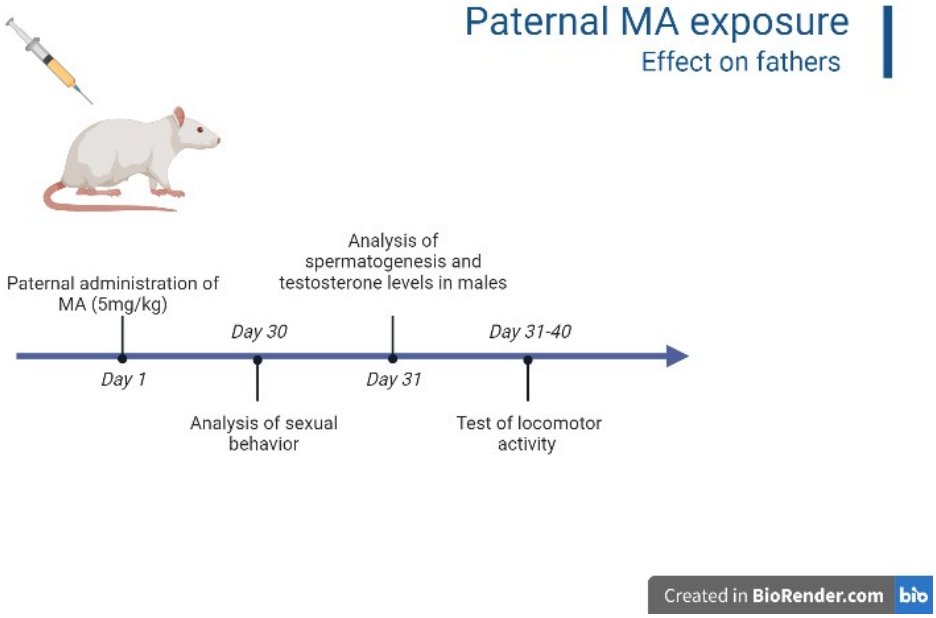
Fertilization

After 30 days of MA administration, male rats were mated with non-treated females. As behavior in females can differ depending on phase of the estrous cycle, the phase of the cycle was determined by vaginal lavage smears subsequently 2-3 days before mating. The smears were examined by light microscopy. At the onset of the estrous phase of estrous cycle (Turner and Bagnara, 1976), the males and receptive females (females in proestrus or estrus) were introduced into transparent plexiglass cages (40x20x20 cm), with one male and one female ratio.

After a week, females were separated from males and left undisturbed until the day of delivery, postnatal day (PD) 0. On PD 1, the number of pups in each litter was adjusted to 12 (whenever possible, half males and half females).

Methamphetamine administration

The effect of MA exposure on male rats were examined by behavioral experiments, microscopical analysis of spermatogenesis and biochemical analysis of testosterone levels. The effect of paternal and acute MA administration in adulthood were examined by behavioral tests on offspring of MA/SA-exposed fathers. To clarify the gender differences affected by MA exposure, both males and females were examined. Prior to behavioral testing, animals were exposed to acute challenge dose of MA (1 mg/ml/kg) in adulthood to examine the possible sensitizing effect of the paternal treatment. This dose of MA, as compared to higher doses, is not responsible for stereotyped patterns of behavior and does not affect the test performance (Kelly et al., 1975). The other half of animals were administered SA (1 ml/kg) at the same time.



Phase of estrous cycle

Previous studies show that female sex hormones (estrogen and progesterone) increase sensitivity to the drug (White et al., 2002). The effect of drug was more potent during the follicular phase compared to the luteal phase, thus sensitivity to MA positively correlates with increased estradiol levels. Due to fluctuating levels of estrogen and progesterone, the proportion of cells in the cytological sample has changing. The levels of estrogen and progesterone are reduced in the metestrus to diestrus phase (M/D). The sample consists of leukocytes and very low presence of epithelial cells. In the proestrus to estrus (P/E) phase, sex hormone levels are highest, there are no or very few leukocytes and numerous of epithelial cells have a regular cubic shape (Hubscher et al., 2005, Marcondes et al., 2002, Simpson and Kelly, 2012).

Vaginal lavage was performed on the morning of the experimental day and a light microscope (40x magnification) was used to determine the phase of the estrous cycle. Females were assigned to the M/D or P/E phase group and then used in experiment.

BEHAVIORAL TESTS OF FATHERS

Sexual behavior testing

The experiment was initiated after 30 days of MA administration, when 8 MA-treated and 8 SA-treated male rats were mated with non-treated females. The experiment has conducted after 5 pm in the same laboratory under dim light, when animals are becoming more active. The occurrence and disappearance of phases of sexual mating were determined by following parameters of mating behavior (Agmo et al.,1995, Zanoli at al.,2005): numbers of mounts before ejaculation or mounting frequency (MF), numbers of intromissions before ejaculation or intromission frequency (IF), time from the introduction of the female up to the first intromission by male or intromission latency (IL), ejaculatory frequency (EF) and pre-coital sexual behaviors such as sniffing and nosing time (sec). The observed parameters were recorded up to 2 hours of pairing and used for further analysis (Kataria et al., 2013). Each parameter of sexual mating was analyzed separately by *t-test.* Differences considered as significant if $p<0.05$.

Test of locomotor activity - Laboras test

The Laboras apparatus (Metris B.V., Netherlands) is a fully automated system for continuous behavior recognition and tracking of small rodents (Schutová et al., 2013). It consists of a triangular-shaped sensor platform connected to a computer. The platform transforms the mechanical vibrations from movements of the animal into electrical signals. Each movement pattern has its own unique frequency and amplitude, and thus separate behavioral categories can be easily distinguished and classified by the computer. Behavioral testing was performed from 12 pm to 6 pm in a darkened room. The test is conducted under low light intensity and animals are tested in the unknown environment.

Two weeks before the experiment, the light- dark cycle was reversed in the colony room (lights on from 6:00 pm for 12 hours) to achieve higher activity in animals. Each animal was tested and recorded for 1 hour. Lately, the 1-hour period was divided into six consecutive 10-minute intervals to follow the changes in behavior during the habituation time in the Laboras apparatus (Šlamberova et al., 2018). The following parameters were automatically evaluated by software: time spent in locomotion (s), time spent immobile (s), time spent rearing (s), time spent grooming (s), distance travelled (m) and average speed (mm/s). Each measured parameter was evaluated separately by using a two-way ANOVA (*paternal exposure x acute exposure)* with repeated measure (*intervals).* The Bonferroni test was used for *post-hoc* comparisons. Differences considered significant if $p<0.05$.

Laboras Metris Netherlands

Laboras test in adult male rats

16 MA-exposed and 16 SA exposed male rats were used in the experiment. Prior to testing, acute dose of MA (1 mg/kg) or SA in a volume of 1 ml/kg was administered to adult male rats to determinate the sensitizing effect of the drug. We have 4 experimental groups in total, in each group was 8 animals.

Table 1 Experimental groups

	Experimental groups			
30-day application period	MA	MA	SA	SA
Acute application	MA	SA	MA	SA
Number of animals	8	8	8	8

ANALYSIS OF TESTOSTERONE LEVEL AND SPERMATOGENESIS IN FATHERS

Analysis of testosterone level

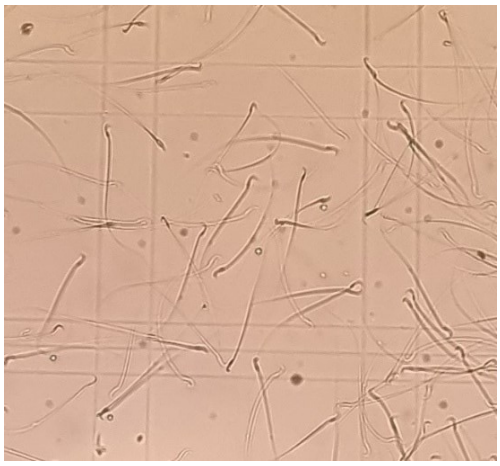
Adult male rats (n=18) after 30 days of MA administration were used to determine the testosterone plasma levels by ELISA analysis. 24 hours after the last MA application, the rats were sacrificed by decapitation and the blood was collected into precooled plastic K3 EDTA tubes (Monovette). The plasma samples were collected in morning hours (8-10 am), when the testosterone levels are increased. Afterwards, the plasma was separated in centrifuge for 20 minutes. The plasma samples were collected and stored at the temperature -80 degrees of Celsius until the test was provided.

Levels of testosterone were measured by competitive ELISA method. ELISA is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the principle of competitive binding (Microsoft Word - IFU AA E-1300 V9.0 (ldn.de)). The microtiter wells are coated with a monoclonal (mouse) antibody directed towards a unique antigenic site of the testosterone molecule. At the first incubation that lasts for 60 minutes, the testosterone in the 25 µl of each sample competes with the 200 µl of added enzyme conjugate (testosterone conjugated with horseradish peroxidase) for binding to the coated antibody. After the washing procedure removed all unbound substances, the solid phase was incubated for 15 minutes with 200 µl of substrate solution (*Tetrametylbenzidine- TMB*) at the room temperature. The colorimetric reaction was stopped by adding 100 µl of stop solution (*0.5M H2SO4*). Approximately 10 minutes after adding the stop solution, the optical density of the resulting yellow product was determined at 450 nm (reading) and at 620 nm to 630 nm (background subtraction) with a microtiter plate reader. The color intensity of the product is inversely proportional to the concentration of the analyte in the sample.

Analysis of spermatogenesis - sperm count

6 MA-treated and 6 SA-treated adult male rats were used to examine the effect of the long-term abuse of MA on spermatogenesis. The total sperm amount per milliliter (mL) was evaluated by using a bright field microscope. After 30 days of MA (or SA) exposure, males were anesthetized with xylazine (10 mg/kg)-ketamine (90 mg/kg) combination. The sperms were released by cutting the cauda epididymitis longitudinally with a pair of fine-pointed scissors and compressing with forceps (Kempinas and Carvalho, 1988). The sperm suspension (0.5 µl) was drawn into a red blood cell pipette and diluted to 1:200 with saline solution. To prevent the formation of gross spermatozoa clusters, the procedure has to be done rapidly. A *Bürker hemocytometer* was used for counting of spermatozoa. The physiological sperm concentration in rats is ranged from 152.5 to 230.0 x 10^7 spermatozoa/ml.

Sperm count in light microscope

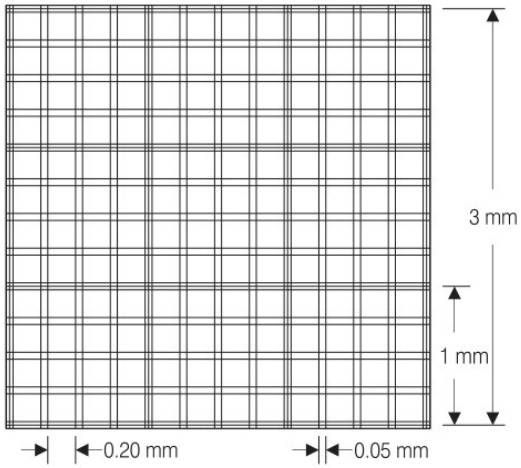


Calculation of cauda epididymal sperm concentration

The formula for calculating the sperm count, when 5 small squares within the large center square are counted:

$$\left\{ \frac{number\ of\ sperms\ counted\ in\ 5\ squares\ (Y)}{volume\ of\ 5\ counting\ squares} = \frac{number\ of\ sperms\ (X)}{1ml} \right\} \text{ x dilution (200x)}$$

Volume of 1 counting square: **Height** × **width** × **depth** [mm^3]



Calculation – volume of 1 counting square (H x W x D):

0.2 mm (*H*) x 0.2 mm (*W*) x 0.1 mm (*D*) = 0.004 mm^3 = 4x 10^{-6} ml

Calculation- sperm concentration (number of sperms/ ml):

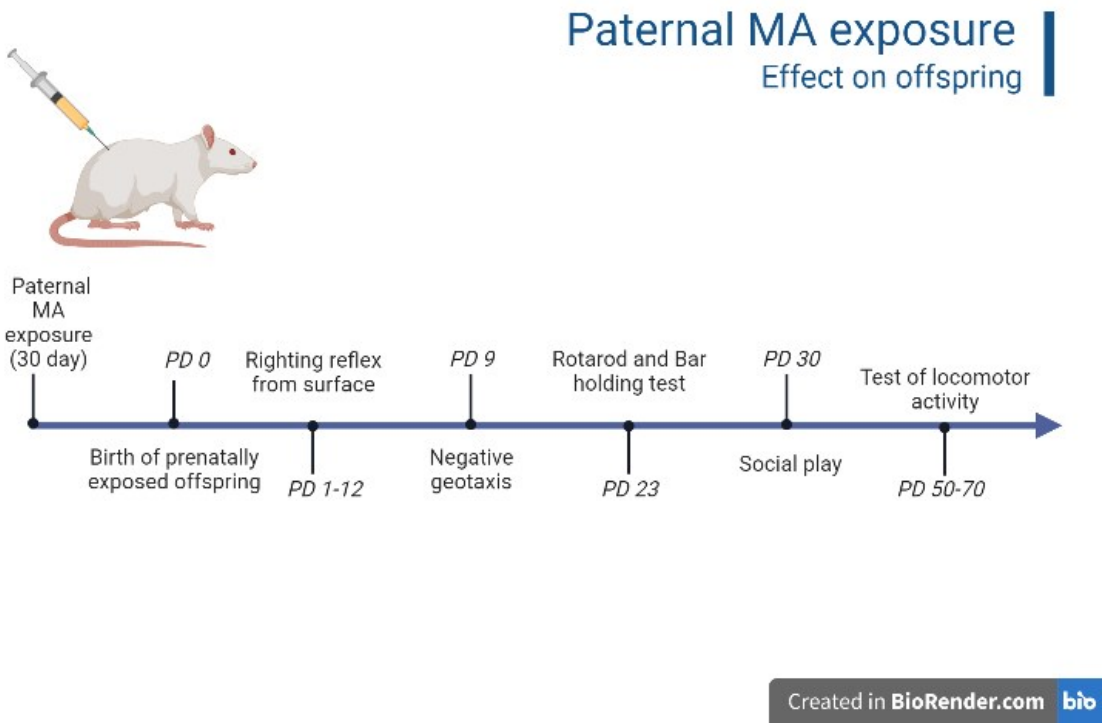
$$\frac{Y}{5x\ Volume\ of\ 1\ couting\ square} = \frac{X}{1ml} \Rightarrow X = \frac{1ml\ x\ Y}{0.00002} = Y \text{ x } 50000 \text{ x } 200 \text{ (dilution)}$$

$$X = Y \times 10^7 /ml$$

BEHAVIORAL TESTS OF OFFSPRING

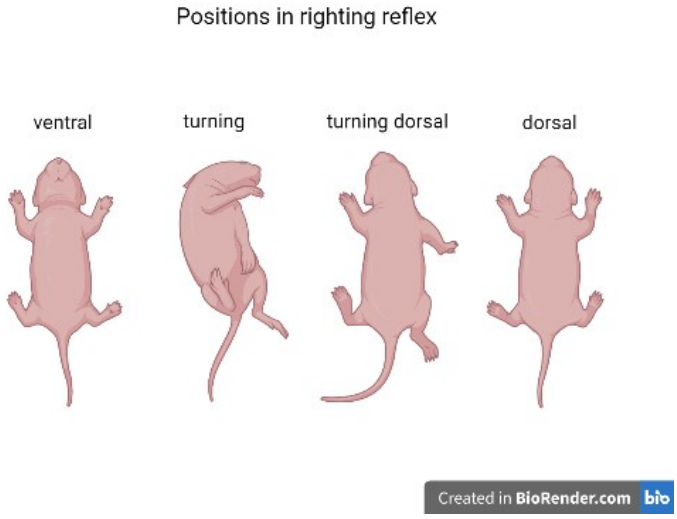
Behavioral experiments during development

Pups from 20 MA litters and 20 SA litters were tested in the following experiments. Average from males and females, respectively, from each MA or SA litters were counted as one unit.



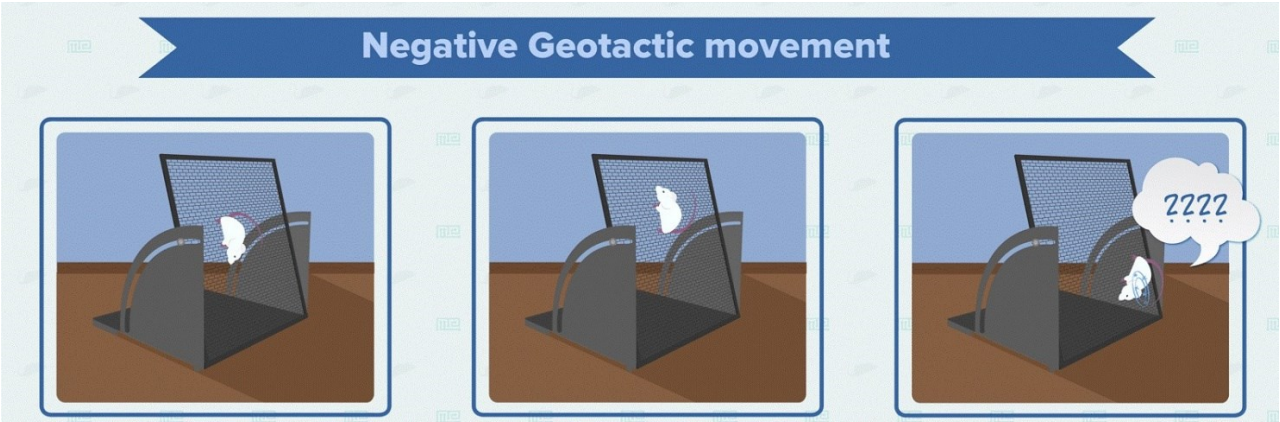
Righting reflex on surface

The surface righting reflex was tested daily within PD 1- 12 (Altman and Sudarshan, 1975, Hrubá et al., 2009, Ševčíková et al., 2017). Each pup was turned to supine position and the time that it took for the pup to right itself with all four paws contacting the surface of the testing table was recorded. Two-way ANOVA (*Paternal treatment* x *Sex)* with repeated measure (*Days)* was used to analyze differences in righting reflex on surface. Differences were considered significant if $p<0.05$.



Negative geotaxis

The Negative geotaxis was performed on PD 9 (Altman and Sudarshan, 1975, Hrubá et al., 2009, Ševčíková et al., 2017). Each pup was placed facing downward on a board inclined at 30º angle. Each animal was given three trials and the best latency of turning their face upward (180º rotation) was recorded. If the pup was slid off the board, it was replaced with head in downward position. Two-way ANOVA (*Paternal treatment* x *Sex)* was used to analyze the differences in negative geotaxis. The Bonferroni post-hoc test was used for comparisons of ANOVA analyses. Differences were considered significant if $p<0.05$.



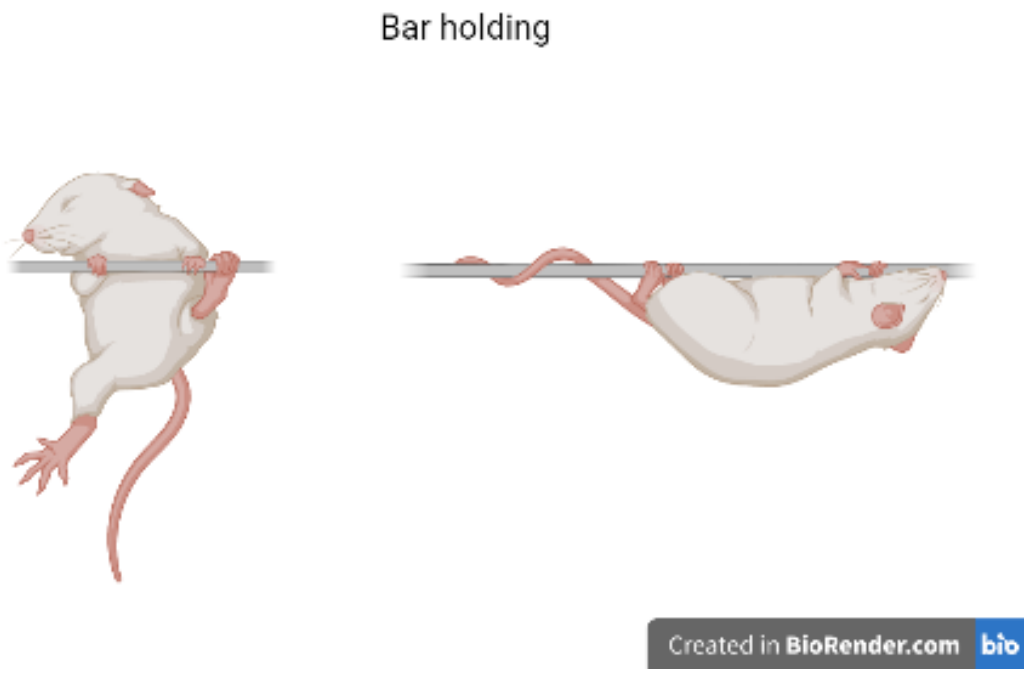
Starting position *Negative Geotaxis* *Failed Trial*

Source: https://conductscience.com/maze/portfolio/geotaxis-test/

Bar holding test

The Bar holding test on PD 23 was performed to examine vestibular function and sensorimotor coordination to achieve the maintenance of the balance on the narrow bar (Murphy et al., 1995, Hrubá et al., 2009, Ševčíková et al., 2017).

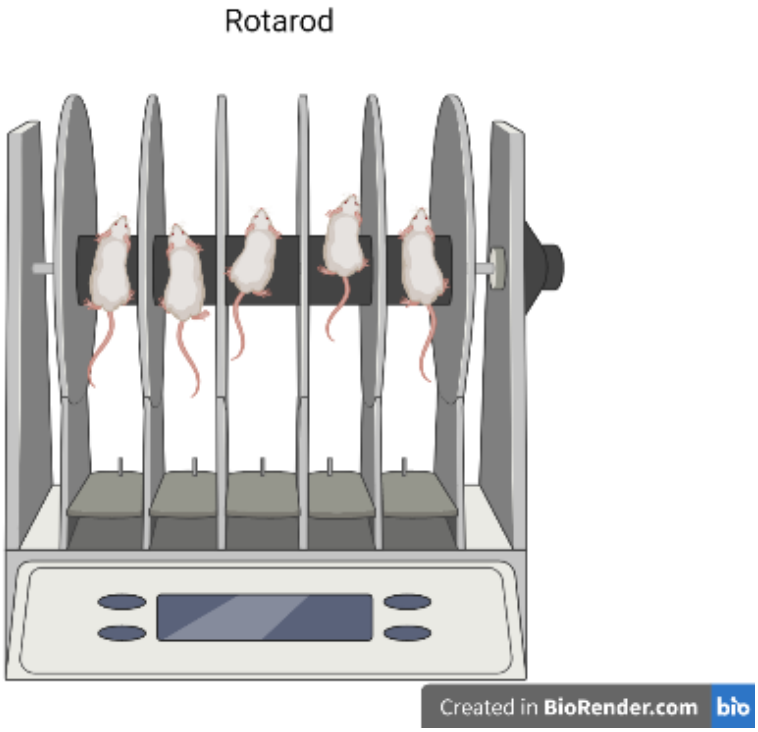
A wooden bar 40 cm long with a diameter of 1 cm was suspended 80 cm above a soft, padded surface. The pup was placed on the bar being held by the nape of its neck and its forepaws were allowed to touch the bar. The time of the fore- and hind-limb grasping reflex with a limit of 120s was recorded. Animals were subjected to three consecutive trials. The two- way ANOVA (*Paternal treatment* x *Sex)* with repeated measure (*Trials*) was used to analyze the differences in performance of Bar Holding Test. The Bonferroni post- hoc test was used for comparisons of ANOVA analyses. Differences were considered significant if $p<0.05$.



Rotarod test

Rotarod test was performed on PD 23 to examine the sensorimotor coordination and dynamic postural reaction necessary for active moving to maintain balance on a rotating cylinder (Šlamberová et al., 2006, Hrubá et al., 2009, Ševčíková et al., 2017). Pups were placed on a rugged cylinder (11.5 cm in diameter, rotating at constant speed of 6 rpm) oriented in opposite direction of cylinder rotation, so they were able to walk forward. The duration of balance on rotarod was determined for 120 s. Trials were repeated until the rats successfully accomplished the task, or until there were 6 failures. Overall number of falls was recorded. The two-way ANOVA (*Paternal treatment* x *Sex)* with repeated measure (*Trials*) was used to analyze the differences in Rotarod Test. The Bonferroni post-hoc test was used for comparisons of ANOVA analyses. Differences were considered significant if $p<0.05$.

On the PD 23, the pups were weaned and split into cages according to sex and left undisturbed until next experiment.



Social play

Behavioral procedures of social play used in my study were provided according to studies by Vanderschuren, Achterberg, Trezza and Ševčíková (Ševčíková et al., 2020, Achterberg et al., 2014, Trezza et al., 2009, Vanderschuren et al., 2008). Two weeks before the experiment, the light/dark cycle was reversed in the colony room (lights on from 6:00 p.m. for 12 hours). The experiments were performed in a transparent plexiglass cage measuring 40x20x20 cm (l×w×h), under dim light conditions. On PD 28 and 29, each animal was separately habituated to the test cage for 10 min. Rats were socially isolated the night before the experiment (approximately for 16 hours) to enhance the expression of social play behavior (Ševčíková et al., 2020). On PD 30, an acute dose of MA (1 mg/kg) or SA at the same volume (1 ml/kg) was administered to pairs of rats approximately 45 min before testing. This dose of MA has been shown to peak in plasma and brain concentrations 45–60 min after exposure (Rambousek et al., 2014). For the experiment, two rats with the same age, gender, paternal and acute drug exposure were placed into the test cage and recorded for 15 min.

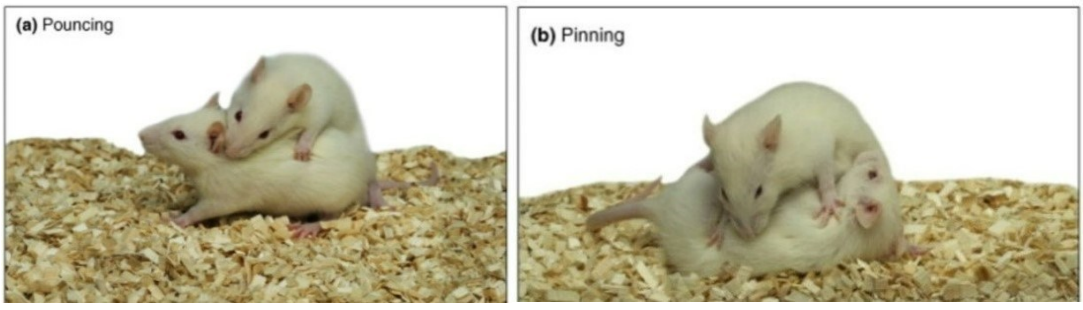
Rats were paired with a similar body weight and with rats that were not cage mates to minimalize the influence of dominant behavior. The video recordings were analyzed using the ODLog (Macropod Software) program. The frequency of pinning (i.e., one of the animals laying in supine position on the floor with the other animal standing over it, which serves as a social releaser of a continuing play round) and the frequency of pouncing (i.e., one of animals soliciting the partner by attempting to nose or rub the nape of its neck) were considered as major elements of social play behavior in rats (Vanderschuren et al., 2016, Vanderschuren et al., 1997, Ševčíková et al., 2020). Also, appearance of non-playful forms of social behavior was measured (i.e., climbing over the partner, mutual sniffing). The rest of the 15 min, during which rats did not play or explore each other, represent other activities, such as space exploration (i.e., rearing

or sniffing). Other activities were not measured, since they are not considered as elements of social play behavior (Vanderschuren et al., 1997, Ševčíková et al., 2020).

Eight pairs of pups were used in each group. Frequencies and duration of social play behavior patterns were calculated per each pair and expressed as a mean ± SEM. To determine whether the paternal or acute impact of MA administration on social play behavior altered over time, the 15-min test session was divided into three 5-min intervals, which were analyzed using a three-way ANOVA *(paternal drug × acute drug × sex)* with repeated measures *(intervals)*. Differences were considered significant if $p<0.05$.

Table 2 Experimental groups

	Experimental groups in social play test							
Paternal treatment	MA				SA			
Gender	male		female		male		female	
Acute treatment	MA	SA	MA	SA	MA	SA	MA	SA
Number of animals per group	16	16	16	16	16	16	16	16
Total number of animals	128 (64 pairs)							



Source: https://www.semanticscholar.org/ (Weiss 2018)

Laboras test in offspring

Pups from 16 MA litters and 16 SA litters were tested in the Laboras experiments. We tested both male and female adult rats (PD 50-70) to determine the gender differences during the test performance. Eight animals were tested in each group (*Table 3).*

Prior to experiment, animals were exposed to acute dose of MA (1 mg/ml/kg), SA (1 ml/kg) or SHAM (single injection) to probe the sensitizing effect of the paternal treatment (MA or SA), eventually to determine the stress response after single injection. The dose 1 mg/kg of MA was chosen based on the finding that this dose induces the concentration of drug in the plasma and brain that peaks 45-60 min after MA administration (Rambousek et al., 2014). As behavior in females can differ depending on the phase of the estrous cycle, the phase of the cycle was determined by vaginal lavage smears (Turner and Bagnara, 1976). Each measured parameter was evaluated separately by using three-way ANOVA *(paternal drug × acute drug × sex)* with repeated measures *(intervals)*. Differences were considered significant if $p<0.05$.

Table 3 Experimental groups

Paternal treatment	Acute treatment in adulthood	Sex		
MA (5mg/ml/kg)	MA (1mg/kg)	Males	Females (estrus/ proestrus)	Females (diestrus/ metestrus
	SA (1mg/kg)	Males	Females (estrus/ proestrus)	Females (diestrus/ metestrus
	SHAM	Males	Females (estrus/ proestrus)	Females (diestrus/ metestrus
SA (1ml/kg)	MA (1mg/kg)	Males	Females (estrus/ proestrus)	Females (diestrus/ metestrus
	SA (1mg/kg)	Males	Females (estrus/ proestrus)	Females (diestrus/ metestrus
	SHAM	Males	Females (estrus/ proestrus)	Females (diestrus/ metestrus

RESULTS

BEHAVIORAL TESTS OF FATHERS

Sexual behavior testing

Our results indicate that long-term MA administration does not influence the sexual behavior in adult male rats. As shown in *Figure 1* chronic MA application (30 days) did not induced any statistically significant differences between MA males relative to mounting frequency [$p = 0.17$], intromission frequency [$p = 0.59$], and number of ejaculations [$p = 0.73$] compared to saline controls. There were also no significant differences in time spent sniffing [$p = 0.66$]. Moreover, our data showed that MA exposure does not affect the length of post-ejaculatory interval [$p = 0.83$] and intromission latency [$p = 0.22$].

Test of locomotor activity -Laboras test

Experimental data of Laboras test show that 30-day MA administration has impact on locomotory activity of male rats. As shown in *Figure 2*, there was a main effect of acute application in all measures showing increase in: locomotion [$F(1, 31)= 30.63$; $p<0.05$], average speed [$F(1, 31)=27.57$; $p<0.05$], distance traveled [$F(1, 31)= 27.57$; $p<0.05$], rearing [$F(1, 31)= 38.93$; $p<0.05$], grooming [$F(1, 31)= 23.03$; $p<0.05$], while decreasing immobility [$F(1, 31)= 36.52$; $p<0.05$]. Moreover, there was an interaction between chronic 30-day MA exposure, acute application and time the way that acute MA application increased locomotion [$F(5, 155)= 3.77$; $p<0.05$], average speed [$F(5, 155) = 2.92$; $p<0.05$], and distance traveled [$F(5, 155)= 2.92$; $p<0.05$] more in animals treated 30-days with saline than in animals with MA chronic treatment within the 20^{th} and 50^{th} minute of measure.

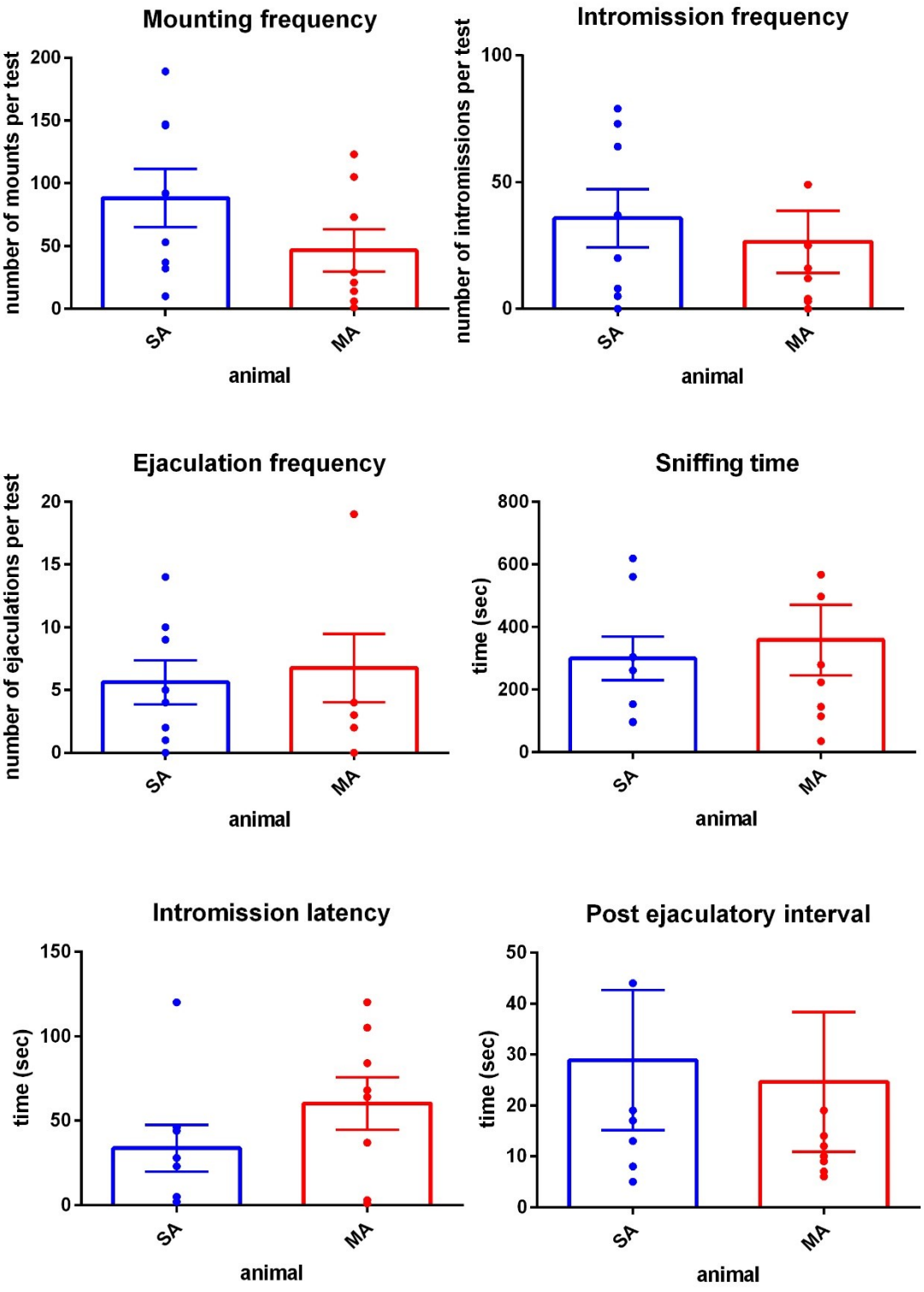


Figure 1. The effect of 30-day MA exposure on sexual behavior. There were no significant differences between MA and SA exposed groups. MA= methamphetamine, SA= saline. Values are mean ±SEM (n=8).

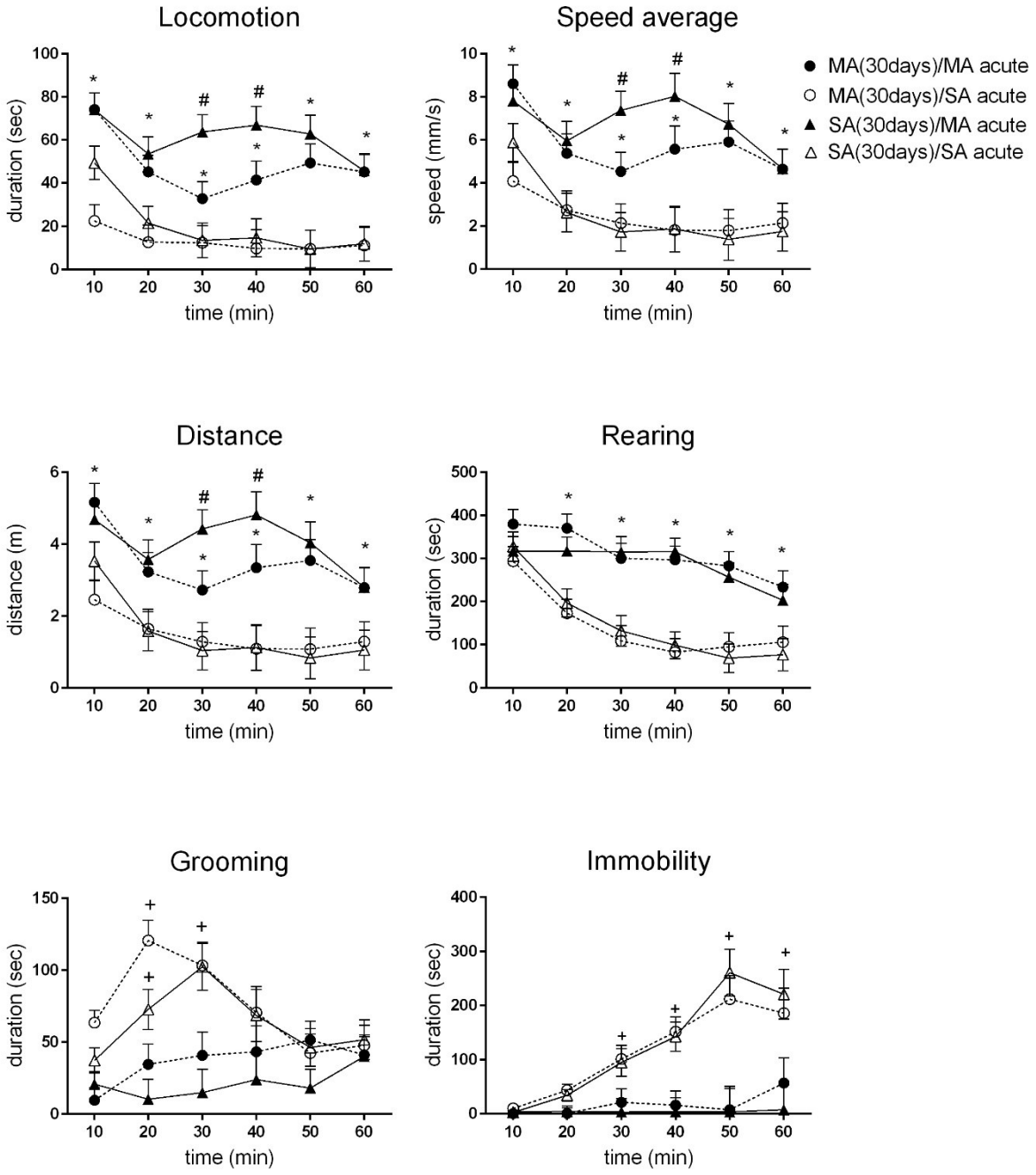


Figure 2. The effect of acute and chronic (30 days) MA exposure on locomotor and exploratory behavior in the Laboras test. MA= methamphetamine, SA= saline. Values are mean ±SEM (n=8).

* $p<0.05$ acute MA > acute SA

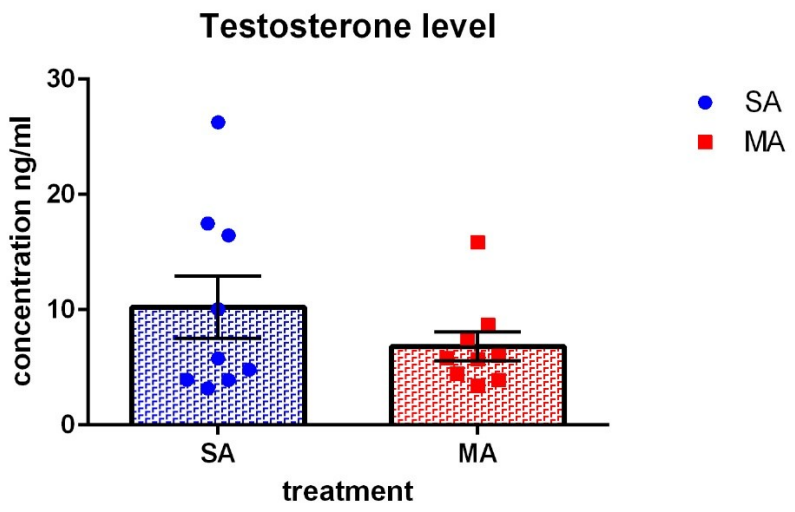
# $p<0.05$ chronic SA > chronic MA

+ $p<0.05$ acute SA > acute MA

ANALYSIS OF TESTOSTERONE LEVEL AND SPERMATOGENESIS IN FATHERS

Analysis of testosterone level

Our results showed that MA exposure does not affect the testosterone level in adult male rats. As shown in *Figure 3,* the testosterone concentration in plasma indicate decreasing trend of values compared to control group. However, our data demonstrate that chronic (30 day) MA administration did not significantly decrease the testosterone levels compared to control group. The blood samples were collected during morning hours; thus, these concentrations reflect the highest levels of testosterone during the day. Values are mean ±SEM (n=9).

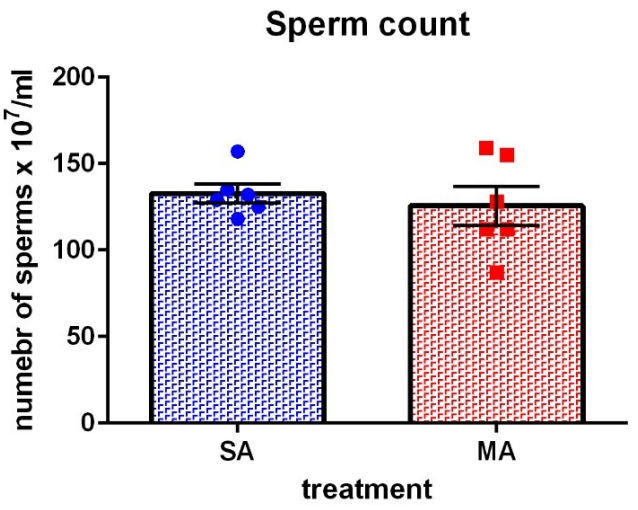


Concentration of Testosterone [ng/ml]	
SA	MA
3,198	3,406
10,064	15,844
4,801	5,783
5,783	8,694
17,486	3,906
3,938	4,459
16,456	5,709
26,25	6,004
3,924	7,553

Figure 3. Chronic MA exposure did not result into significant changes of testosterone levels in adult male rats. MA= methamphetamine, SA= saline. Values are mean ±SEM (n=9).

Analysis of spermatogenesis

The analysis of spermatogenesis in adult male rats exposed to MA did not show any significant differences in sperm production compared to control group. However, the data from SA- treated and MA-treated males did not differ significantly, our results indicate that both treated groups had lower sperm levels compared to physiological level of non-treated healthy male rats. The average amount of spermatozoa/ml in MA-treated group was 125,5 ± 11,32 $\times 10^7$/ml (Mean ± SEM) and in SA-treated group was 132,7 ± 5,432 $\times 10^7$/ml (Mean ± SEM).



NUMBER OF COUNTED SPERMS [×10^7/ML]	
SA	MA
118	87
135	112
132	112
125	128
129	155
157	159

Figure 4. Chronic MA administration did not influence the sperm production of MA- exposed males compared to saline controls. MA= methamphetamine, SA= saline. Values are mean±SEM (n=6).

BEHAVIORAL TESTS OF OFFSPRING

Behavioral experiments during development

Righting reflex on surface

No significant differences were found between MA- and SA - treated groups of pups in any of the test days (PD 1-12). As shown in *Figure 5*, paternal MA exposure did not impair the performance in righting reflex. However, righting reflex on surface determinates significant sex differences [F (1,229) = 5.98, $p<0.05$]. As shown in *Figure 6* male pups were faster in righting than female pups on PD 2.

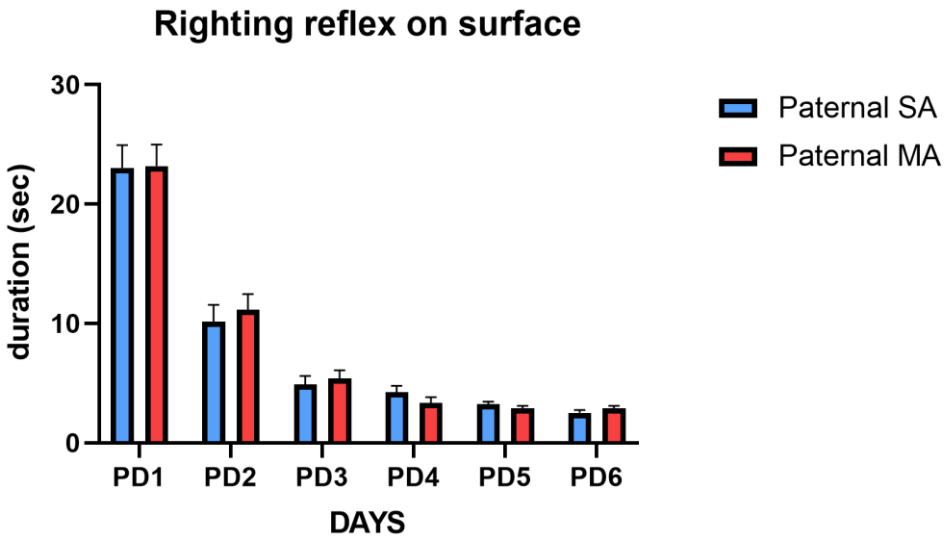


Figure 5. Paternal MA exposure did not affect the righting reflex in pups. MA= methamphetamine, SA= saline. Values are mean ±SEM (n[SA]=109, n[MA]=124).

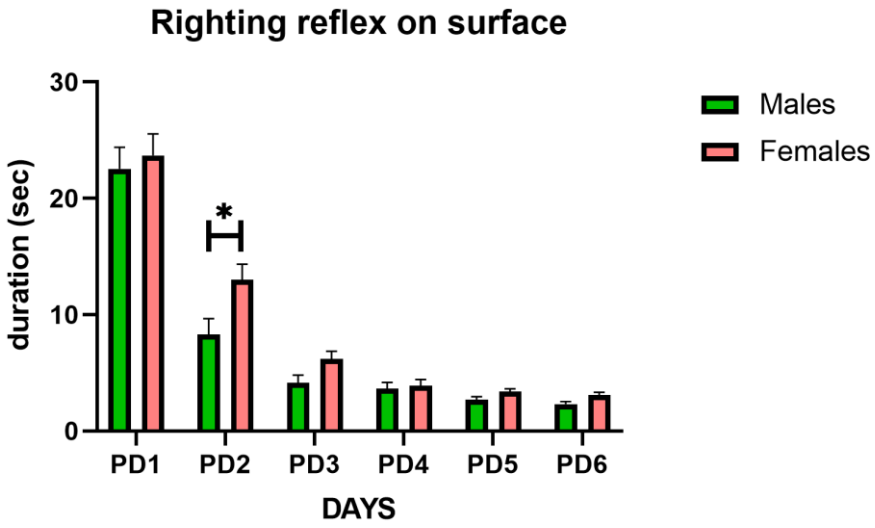


Figure 6. Sex differences in surface righting reflex on the second postnatal day. Values represent the time [s] required for rotating from the supine to the on all four paws position and are shown as mean ± SEM (n[males]=117, n[females]=116) *p<0.05.

Negative geotaxis

Paternal MA administration or gender did not induce any significant differences in negative geotaxis on PD 9.

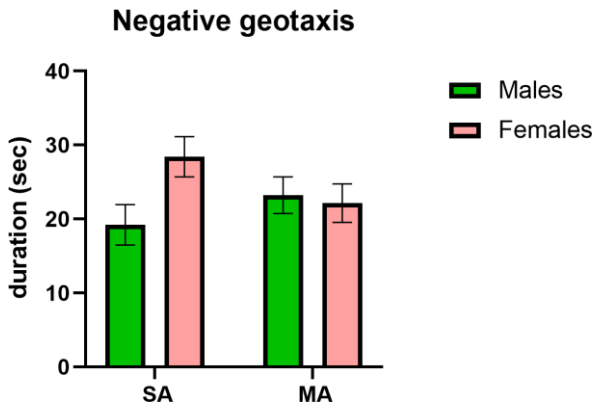


Figure 7. Paternal MA administration did not impact the negative geotaxis on PD 9. No sex differences were found in test performance. MA= methamphetamine, SA= saline. Values are mean ±SEM (n[males]=117, n[females]=112).

Bar holding test

No differences in time spent on the narrow bar were shown between MA- and SA-treated groups of pups on PD 23. As shown in *Figure 8*, bar holding test indicate significant sex differences on the first and second testing trial. Males had poor performance in the test compared to females [F (2, 458)= 3.66, p<0.05].

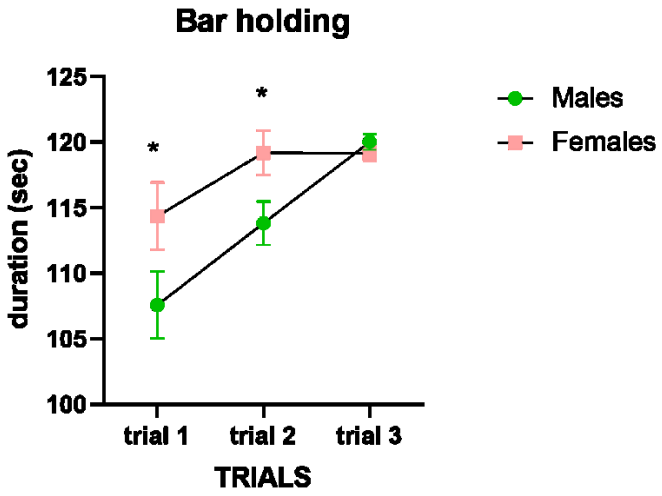


Figure 8. Sex differences in bar holding test on PD 23. The graph shows the time [s] that animals endure to balance on the bar. Values are means±SEM (n[males]=117, n[females]=116) *p<0.05.

Rotarod test

Rotarod test did not show any significant differences in the time spent on the rotating cylinder between MA- and SA-treated pups on PD 23 (*Figure 9*). The test did not show any sex differences between groups.

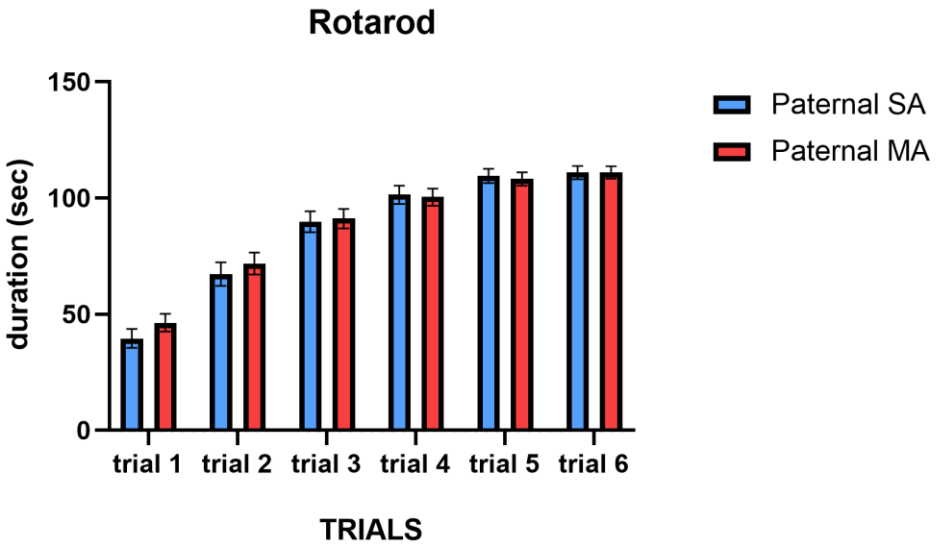


Figure 9. Paternal MA administration did not influence the performance on Rotarod in offspring. Also, no sex differences were found in test performance. MA= methamphetamine, SA= saline. Values are mean±SEM (n[SA]=109, n[MA]=124).

Social play

The following parameters were scored per pair of rats in social play experiment:

1. **Social** behaviors **related** to play:
   - Frequency and duration of pinning
   - Frequency and duration of pouncing
2. **Social** behaviors **unrelated** to play:
   - Social exploration: duration of mutual sniffing [s] and frequency of climbing over
3. **Non-social** behaviors: duration of rearing [s]

Only significant results evaluated in social play experiment are described in further sections.

Our results demonstrate that paternal MA exposure significantly decreased frequency of pouncing [$F(1, 28)= 8.07$, $p<0.05$], decreased duration of mutual sniffing [$F(1, 27)= 6.47$, $p<0.05$] and increased duration of rearing [$F(1, 27)= 6.73$, $p<0.05$] in male offspring. Moreover, paternal MA administration significantly decreased [$F(1, 27)= 6.25$, $p<0.05$] duration of mutual sniffing in female progeny. Acute MA administration significantly decreased frequency of pinning [$F(1, 28)= 92.65$, $p<0.0001$], decreased frequency of pouncing [$F(1, 28)= 162.10$, $p<0.0001$], decreased duration of mutual sniffing $F(1, 27)= 8.496$, $p<0.05$] in males. Acute dose of MA also significantly decreased frequency of pinning [$F(1, 26)= 121.53$, $p<0.0001$], decreased frequency of pouncing [$F(1, 26)= 253.92$, $p<0.0001$] and decreased duration of mutual sniffing [$F(1, 27)= 20.36$, $p<0.0001$] in female offspring.

Sex differences were observed after paternal MA administration in frequency of pouncing. Our results show that paternal MA exposure significantly decrease the frequency of pouncing in male offspring [$F(1, 54)= 4.59$, $p<0.05$] compared to females. Additionally, acute MA administration significantly decreased frequency of pinning [$F(1, 54)= 4.34$, $p<0.05$] compared to saline control groups in both genders.

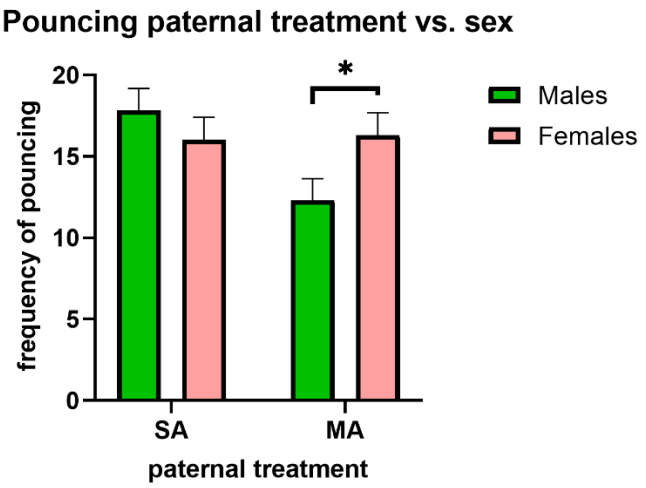


Figure 10. Paternal MA exposure significantly decrease the frequency of pouncing in male offspring compared to females. Values are mean±SEM (n = 16) *p<0.05.

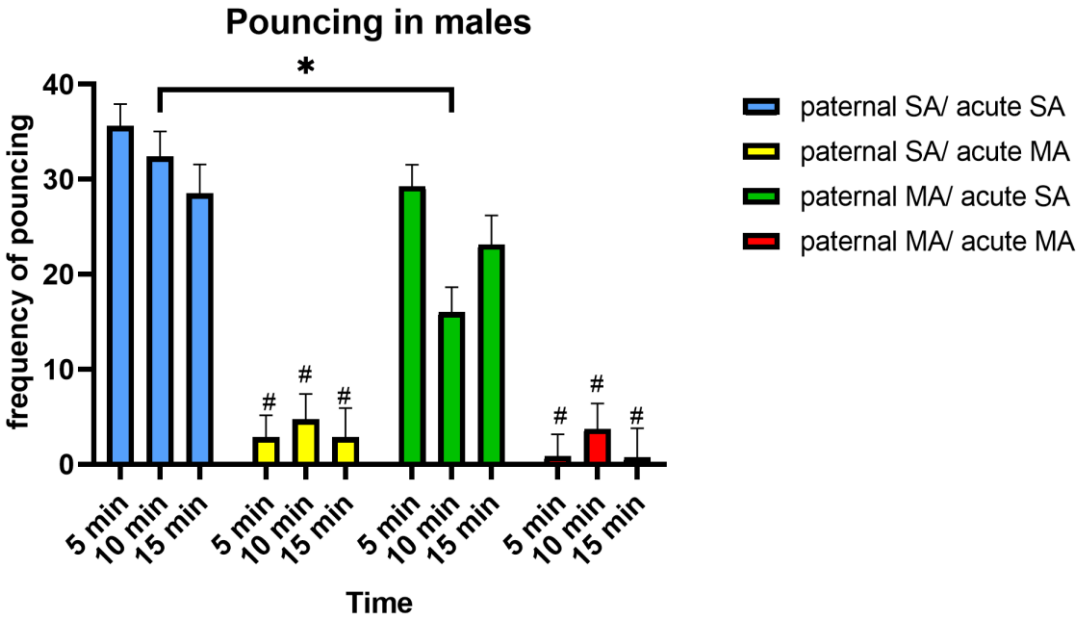


Figure 11. Paternal MA exposure significantly decreased frequency of pouncing in second period of experiment (5^{th} – 10^{th} minute) * p<0.05. The figure also demonstrates the strong effect of acute MA administration which significantly suppress the major pattern of social behavior in juvenile male rats (PD 30). # p<0.05 acute SA > acute MA. Values are mean±SEM (n=8).

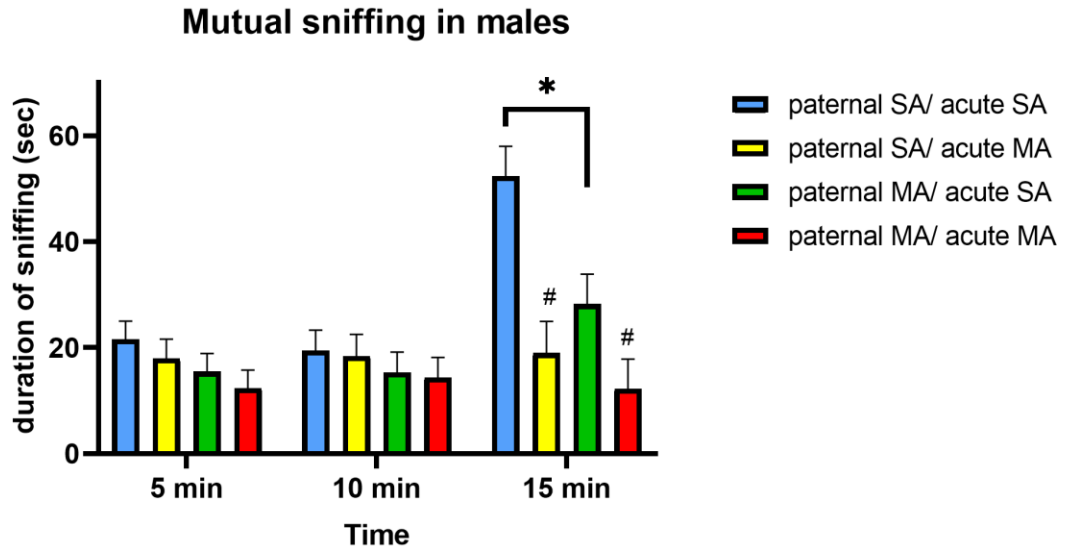


Figure 12. Paternal MA exposure significantly decrease the time spent mutual sniffing [s] in male offspring in the last period of test * $p<0.05$. As well as, acute MA administration significantly decreased duration of mutual sniffing [s] compared to saline group. # $p<0.05$ acute SA>acute MA. Values are mean±SEM (n=8).

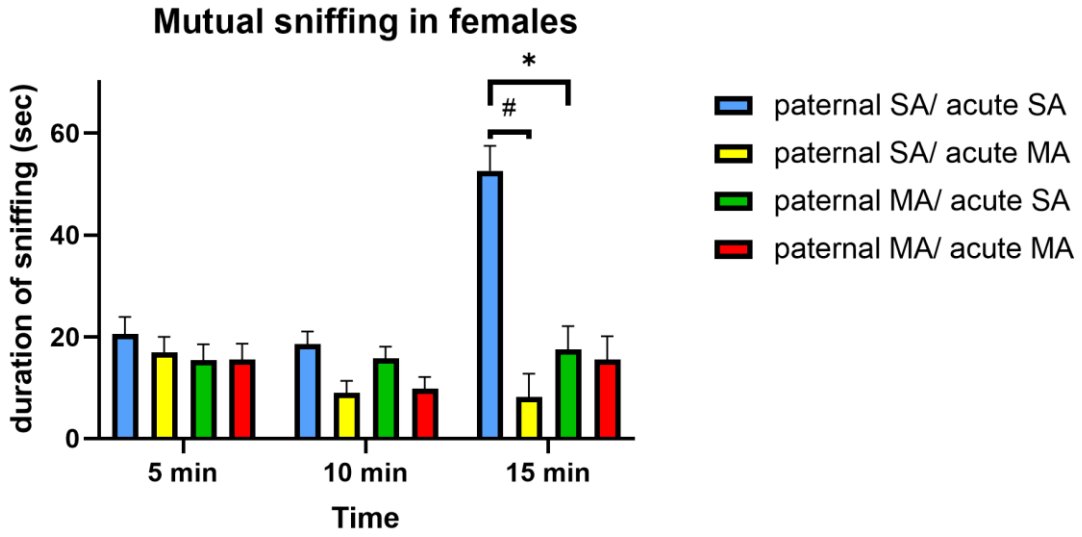


Figure 13. Paternal MA exposure significantly decreased the time spent mutual sniffing [s] in female offspring in the last period of experiment *$p<0.05$. Moreover, acute MA administration significantly decreased the time spent mutual sniffing [s] compared to SA group # $p<0.05$. Values are mean±SEM (n=8).

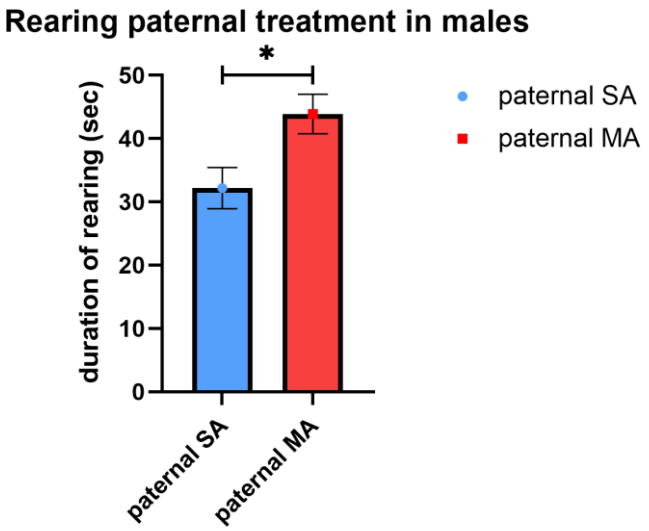


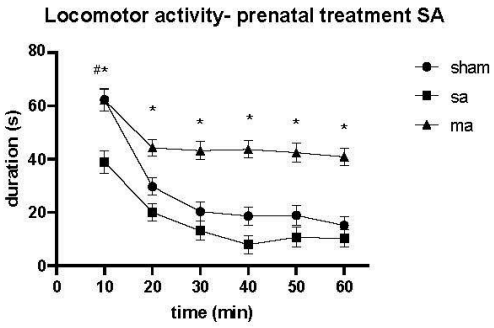
Figure 14. Paternal MA exposure significantly increase the exploratory activity of male offspring represented by the increased time spent rearing [s] compared to saline group. MA= methamphetamine, SA= saline. Values are mean ±SEM (n=16). *p<0.05.

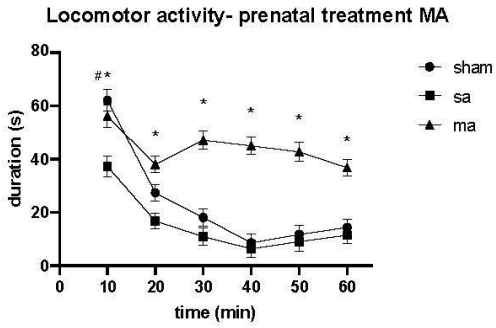
Laboras test in offspring

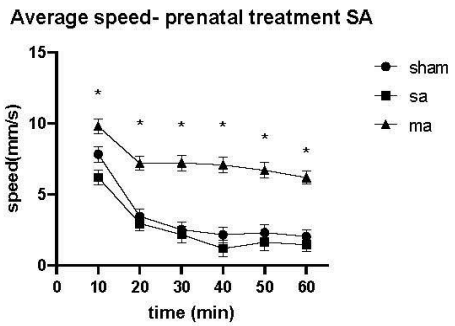
Our results do not demonstrate any significant differences in the parameters of Laboras test regarding the effect of paternal MA exposure on locomotor activity of offspring. However, as shown in *Figure 15*, acute MA exposure significantly increased locomotion [F (10,660)= 14.34, p<0.0001], increased average speed [F (10,660)= 9.89, p<0.0001], increased distance traveled [F (10, 660)= 9.89, p<0.0001], increased rearing [F (10,660)= 30.62, p<0.0001] and decreased immobility [F (10, 660)= 12.58, p<0.0001] relative to control groups. In addition, our results show that SA-treated animals had decreased locomotion [F (2,132)= 19.61, p<0.0001] relative to SHAM-injected offspring in both of prenatally treated groups but only within the first 10-minute interval of the Laboras test.

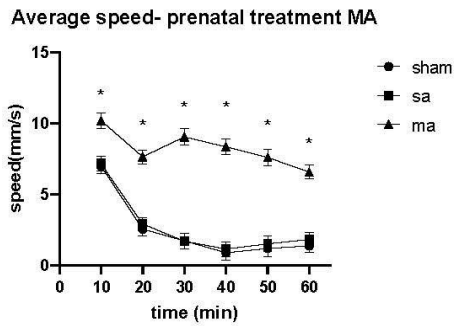
Moreover, SA-treated offspring show increased immobility [F (2,132) = 55.15, p<0.0001] relative to SHAM-injected animals but only within 40- 50 minutes interval of Laboras session. As shown in *Figure 16*, sex differences were observed after acute MA administration in

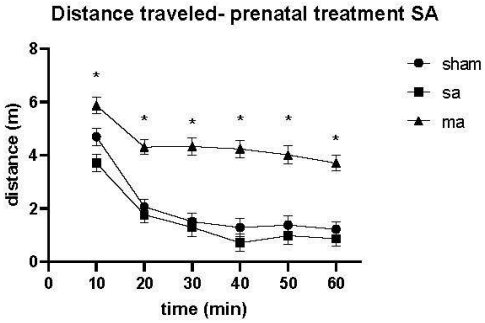
locomotion, average speed, rearing and distance travelled. Our results indicate that MA-treated males had decreased locomotion [$F(4,132) = 2.69$, $p<0.05$], decreased speed average [$F(4,132) = 4.01$, $p<0.01$], decreased distance traveled [$F(4,132) = 4.01$, $p<0.01$] and decreased rearing activity [$F(4,132) = 3.79$, $p<0.01$] relative to females regardless of their estrous cycle. Additionally, sex differences were also found in grooming. SHAM- injected males spent more time grooming [$F(4,132) = 2.52$, $p<0.05$] compared to SHAM-injected females regardless of estrous cycle.

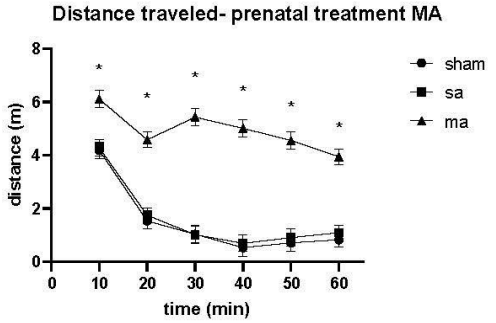












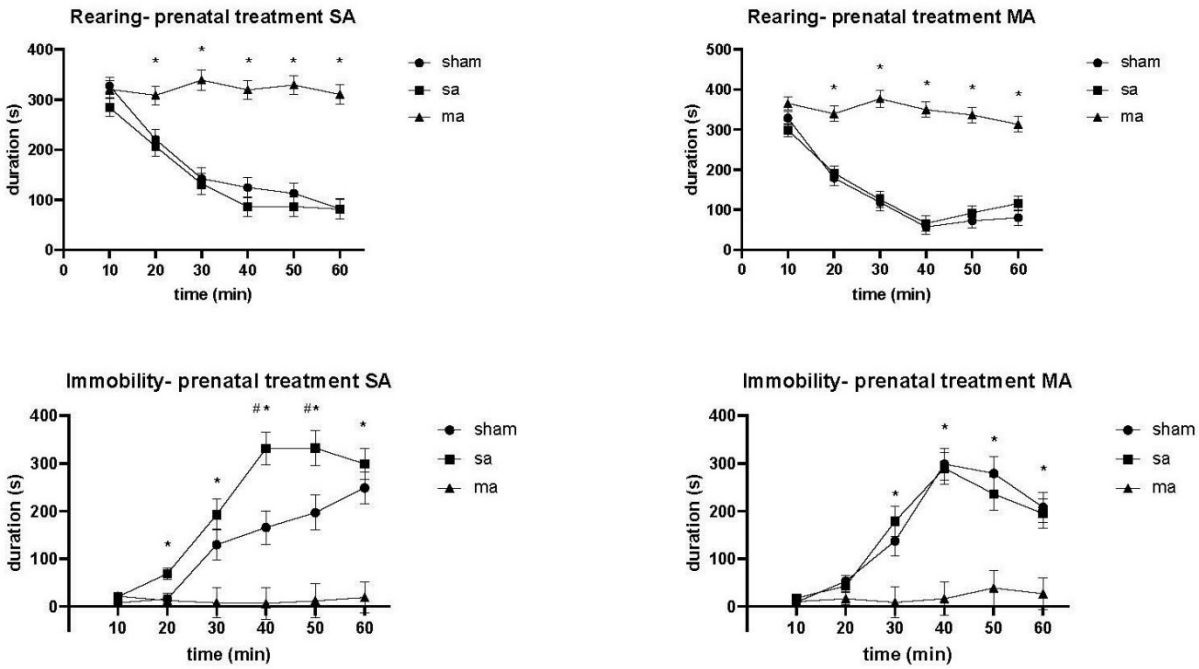


Figure 15. The effect of paternal and acute MA exposure on locomotor activity of offspring in the Laboras test. MA = methamphetamine, SA-saline, SHAM-single injection.

Values are mean ±SEM (n=8).

*p<0.05 acute MA > acute SA, SHAM;

# p<0.05 acute SA < acute SHAM (locomotion);

# p<0.05 acute SA > acute SHAM (immobility)

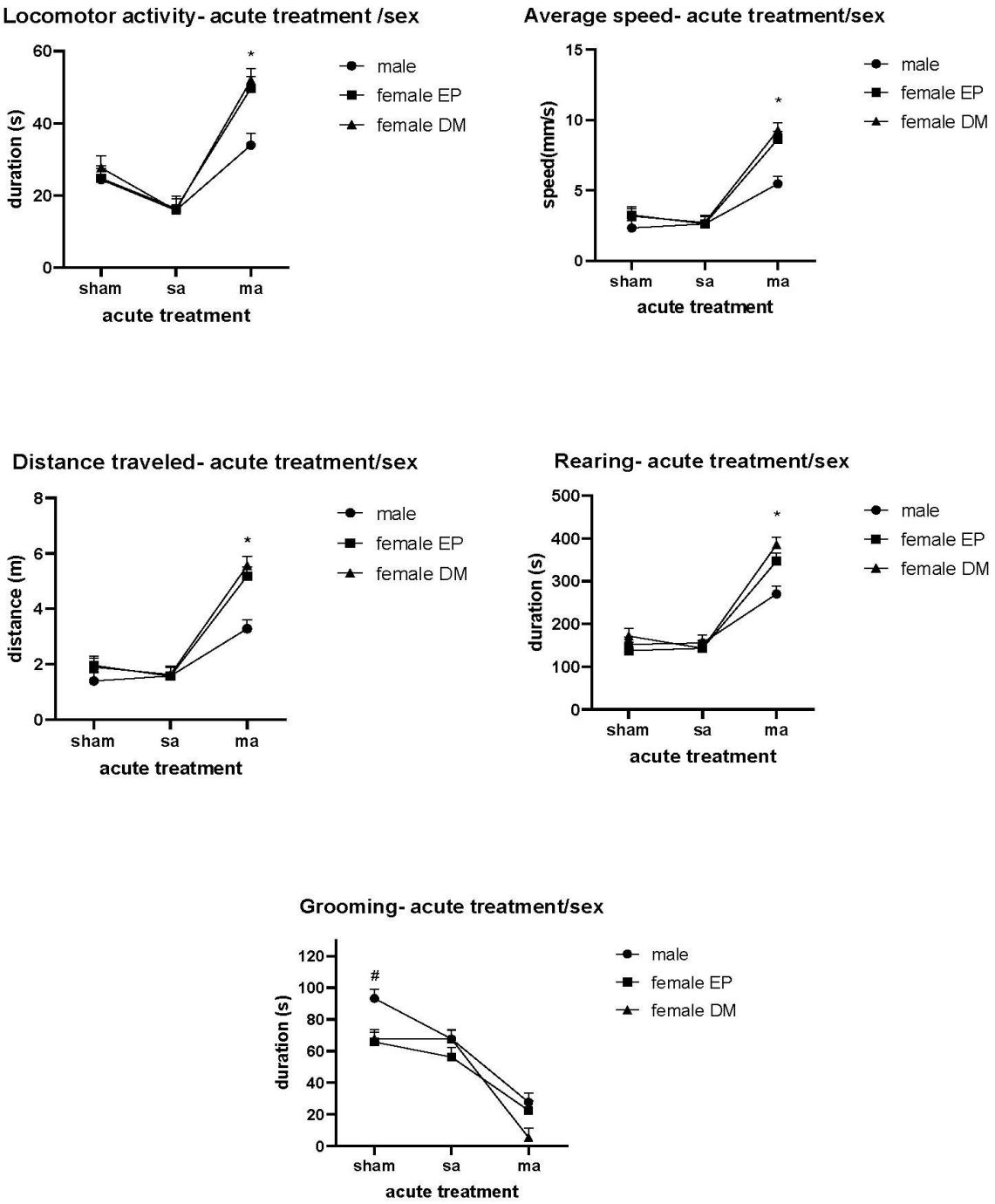


Figure 16. Sex differences in locomotor activity after acute MA exposure of offspring in Laboras test. MA = methamphetamine, SA =saline, SHAM = single injection Values are mean ±SEM(n=8). *p<0.05 males < females (MA administration); # p<0.05 males > females (SHAM injection)

DISCUSSION

BEHAVIORAL TESTS OF FATHERS

Sexual behavior testing

Our results demonstrate that chronic MA exposure did not influence the sexual behavior of adult male rats, which contrasts with some previously published studies. MA abuse is commonly associated with sexually compulsive behavior (Rawson et al., 2002) and increased sexual risk behavior in humans (Frohmader et al., 2010). An animal study by Bolin and Akins (2009) demonstrates that chronic pre-exposure to MA impairs sexual motivation but not sexual performance. However, MA is also associated with decreased sexual function, as chronic MA abuse results in an inability to reach full erection, delayed ejaculation, and orgasm (Frohmader et al., 2010). A study by Kuiper et al. (2017) reports that MA administration leads to maladaptive sexual behavior, which was associated with alterations in neural activation of the brain. Studies by Frohmader et al. (2010) found that MA administration in male rats impairs sexual motivation and performance in a dose-dependent matter. Low doses of MA did not disrupt sexual functions, and acute MA administration failed to impair sexual interest and activity.

Moreover, they (Frohmader et al., 2011) found that MA pretreatment did not affect the expression of sexual behavior; however, they did find that the association between MA and mating was essential for the development of compulsive sexual behavior and changes in sex and drug reward systems. Despite the above-cited works, studies using chronic MA exposure to examine male rodent sexual behavior are still lacking. Our results showed no effect of MA on sexual activity and correlated with previous findings that MA exposure does not affect sexual motivation and performance in pre-treated male rats (Mihalčíková et al., 2019).

Test of locomotor activity - Laboras test

Regarding the effect of MA on locomotor activity in an unknown environment, our results demonstrate that 30-day MA administration has impact on locomotory activity of male rats. Our results show that acute MA application increased locomotion more in animals treated 30-days with SA than in animals with chronic MA treatment. This finding indicates that chronic MA administration decreased the baseline level of locomotor activity in male rats compared to chronic SA-treated group. Previous study by Segal and Mandell (1974) demonstrated that the first amphetamine administration significantly increased locomotor activity in rats; however, this was gradually replaced by progressive increase in stereotypy and decrease in locomotion during 36 days of drug exposure. Moreover, the same impact on locomotor activity has been reported after long-term MA administration (Nazari et al., 2020). Thus, our results correlate with previous findings regarding the effect of chronic MA exposure on locomotor activity in adult male rats.

In addition, significant increase in overall activity (increased locomotion, rearing, grooming, speed average, and distance traveled); decreased immobility after acute MA (1 mg/kg) administration was observed in adulthood (Laboras test). The increased locomotor activity seen in MA-exposed rats is mainly associated with increased levels of dopamine, especially in the nucleus accumbens (Bubeníková-Valešová et al., 2009). Dopaminergic neurotransmission in the nucleus accumbens and the caudate nucleus mediates MA-induced hyperlocomotion and stereotypy, respectively (Kelly et al., 1975, Kelly and Iversen, 1976, Lucot et al., 1980, Wallace et al., 1999). Previous studies demonstrate that the effect of MA exposure on locomotor activity is dose-dependent.

Very low doses of MA (0.1 mg/kg) did not lead to a statistically significant increase in locomotion compared with saline controls. However, higher doses (0.3 and 1.0 mg/kg) caused a statistically significant increase in locomotor activity ($p < 0.05$), which lasted for up to 3 hours (Riviere et al., 1999). Acute MA administration increases locomotor activity when administered at lower doses (1 mg/kg) and elicits stereotypic behavior when administered at higher doses (5 mg/kg) (Kelly et al., 1975, Cho and Segal, 1994). In addition, female rats have been shown to be more sensitive to the locomotor activating effect of i.p. MA administration (0.1–3.0 mg/kg) than male rats (Schindler et al., 2002). Interestingly, previous studies indicate that increased sensitivity to MA exposure is age-related. A study by Zakharova et al. (2009) demonstrated that daily administration of MA increased locomotor activity in both adolescent and adult rats, with a more significant effect seen in adults. Our findings of increased locomotion and exploration, induced by acute MA exposure, agree with other studies that found that psychostimulants, such as MA, increase locomotor activity (Glatt et al., 2000, Hall et al., 2008, Schutová et al., 2013).

ANALYSIS OF TESTOSTERONE LEVEL AND SPERMATOGENESIS IN FATHERS

Analysis of testosterone level

Our results demonstrated that chronic MA exposure did not influence testosterone concentrations compared to the saline controls, which is in contrast with a study by Lin et al. (2014), showing that chronic administration of MA for 15, 30, 60, and 90 days significantly decreased total testosterone secretion compared to the control treatment. Also, other researchers reported that illicit use of MA decreased plasma testosterone concentrations (Nudmamud-Thanoi and Thanoi, 2011). However, there are also studies showing the opposite. A study by Heidari-Rarani et al. (2014) showed that 14 days of MA increased serum testosterone levels in adult male rats. Furthermore, a study by Yamamoto et al. (1999) demonstrated that serum testosterone concentrations showed a biphasic change after MA exposure in mice. An initial significant decrease was followed by an increase, which 48 hours after drug injection showed testosterone levels higher than the control group. Thus, the previous studies are inconsistent in how MA administration affects testosterone levels. We considered how our data correlated or contradicted previously published studies relative to measured values of testosterone. Interestingly, our raw testosterone data show differences in measured values of testosterone concentration among the same treatment group.

We suggest that dominance-subordinate relationships between male rats could influence these differences. During the MA application period (30 days), male rats were housed 2 per cage. It has been demonstrated that adult male rats living together form dominance relationships, with one dominant and the others adopting subordinate roles (Pells et al., 1993). Animal studies on rats demonstrate that testosterone plays a primary role in intermale social aggression and

dominant behavior and that castration, thus the loss of testosterone, is typically accompanied by a loss of social dominance. Therefore, we suggest that the variety of measured testosterone levels shown in *Figure 3* could have been influenced by animal hierarchy (Albert et al., 1986).

Analysis of spermatogenesis - sperm count

Our results demonstrated that chronic MA exposure did not significantly affect sperm production relative to saline controls. Several studies have reported that MA administration induces apoptosis of spermatogenic cells, lower sperm quality, as well as damage to Leydig cells and their functions (Nudmamud-Thanoi and Thanoi, 2011, Lin et al., 2014, Kaewman et al., 2018). Recent studies indicate that MA exposure leads to changes in GABAergic activity, which is involved in the proliferation of Leydig cells, testosterone production, and spermatogenesis (Kaewman et al., 2018). A study by Saberi et al. (2017) demonstrated that MA abuse causes a significant decrease in the number of seminiferous tubule cells and lower sperm production in the MA-treated group compared to controls. Additionally, MA abuse in a dose-dependent manner showed detrimental effects on male reproductive functions, including impaired sperm parameters and sperm chromatin/DNA integrity (Sabour et al., 2017). Thus, our results showing no differences in spermatogenesis between the MA and SA- treated male rats are inconsistent with the abovementioned studies. Surprisingly, our MA- and SA-treated data show lower spermatozoa concentrations than physiological levels. The average number of sperm cells ranged from 152.5–230.0 $\times 10^7$ spermatozoa/ml (Kempinas and Carvalho, 1988). If we found reduced sperm concentration only in the MA group, we could argue that this was a drug effect; however, since the same reduction was also seen in the control group exposed to SA injections, then the explanation may be due to the effect of stress induced by repeated injections.

Regardless of the injected substance, the injection itself may be associated with a stress reaction (Šlamberová et al., 2018) and activation of the hypothalamic-pituitary-adrenal axis. In animals, social stress, high altitude, surgery or injections, and immobilization stress were shown to affect body weight, testosterone levels, and copulatory behavior with variable effects on testicular morphology (McGrady, 1984). In addition, a study by Drude et al. (2011) showed that mice that received a single intraperitoneal injection of harmless saline had an increased corticoid stress response to a second saline injection. Thus, we suggest that the same effect occurred in our experiment too.

BEHAVIORAL TESTS OF OFFSPRING

Behavioral experiments during development

Our data did not show any significant effects of paternal MA exposure on sensorimotor development of the surface righting reflex, Negative geotaxis, Bar holding test, or Rotarod test. These results are in contrast to the effects induced by maternal MA administration, which showed that after maternal MA exposure, the surface righting reflex (on PD 1–5) was slowed (Hrubá et al., 2008, Šlamberová et al., 2007, Malinová-Ševčíková et al., 2014), the Negative geotaxis on PD 9 was unchanged (Malinová-Ševčíková et al., 2014), and performance on the Rotarod, but not Bar holding, was impaired on PD 23. Thus, it seems paternal MA exposure does not influence the sensorimotor development of rat pups, as does maternal MA exposure. The explanation may be that while maternal exposure can directly affect the development of pups (since MA crosses the placenta and enters breast milk during lactation) (Dattel, 1990; Rambousek et al., 2014), paternal exposure would need to change the genetics of the pup, which does not appear to occur.

Because there is a lack of relevant studies for comparison with our results, we can discuss our data only in comparison to studies focused on paternal exposure to different psychoactive drugs (such as cocaine or cannabinoids). These studies demonstrated that paternal cocaine administration did not affect litter size or birth weight but resulted in pup hyperactivity (Abel et al., 1989). A similar study by George et al. (1996) showed reduced litter size and increased prenatal and postnatal mortality in cocaine-exposed rat offspring. In addition, a study by Dalterio et al. (1984) showed that paternal THC (delta-9-tetrahydrocannabinol) exposure significantly impaired rat pup development. Our results demonstrate that paternal MA administration does not result in such a severe impairment of offspring development compared to paternal cocaine and cannabinoid exposure.

In addition, our data indicate sex differences in tests performed during development. When examining sensorimotor development, male rats were faster at righting on the second postnatal day than females. In contrast, females were more capable of balancing on the narrow bar than males and achieved a better score on the Bar holding test on the first and second testing trial. There were no sex differences on the Negative geotaxis or the Rotarod test. Based on our data, males were faster, while females were better at maintaining balance. The sex differences relative to test performance can be caused by the age at which the test was performed and because males and females differ in developing sensorimotor skills. Tests used in our study revealed different skills. While the surface righting reflex on PD1–12 examines tactile maturation of motor skills of pups and is under the control of the brain stem (Pellis and Pellis, 1994), the Negative geotaxis test examined on PD 9 is an automatic, stimulus-bound orientation movement considered diagnostic of vestibular and proprioceptive function (de Castro et al., 2007). The Rotarod and Bar holding tests examined on PD 23 are related to sensorimotor development that requires fully developed cerebellar coordination (Murphy et al., 1995).

Social play

Effect of paternal MA exposure on the social behavior of offspring

Our results demonstrate that paternal MA administration significantly decreased social play and social exploration in juvenile rat offspring (PD 30) by decreasing the frequency of pouncing in males and decreasing the time spent in mutual sniffing in both genders. Moreover, paternal MA exposure significantly increased exploration of the environment by increasing the time spent rearing by male offspring. Social play in rats is characterized by behavioral patterns including pinning, pouncing, nape attacks, boxing, and social exploration (Panksepp et al., 1984, Vaderschuren et al., 1997), which can be disrupted by acute exposure to a variety of drugs (with the notable exceptions of morphine and ethanol) (Young et al., 2011). A study by Goldberg and Gould (2019) demonstrated that acute treatment and repeated prenatal drug exposure alter juvenile social play behavior. Our results indicate that paternal MA administration alters specific patterns of social play in offspring. Specifically, paternal MA exposure significantly decreased the frequency of pouncing and the duration of mutual sniffing. Each social play-related pattern has its own mechanism and interpretation. In rats, an episode of social play behavior usually starts when a rat approaches a mate and attempts to touch the mate's neck with its own snout (Panksepp and Beatty, 1980, Pellis and Pellis, 1987, Poole and Fish, 1975, Vanderschuren et al., 1997). This behavior, called pouncing or nape contact, is considered the most critical parameter of play initiation, perhaps reflecting the motivational aspect of social play. The most characteristic response to this play initiation is when the recipient rat rolls onto its dorsal surface, commonly known as pinning (Vanderschuren et al., 2016). Mutual sniffing indicates social exploration of a partner and represents a non-playful pattern of social behavior.

Since our results showed a decreased frequency of pouncing, we suggest that paternal MA impaired mainly the initiation of social behavior, which also resulted in the suppression of non-playful patterns of social play (mutual sniffing). It is necessary to review the previous findings of psychostimulant-induced alterations in social behavior to understand the mechanism of MA exposure on social behavior. Since psychostimulants, such as MA, directly increase dopamine levels in the nucleus accumbens, the behavioral effects of these drugs are mainly attributed to their impact on dopamine neurotransmission. However, pretreatment with DA-receptor antagonists does not influence MA-induced impairment of social play behavior (Beatty, 1984, Vanderschuren et al., 2008). This finding indicates that altered dopamine neurotransmission may not be the only thing responsible for the effect of psychostimulants on social play. We suggest that chronic paternal MA administration can alter mechanisms of neurotransmission in the CNS, which could lead to impairment of specific social behavior patterns in offspring.

Since there is a lack of studies examining paternal MA exposure on the social play behavior of offspring, studies on the paternal effect of other psychostimulants (cocaine and nicotine) might be explicatory. These studies demonstrate that paternal cocaine exposure does not impair the social behavior of cocaine-sired male and female offspring (Fischer et al., 2017, Vestegren et al., 2020). A study by Yaw et al. (2022) indicated that paternal cocaine exposure alters patterns of social behavior and the density of oxytocin receptors in the first generation of offspring. Prenatal exposure to drugs, particularly cocaine, results in lasting alterations in central dopaminergic systems, which may underlie impaired behavior later in life (Spear et al., 1989). Additionally, the genetic background (inbred vs. outbred rats) may significantly affect the response to cocaine in the sired rats and, thus, the behavioral effects seen in the offspring. Other recent studies suggest that paternal nicotine exposure affects behavioral and neural development in offspring (Goldberg and Gould, 2019).

A study by Dai et al. (2017) indicates that the effect of chronic nicotine exposure on social behavior depends on the method of administration. Daily exposure to tobacco smoke for 1 hour significantly increased the time spent in the social chamber test. However, when nicotine was administered intraperitoneally, the social behavior of the offspring was unaffected. Nicotine's reinforcing and rewarding properties are induced by binding to nicotinic acetylcholine receptors on dopaminergic neurons in the mesolimbic dopaminergic system, resulting in increased dopamine release in the nucleus accumbens (Barrett et al., 2004, Balfour, 2009). However, a significant genetic component may be involved relative to the effect of nicotine exposure on behavior. Based on previous studies, paternal cocaine and nicotine exposure could lead to alterations in the social behavior of offspring, as seen in paternal MA exposure in our experiment. We suggest that psychostimulants might similarly affect neurotransmitter system regulation, which may play a role in altered social behavior.

Effect of acute MA exposure on the social behavior of offspring

Acute MA exposure 45 minutes before the social play test was conducted significantly decreased all social behavior patterns and social exploration of offspring. These results correlate with previous findings of our laboratory that acute MA administration significantly decreased all patterns of social play behavior in juvenile rats compared to controls (Ševčíková et al., 2020). Moreover, acute MA exposure also significantly decreased all parameters of social interaction in adulthood and increased non-social activities compared to the acute SA group (Hrebíčková et al., 2017). Other experimental studies have also reported significantly decreased social contact after acute MA administration both in adolescence and adulthood (Davidson et al., 2001, Manduca et al., 2014, Šlamberová et al., 2015). Acute administration of other psychostimulants, such as cocaine, amphetamine, or MDMA, was also shown to decrease social behavior in rats (Achterberg, 2014, Vanderschuren et al., 2008).

A previous study by File et al. (1998) showed that specific experimental conditions can generate moderate levels of anxiety (e.g., light set-up in the experiment, unfamiliar arena, or anxiogenic drug exposure), which decreases social interaction patterns in rats. Thus, psychostimulant suppression of social behavior could be related to the anxiogenic effects of these drugs (File and Seth, 2003). Previous studies concluded that MA, cocaine, nicotine, and amphetamine exposure caused persistent anxiety-related behavioral symptoms (Hayase et al., 2005, Biala and Kruk, 2008). Other studies indicate that the mechanism by which an acute dose of amphetamine decreases social play likely depends on the dopaminergic system, which is associated with reward circuits, as well as noradrenergic neurotransmission (Achterberg et al., 2014, Achterberg et al., 2016, Veeneman et al., 2012). Increased dopamine levels after acute MA exposure can alter social play motivation while not influencing the expression of the social play itself.

Moreover, a study by Achterberg et al. (2016) indicated that decreased motivation and expression of social play are mainly modulated by noradrenergic neurotransmission. Their study showed that acute administration of atomoxetine (a direct inhibitor of noradrenaline reuptake) significantly decreased social play behavior (Achterberg et al., 2014). These findings thus indicate that decreased expression of social play behavior after MA exposure can also be induced by noradrenergic stimulation of α2-adrenoceptors. Although, the suppressant effect of psychostimulants on social behavior is probably far more complex. It can be hypothesized that the effects of psychostimulants, such as MA, result in enhanced or exaggerated behavioral inhibition (Achterberg et al., 2014). Because of increased inhibition of behavior, psychostimulant drugs may increase attention toward non-social stimuli in the environment and suppress patterns of social play.

Sex differences in social behavior

Prenatal and acute MA exposure significantly decreased specific patterns of social play behavior in both genders. In our experiment, juvenile male rats show decreased activity during social play, relative to the frequency of pouncing, compared to females. Previous studies of non-treated juvenile rats showed that juvenile social play behavior is sexually dimorphic, with males exhibiting higher levels than females (Auger and Olensen, 2009). This sex difference has been attributed to increased play initiation by males, represented by an increased frequency of pouncing (Thor and Holloway, 1983, Auger and Olensen, 2009). In addition to play initiation, other components of social play are also sexually dimorphic. Healthy male rats engage more frequently in boxing and pinning their play partners than females (Auger and Olensen, 2009). Sex differences in social behavior have been found to be under the control of gonadal hormones acting during the neonatal period (Meaney et al., 1985, Auger and Olensen, 2009). Other previous studies indicate that the modulation of social play behavior may involve both androgen and estrogen receptors (Amateau et al., 2004, Beatty et al., 1981). However, since neonatal estrogen exposure increases androgen receptor mRNA expression (McAbee and Doncarlos, 1999), it is possible that estrogens influence social play expression by increasing androgen receptor sensitivity. A previous study by Šlamberová et al. (2011) examined the effect of MA exposure and its interaction with gonadal hormones on social interaction in adulthood. Results showed that an acute methamphetamine administration decreased the frequency and duration of social interaction patterns (especially mutual sniffing and allogrooming) in adult female rats relative to gonadectomized male rats (Šlamberová et al., 2011). Other studies indicate that not only gonadal function but also dopaminergic neurotransmission plays a vital role in sexual differentiation of social play behavior (Hull et al., 1984, Gonzales et al., 2000, Gotz et al., 1991).

Neonatal treatment of females with a dopamine receptor agonist (lisuride) masculinizes juvenile play behavior (Gotz et al., 1991). Thus, it appears that alterations in the dopaminergic system may influence the expression of social play in juvenile rats. Since the mechanism of action of MA is modulated by dopaminergic neurotransmission, MA administration may influence sex differences in social play behavior. A recent study by Hrebíčková et al. (2017) showed that an acute dose of MA decreases social interaction in adult male rats to a greater extent than in females, which correlates with our findings. However, previous studies of other psychostimulants exposure have shown the opposite results. Prenatal cocaine exposure was shown to significantly increase the pinning frequency of males compared to females (Wood et al., 1994). A study by Weiss et al. (2015) showed that amphetamine exposure significantly decreased social interaction in the conditioned place preference test in female rats compared to males.

In our experiment, we hypothesized that MA exposure results in an attention deficit and locomotor hyperactivity, which leads to reduced social play behavior in rats (Parvopassu et al., 2021). Furthermore, previous studies have found that females are more sensitive to MA-induced locomotor hyperactivity (Becker, 1999, Bisagno et al., 2003, Páleníček et al., 2005). Therefore, we suggest that the increased activity of females in social play can be attributed to this effect of MA. We suggest that sex differences seen in social play behavior are not affected only by MA exposure but also by complex interactions between neurotransmission and gonadal function. Evidence shows that the dopaminergic system plays a role in the reward aspect of social play behavior. The role of cholinergic, noradrenergic, and opioid systems is also essential to attentional processes, which enhance the expression of social play behavior; androgens are also vital to the sexual differentiation of social play behavior (Vanderschuren et al., 1997, Vanderschuren et al., 1995).

Laboras test in offspring

The Laboras test results demonstrate that paternal MA exposure does not affect the locomotor activity and exploratory behavior of offspring in adulthood. Because no studies have investigated the effect of paternal MA exposure, we compare our results with the effects of other illicit drugs. However, these studies are inconsistent in their outcomes. Similar to our results, a study by Killinger et al. (2012) showed that spontaneous locomotor activity after paternal cocaine exposure was unaffected. A study by Fisher et al. (2017) reported increased locomotor activity after paternal cocaine exposure, and a study by Levin et al. (2019) showed that paternal THC treatment does not affect the spontaneous locomotion of offspring. However, it increases habituation during locomotor activity. In addition, another study with paternal alcohol exposure showed that the locomotor activity of offspring increased by 30% compared to controls (Ledig et al., 1998). The inconsistent outcomes of previous studies could be the result of different experimental conditions as well as related to varying drug doses, methods of administration, and duration of the exposure period.

In comparing acute MA administration relative to paternal MA exposure, our data showed that acute MA administration increased overall activity in the Laboras cage, as demonstrated by increased locomotor activity, rearing, average speed, distance traveled, and decreased immobility. However, this effect was not dependent on paternal MA exposure. Our findings agree with previous studies (Hrubá et al., 2012, Šlamberová et al., 2014) showing that maternal MA exposure during gestation did not affect the baseline level of locomotor activity in adult offspring, while acute MA treatment increased it. (Glatt et al., 2000, Hall et al., 2008, Schutová et al., 2010). The increased overall activity in the Laboras test, induced by an acute MA application of 1 mg/kg, was mainly associated with increased levels of dopamine, especially in the nucleus accumbens (Bubeníková-Valešová et al., 2009).

Apart from the effects of acute MA administration, our data showed that saline injections significantly decreased locomotor activity relative to SHAM-injected rats, but only during the first 10 minutes of the experiment. Moreover, SA-treated offspring showed increased immobility relative to SHAM-injected animals during the 40–50 minutes of the Laboras test. The explanation for this finding may be that the injection itself, regardless of the injected substance, induces behavioral changes in animals in an unknown environment (Šlamberová et al., 2018), which may be associated with stress and activation of the hypothalamic-pituitary-adrenal axis (Gomez and Garcia-Garcia, 2017).

Sex differences were also observed during the Laboras experiment after an acute dose of MA was administrated. Our data showed that MA-treated males were significantly less active, as demonstrated by decreased locomotor activity, rearing, average speed, and distance traveled relative to females, regardless of the estrous cycle phase. There is considerable evidence for gender differences in psychostimulant drug abuse (Becker et al., 1982, Dluzen and Liu, 2008, Cox et al., 2013, Reichel et al., 2012, Hrubá et al., 2012). In rodents, acute or chronic treatment with psychostimulants results in higher locomotor activity in females than males (Becker, 1999, Bisagno et al., 2003, Páleníček et al., 2005). In addition, a study by Milesi-Hallé et al. (2007) demonstrated that MA-treated females show greater and longer-lasting locomotor activity than males. Thus, our findings that acute MA exposure increase locomotor activity in females compared with males agree with previous studies.

CONCLUSION

In conclusion, based on our results, we could answer the questions raised by our hypotheses.

1. Hypothesis:

   The long-term application of MA (30 days) to adult male rats should induce changes in their reproductive system. Hypothetically, we expect increased sexual and locomotor activity after MA application. However, it is questionable whether long-term MA administration will induce stereotypic behavior and rejection of a sexual partner. Another unanswered question is whether the MA application will affect spermatogenesis and testosterone levels in males. Since this effect has been demonstrated in other psychostimulants, we expect reduced sperm production and a negative impact on reproductive functions after long-term MA exposure.

Our results demonstrate that MA administration in adult male rats does not affect sexual performance and sexual motivation compared to control group. Thus, our hypothesis that MA exposure may influence the sexual behavior of fathers, as it was seen in other psychostimulants, was not confirmed.

Our assumptions were shown to be wrong regarding the effect of MA exposure on spermatogenesis and testosterone levels in male rats. Chronic MA administration (30 days) did not influence sperm production or testosterone levels compared to saline controls.

Despite these negative results, more detailed studies are needed to thoroughly investigate dose-dependent responses and other factors that may play a role in the possible effect of MA on male reproductive performance.

2. Hypothesis:

Paternal MA administration (30 days) could also lead to changes in rat pup functional development, social behavior, and locomotor activity, as occurs with maternal MA administration.

The present study's data did not show any significant effects of paternal MA exposure on sensorimotor development in the offspring. There is a lack of relevant studies on paternal MA exposure and its consequences on offspring development. However, previous studies of maternal MA exposure showed significant impairment in the sensorimotor development of offspring. Our hypothesis that paternal MA exposure could influence the sensorimotor development of rat pups, as does maternal MA exposure, was not confirmed. Although we did not find any significant effects of paternal MA exposure, our study was significant because it is one of the first to determine whether paternal MA exposure had similar adverse effects on offspring development as maternal exposure.

The present study demonstrates that paternal MA exposure significantly impaired social play behavior in offspring, which corresponds with our hypothesis that paternal MA exposure could impair social behavior in offspring. However, the mechanism by which paternal MA exposure alters play behavior in offspring remains unknown. More experiments are needed to clarify the mechanisms of drug addiction and its influence on future generations, which appear to involve complex modulations of neurotransmitter systems. The following studies suggest a path for future research. Study by Vanderschuren et al. (1997) showed that the dopaminergic system plays an essential role in the reward aspect of social play behavior. In addition, serotonin plays an important role in regulating mood and emotional states, particularly anxiety (Guimarães et al., 2010), and regulates social and sexual behavior (Duman and Canli, 2010). Further, the role of cholinergic, noradrenergic, and opioid systems is essential in attentional processes that

facilitate the expression of social play behavior in juvenile rats (Vanderschuren et al., 1997, Vanderschuren et al., 1995).

Another explanation for our findings regarding the effect of MA on the social play of offspring may be that MA-induced locomotor hyperactivity and increased exploration results in attention deficits and, thus, decreased interest in social play. Additionally, the anxiogenic effect of MA could also result in the suppression of social play behavior.

Regarding sex differences that occurred in social play test after MA exposure, we suggest that patterns of social behavior are affected by MA exposure as well as complex interactions between neurotransmission systems and gonadal function, which underlie the critical role of androgens and estrogens in sexual differentiation. When we consider the possible mechanism of action by which paternal exposure to MA could affect the social play behavior of the first generation of offspring, we can assume that it involves altered dopaminergic, serotoninergic, or noradrenergic neurotransmission in CNS as well as the involvement of the endocrine system. However, many genetic factors underlie drug-related behaviors.

Finally, our results show that MA administration to male rats does not influence the locomotor activity and exploratory behavior of their adult offspring. These findings agree with previous studies showing that maternal MA exposure did not influence the locomotor activity of their adult offspring. In addition, an acute dose of MA significantly increased all parameters of locomotor activity, which confirmed one of the significant effects of MA exposure.

To conclude, our study demonstrated that the effect of paternal MA exposure on offspring was not as significant as that observed after maternal MA exposure. Nevertheless, our results indicated that paternal MA exposure alters specific patterns of social behavior that could seriously impact the social adaptation, mental health, and social life of their offspring.

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AUTHOR´S PUBLICATION

Mihalčíková, L., Ochozková, A., & Šlamberová, R. (2021). Does paternal methamphetamine exposure affect the behavior of rat offspring during development and in adulthood?. *Physiological Research*, *70*(Suppl 3), S419. **IF 2.139**

Mihalčíková, L., Ochozková, A., & Šlamberová, R. (2019). Effect of methamphetamine exposure on sexual behavior and locomotor activity of adult male rats. *Physiological Research*, *68*, S339-S346. **IF 2.139**

Ochozková, A., Mihalčíková, L., Yamamotová, A., & Šlamberová, R. (2019). ADHD symptoms induced by prenatal methamphetamine exposure. *Physiol Res*, *68*(Suppl 3), S347-S352. **IF 2.139**

Ochozková, A., Mihalčíková, L., Yamamotová, A., & Šlamberová, R. (2021). Can prenatal methamphetamine exposure be considered a good animal model for ADHD? *Physiological Research*, *70*(Suppl 3), S431. **IF 2.139**

Šlamberová, R., Nohejlová, K., Ochozková, A., & Mihalčíková, L. (2018). What is the role of subcutaneous single injections on the behavior of adult male rats exposed to drugs? *Physiological Research*, *67*, S665-S672. **IF 2.139**

AUTHOR´S PRESENTATION

17th Biennial Meeting of the European Behavioural Pharmacology Society, 31.8.-3.9.2017, Heraklion, Greece: *Šlamberová R., Nohejlová K., Ochozková A., Mihalčíková L.: Dose dependent effect of THC on behavior in unknown environment in adult male rats (poster).*

Progres Q 35 Meeting, Brandýs nad Labem, 2017: *Paternal Methamphetamine exposure- effect on offspring (oral presentation).*

11th Conference of the Czech Neuroscience Society, 28.- 29.11. 2017, Prague*: Mihalčíková L., Ochozková A., Šlamberová R.: Paternal methamphetamine exposure affects development of rat offspring (poster).*

94th Physiological days of Czech and Slovak Physiological Society 6.-8. February 2018, Faculty of Medicine in Plzeň: *Mihalčíková L., Ochozková A., Šlamberová R.: Effect of paternal methamphetamine exposure- effect on development of offspring (poster).*

60th Czech and Slovak Psychopharmacological Conference 9.-14.1.2018 Jeseník: *Mihalčíková L., Ochozková A., Šlamberová R.: Paternal methamphetamine exposure affects development of rat offspring (poster).*

11th FENS Forum of Neuroscience 7.-11. July 2018 Berlin, Germany**:** *Mihalčíková L., Ochozková A., Šlamberová R.: Paternal methamphetamine exposure affects development of rat offspring (poster).*

Student science conference Third Faculty of Medicine 22.5.2018 Prague: *Mihalčíková L., Ochozková A., Šlamberová R.: Paternal methamphetamine exposure- effect on offspring (poster).*

Progres Q 35 Meeting, Velké Popovice 23.-24. November 2018: *Paternal methamphetamine exposure- effect on locomotor activity of male rats (oral presentation).*

The 48th annual meeting of Society for Neuroscience, 3.- 7. of November 2018, San Diego, California, USA: *Mihalčíková L., Ochozková A., Šlamberová R.: Effect of paternal methamphetamine exposure on development of rat offspring (poster).*

95th Physiological days of Czech and Slovak Physiological Society 5.-7. February 2019, Prague: *Mihalčíková L., Ochozková A., Šlamberová R.: Effect of methamphetamine exposure on sexual behavior and locomotor activity of adult male rats"(poster).*

EBPS Biennal meeting, 28.- 31. August 2019, Porto- Braga, Portugal: *Mihalčíková L., Ochozková A., Šlamberová R.: How methamphetamine exposure affects sexual behavior and locomotor activity in male rats? (poster).*

The 10th IBRO World congress of Neuroscience 21.- 25. September 2019, Daegu, South Korea: *Mihalčíková L., Ochozková A., Šlamberová R.: Does methamphetamine exposure affect sexual behavior and locomotor activity in male rats? (poster).*

The 12th Conference of the Czech Neuroscience Society, 26.-27. November 2019 Prague: *Mihalčíková L., Ochozková A., Šlamberová R.: Paternal methamphetamine exposure- effect on locomotor activity of male rats (poster).*

96th Physiological days of Czech and Slovak Physiological Society 4.- 6. February 2020, Martin, Slovakia: *Mihalčíková L., Ochozková A., Šlamberová R.: Paternal methamphetamine exposure- effect on locomotor activity of male rats (poster).*

Progres Q 35 Meeting, Velké Popovice 22.- 23. November 2019: *Paternal methamphetamine exposure- effect on development of offspring (oral presentation)*

Student science conference Third Faculty of Medicine, 20.10. 2020, Prague: *Mihalčíková L., Ochozková A., Šlamberová R.: Does paternal methamphetamine exposure affect locomotor activity of offspring? (poster).*

13th Conference of the Czech Neuroscience Society 2021, 24.- 25. November 2021, Prague: *Mihalčíková L., Ochozková A., Šlamberová R.: Paternal methamphetamine exposure- effect on locomotor activity of offspring (poster).*

97th Physiological days of Czech and Slovak Physiological Society 8.- 10. February 2022, Prague: *Mihalčíková L., Ochozková A., Šlamberová R.: Does paternal methamphetamine exposure affect locomotor activity of offspring?*

31st Annual Meeting of the International Behavioral Neuroscience Society v Glasgow, Great Britain: *Šlamberová R., Mihalčíková L., Bednaříková A.: Paternal methamphetamine administration does not cause such a serious effect to rat offspring during development and in adulthood as maternal administration (poster).*

FENS Forum of Neuroscience 9.-13. July 2022 Paris, France: *Mihalčíková L., Ochozková A., Šlamberová R.: Does paternal methamphetamine exposure cause such a serious impact to rat offspring during development and in adulthood as maternal drug exposure?" (poster).*

LIST OF ATTACHMENTS

1. **Mihalčíková, L., Ochozková, A., & Šlamberová, R.** (2019). Effect of methamphetamine exposure on sexual behavior and locomotor activity of adult male rats. *Physiological Research*, *68*, S339-S346.
2. **Mihalčíková, L., Ochozková, A., & Šlamberová, R.** (2021). Does paternal methamphetamine exposure affect the behavior of rat offspring during development and in adulthood?. *Physiological Research*, *70*(Suppl 3), S419.