MASARYK UNIVERSITY
FACULTY OF MEDICINE
DEPARTMENT OF PHARMACOLOGY

DRUG ADDICTION AS A COMORBIDITY OF PSYCHIATRIC DISORDERS IN ANIMAL MODELS

Habilitation thesis
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Originality and conflict of interest statement

Presented original data were acquired as a part of original research performed by the author together with a team of her colleagues and students.

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1. Introduction

Drug addiction is a serious medical and psychosocial problem which leads to organic harm of the body as well as distortion of the normal functioning of affected persons within the society and family. Effective treatments are scarce because of variability of motivations and causes to abuse drugs, comorbidities and different social environments (Winkler et al., 2013). Drug abuse is clinically a frequent comorbidity of other psychiatric disorders (Testa et al., 2013, Wedekind et al., 2010). This is particularly well documented in affective disorders such as anxiety, depression (Volkow, 2004, Pettinati et al., 2013) and schizophrenia (Koskinen et al., 2009, Mesholam-Gately et al., 2014).

As an explanation of the dual diagnosis of depression and addiction, the self-medication hypothesis is widely accepted (Hall and Queener, 2007, Khantzian, 1985, Markou et al., 1998). It explains drug abuse as an attempt of the patient to relieve the monoaminergic deficits typical for depression. This was clinically confirmed in nicotine (Holma et al., 2013), methamphetamine (McKetin et al., 2011) and other drugs (Tolliver and Anton, 2015). There is a growing evidence that depression and addiction underlie common defective neurobiological regulations shared by major depression and withdrawal syndrome (Markou et al., 1998, Lalanne et al., 2016), namely in dopaminergic (Nestler and Carlezon, 2006), serotonergic, noradrenergic, cholinergic (Zellner et al., 2011), glutamatergic (Tzschentke, 2002) and γ-aminobutyric acid (GABA)-ergic (Koek et al., 2013) systems.

Another psychiatric condition known to be often comorbid with drug addiction is schizophrenia. Almost 50 % of schizophrenic patients suffer comorbid addiction (Lybrand and Caroff, 2009) which is linked with substantially higher burden of the disease, shorter life expectancy (Schmidt et al., 2011, Hartz et al., 2014) and higher suicide attempt rate (McLean et al., 2011, Melle et al., 2010). The most common drug addiction comorbid with schizophrenia is nicotine with a prevalence of 70 to 90 % in the patients compared to 26 % in the general population (Wing et al., 2012, Chambers et al., 2001, Mackowick et al., 2014, Khantzian, 2016). High prevalence with other substances, such as alcohol (Krystal et al., 2006, Kalyoncu et al., 2005, Kerner, 2015, Regier et al., 1990), opiates (Kern et al., 2014), amphetamine psychostimulants (Grant et al., 2012) and cannabis (McLoughlin et al., 2014) is also alarming. Several lines of evidence support the neurochemical association

between schizophrenia and addiction (Volkow, 2009). There is a known risk of triggering schizophrenia by cannabis especially during adolescence (Kucerova et al., 2014, Caspi et al., 2005, Hall and Degenhardt, 2015, Semple et al., 2005) and by psychostimulants, e.g. methamphetamine (Yui et al., 2000, Gururajan et al., 2012) and cocaine (Malave and Broderick, 2014). This suggests a common distortion of neurobiological mechanisms underlying both schizophrenia and substance abuse, namely the dopaminergic system which is known to be aberrant in both schizophrenia and substance abuses. It is possible that the DAergic dysfunction in patients with schizophrenia disrupts normal reward pathways predisposing individuals to higher risks for drug abuses (Chambers et al., 2001). Furthermore, there is a large body of clinical evidence suggesting differential characteristics of drug abuse in men and women. Despite the absolute number of female methamphetamine abusers being lower than the male ones, women usually appear more dependent, show higher escalation rates (Dluzen and Liu, 2008, Becker and Hu, 2008) and most importantly tend to experience more frequent relapses (Bobzean et al., 2014, Fattore et al., 2014). The abuse of psychostimulant drugs (cocaine, methamphetamine, etc.) is currently on the rise among women, and it has been shown that women experience higher cravings and suffer more relapses than men (Becker and Hu, 2008). These gender specific differences require specific treatment strategies for men and women (Brecht et al., 2004, Munro et al., 2006, Terner and de Wit, 2006). This particularly applies to relapse-prevention which represents a key treatment challenge especially for women (Brecht and Herbeck, 2014).

The experimental work included in this habilitation thesis aims to unravel the relationship between drug addiction and its psychiatric comorbidities in animal models. These models may serve in future for testing and development of innovative treatment strategies for dual disorders. Furthermore, the important factor of sex differences is included in order to provide information base for development of gender specific treatments. Therefore, this work is divided into three chapters, first focusing on depression-addiction comorbidity, second on schizophrenia-addiction comorbidity and last on sex-related differences in drug taking behaviours.

2. Depression and addiction comorbidity

2.1. Background

Depression and addiction are frequently comorbid as indicated by a high prevalence of the secondary addictive disorder in patients with history of major depression and other major psychiatric disorders (Langas et al., 2010, Testa et al., 2013, Volkow, 2004). The likelihood of drug addiction and depression to occur together in the same individual is approximately 5 times greater than what would be expected by the prevalence of each disorder alone and leads to increased suicide rates among depressive individuals (Ortiz-Gomez et al., 2014).

One of the theories explaining this comorbidity is the "self-medication hypothesis", arising from common risk factors and similarities in the underlying neurobiology of depression and drug addiction (Hall and Queener, 2007, Khantzian, 1985, Khantzian, 2013, Khantzian and Albanese, 2009). This theory indicates that a potential monoaminergic deficit in depression may be relieved by the drug of abuse, thus individuals with depression have deficits in brain reward systems and may turn to drugs that create euphoric feelings to compensate for their anhedonia and motivational inadequacy (Baicy et al., 2005, Koob and Le Moal, 2008, Markou et al., 1998). There is supporting clinical evidence for methamphetamine (McKetin et al., 2011) and other drugs (McKernan et al., 2015). Consequently, individuals with affective disorders suffer from higher cravings and increased relapse rate (Witkiewitz and Bowen, 2010) which is the most demanding problem faced by clinicians related to the treatment of drug abuse (Schuckit, 2006).

There is growing evidence that common defective neurobiological mechanisms underlie depression and addiction (Markou et al., 1998). Thus a study of these similarities might stimulate the future development of innovative antidepressants acting through the reward circuit of the mesocorticolimbic dopaminergic system (Nestler and Carlezon, 2006). However, it is essential to differentiate such comorbid disorders into those patients with a primary diagnosis of depression from those with a primary substance abuse disorder (Nunes and Rounsaville, 2006).

Despite the high prevalence of drug addiction and depression comorbidity, there are only few animal models examining drug abuse behaviors in depression and relapse of drug

addiction. Conformable with the clinical experience, a positive rewarding effect of amphetamine in rodents subjected to chronic mild stress was shown to be more apparent than in non-stressed rodents. This evidence suggests that stress influences the vulnerability for drug-taking behaviour (Lin et al., 2002). Another model used for the study of dual disorder is the selectively bred rat strain depression-like phenotype with low performance in the forced swim test (SwLo). However, in this model contradictory data were reported showing increased consumption of methamphetamine and cocaine in the SwLo line (Weiss et al., 2008) and opposite effect in a later study (Lin et al., 2012). Unfortunately, this model was not further tested and the findings remain inconclusive.

The most commonly used model of depression for study of the dual disorder has become olfactory bulbectomy (OBX) (Kelly et al., 1997, Filip et al., 2013). Bulbectomized rats were recorded to be hyper-responsive to the locomotor stimulating properties of cocaine administration. This was explained by the hypersensitivity to the drug induced by OBX and the model was suggested to be appropriate for investigation of comorbid depression and addiction disorder (Chambers and Taylor, 2004, Slattery et al., 2007). Validity of the self-medication hypothesis was further confirmed when bulbectomized rats showed decreased behavioural depressive-like symptoms when they were treated with nicotine either intraperitoneally or by self-administration paradigm (Vieyra-Reyes et al., 2008).

Our team has substantially contributed to the research of addiction behaviours in the OBX model. We developed this rat model of depression and addiction dual disorder where olfactory bulbectomized animals showed a significantly higher vulnerability in intravenous drug self-administration of methamphetamine (Kucerova et al., 2012). Later we confirmed also increased tendency of the OBX rats to reinstate the methamphetamine seeking behaviour (Babinska et al., 2016). Furthermore, we tested other drugs, specifically cannabinoid receptor-1 synthetic agonist (Amchova et al., 2014) and ketamine (Babinska and Ruda-Kucerova, 2016) with similar results. We have also attempted to explain the underlying neurochemical variables in the *nucleus accumbens* shell after a drug challenge (Amchova et al., 2014, Ruda-Kucerova et al., 2015b).

2.2. Aims

The research on the animal model of the depression and addiction comorbidity aimed to:

1. Establish the rat model of the dual disorder using olfactory bulbectomy as a model of depression while drug abuse was modelled by intravenous self-administration of methamphetamine
    - Section 2.4.1., Kucerova *et al.*, 2012
2. Further validate the model by assessing the relapse-like behaviour towards methamphetamine
    - Section 2.4.2., Babinska *et al.*, 2016
3. Further validate the model by assessing the basal and methamphetamine influenced profile of extracellular levels of neurotransmitters in the nucleus accumbens shell, i.e. the key area of the reward circuit
    - Section 2.4.3., Ruda-Kucerova *et al.*, 2015
4. Extend the rat model of the dual disorder to intravenous self-administration of synthetic CB1 receptor agonist
    - Section 2.4.4., Amchova *et al.*, 2014
5. Extend the rat model of the dual disorder to intravenous self-administration of potential new antidepressant ketamine
    - Section 2.4.5., Babinska and Ruda-Kucerova, 2016

2.3. Methods

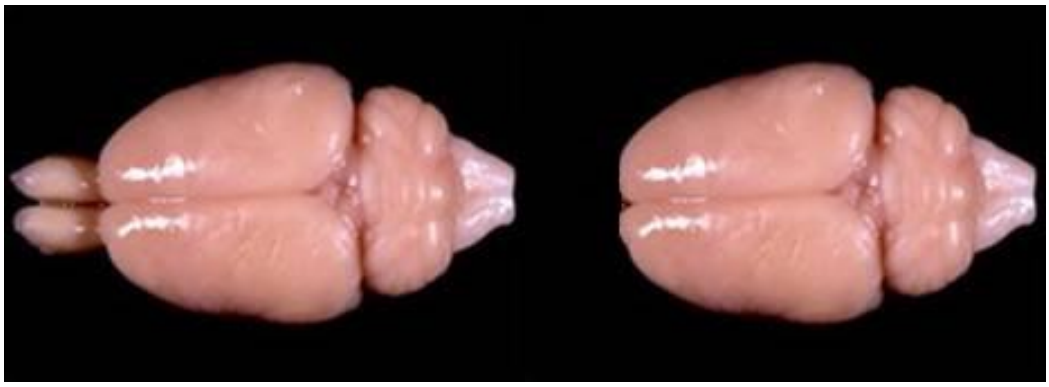
2.3.1. Animals

Adult male rats of Wistar, Sprague-Dawley or Lister-Hooded strain were used in the studies. The rats were housed individually in standard rodent plastic cages. Environmental conditions during the whole study were constant: relative humidity 50-60 %, room temperature 23◦C ± 1◦C, inverted 12-hour light-dark cycle. Food and water were available *ad libitum*. All experiments were conducted in accordance with all relevant laws and regulations of animal care and welfare. The experimental protocol was approved by the Animal Care Committee of the Masaryk University, Faculty of Medicine, Czech Republic, and carried out under the European Community guidelines for the use of experimental animals.

2.3.2. Olfactory bulbectomy model of depression (OBX)

Bilateral olfactory bulbectomy is a well-established model of depression with high validity which closely mimics neurochemical, neuroanatomical, behavioural and endocrine changes seen in patients with major depression (Song and Leonard, 2005, Harkin et al., 2003). The bilateral ablation of the olfactory bulbs (Figure 1) was performed in accordance with the method described by Leonard and Tuite (Leonard and Tuite, 1981) and Kelly et al. (Kelly et al., 1997).

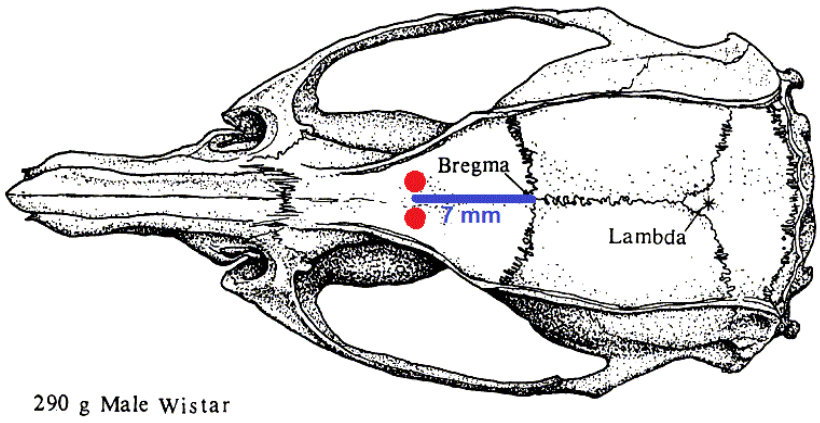
Figure 1: normal rat brain and OBX rat brain



(Source: author's own figure)

As validated at our laboratory (Pistovcakova et al., 2008) animals were anaesthetized with ketamine 50 mg/kg and xylazine 8 mg/kg given intraperitoneally. The top of the skull was shaved and swabbed with an antiseptic solution, after which a midline frontal incision was made in the skin on the skull and the skin was retracted bilaterally. After exposure of the skull, the 2 burr holes 2 mm in diameter were drilled at the points 7 mm anterior to the bregma and 2 mm lateral to bregma suture (Figure 2). Both olfactory bulbs were removed by aspiration. The ablation cavity was filled with a haemostatic sponge. Sham operated rats underwent the identical anaesthetic and drilling procedures as OBX animals, but their bulbs were left intact. Experiments were carried out 3 weeks after the surgery. At the end of the experiment, rats were euthanized by a lethal overdose of ether and their brains were removed for confirmation of the removal of the olfactory bulbs. Rats with an incomplete bilateral olfactory bulbectomy or with damage to other brain structures were excluded from data analysis.

Figure 2: location of burr holes drilled for OBX procedure



(Source: Paxinos and Watson, 2007)

2.3.3. IV self-administration (IVSA) surgery and procedures

Under the general anaesthesia with ketamine 50 mg/kg and xylazine 8 mg/kg given intraperitoneally a permanent intracardiac silastic catheter was implanted through the external jugular vein to the right atrium. The outer part of the catheter exited the skin in the midscapular area. The catheters were flushed daily before all the sessions with heparinized antibiotic to prevent infection and occlusion of the catheter. During this procedure the

blood was aspired daily to assess the patency of the catheter, and changes in general behaviour, weight and other circumstances were recorded. When a catheter was found to be blocked the animal was excluded from the analysis (Thomsen and Caine, 2005).

Standard experimental cages (Figure 3) with two nose-poke holes allocated on one side of the cage were programmed by software L2T2 or later Graphic State Notation 3.03 (Coulbourn Instruments, USA) and the IVSA sessions were conducted under the fixed ratio (FR) schedule of reinforcement[1]. Nose-poking in the active hole led to the activation of the infusion pump and administration of an infusion followed by a timeout, when nose-poking was recorded but not rewarded. The cage was illuminated by a house light during the session. The light was flashing when the system was administering infusion (5 sec) and off during the time-out period to provide environmental cue associated with the drug infusion. IVSA sessions took place 7 days/week between 8 a.m. and 3 p.m. during the dark period of the inverted light-dark cycle and after the end rats were returned to their home cages.

Figure 3: IV self-administration session

(Source: author's own photo)

[1]In our collaborating laboratory in Cagliari (Italy) an analogous system (Med Associates,Vermont, USA) was used. This system is equipped with retractable levers as *operandums*.

After several weeks of stable drug intake the maintenance phase was terminated and rats were kept in their home cages for 14 days of the forced abstinence period. On day 15, rats were placed into self-administration chambers for the reinstatement session. The numbers of responses on the active drug-paired nose-poke and the inactive nose-poke were recorded but the drug was not delivered. Responses on the active nose-poke are considered to reflect the reinstatement of drug seeking behaviour, whilst responses on inactive nose-poke reflect nonspecific locomotor and exploratory activity.

In some studies, self-administration behaviour was initially trained by employing sweet pellets as a reward. The sessions were conducted in the same experimental boxes as IVSA studies (Coulbourn Instruments, USA) under the FR1 schedule of reinforcement, where 1 nose-poke lead to activation of a feeder and delivery of a single palatable pellet (BioServ, sweet dustless rodent pellets, F0021-Purified Casein Based Formula - 45mg). The cage was illuminated by a house light during the whole session. Self-administration sessions lasted 30 minutes during the dark period of the inverted light-dark cycle.

2.3.4. *In vivo* microdialysis

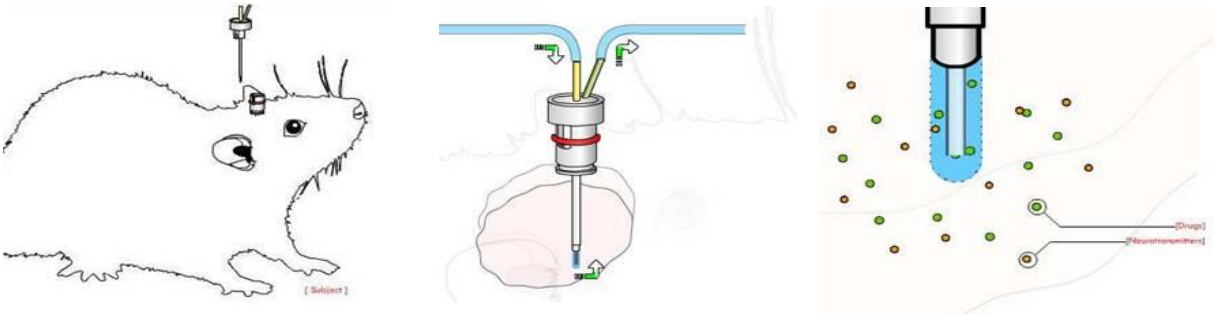
As described in detail earlier (Sustkova-Fiserova et al., 2014, Fiserova et al., 1999), under ketamine – xylazine anaesthesia (ketamine 100 mg/kg i.p., xylazine 10 mg/kg i.p.), rats were implanted with a disposable dialysis guide cannula using a stereotaxic instrument. The guide was randomly alternated on the left and right side. The target site was *nucleus accumbens* shell (Paxinos and Watson, 2007, Paxinos and Watson, 1998).

Forty-eight hours after implantation, the probe was inserted into the guide cannula and artificial cerebrospinal fluid was flushed through the probe at a constant rate of 2.0 μl/min. After 80 min of habituation to the microdialysis set-up (when dialysate was discarded), 40 μl samples were collected at 20-min intervals. After 3 consecutive baseline samples, methamphetamine (5 mg/kg in 2 ml) or vehicle (saline 2 ml/kg) was administered intraperitoneally (at minute 60) and dialysates were collected every 20 minutes (Figure 4). Total duration of the sampled session was 240 min in animals with administration of METH and 180 min in animals after saline injection. This is because administration of the vehicle was not expected to induce any changes so the session was shortened.

The amount of dopamine, serotonin and their metabolites (3-methoxytyramine = 3-MT, 3,4-dihydroxyphenylacetic acid = DOPAC, homovanillic acid = HVA and 5-

hydroxyindoleacetic acid (5-HIAA) resp.), as well as glutamate and GABA in the dialysates were quantified using high-performance liquid chromatography combined with mass spectrometry (HPLC-MS). The appropriate HPLC-MS determination methods are described in detail earlier (Syslova et al., 2011).

Figure 4: in vivo microdialysis principle



(Source: https://www.basinc.com/products/iv/MD.html)

2.3.5. Locomotor activity

In brightly lit room, rats were individually tested for locomotor activity using the Actitrack system (Panlab, Spain)[2]. Each Plexiglas arena (45×45×30 cm) was surrounded by 2 frames equipped with photocells located one above another at 2 and 12 cm over the cage floor. Animals were placed in the centre of arena and the spontaneous behaviour was tracked for 10 minutes. In the test horizontal locomotor activity (the trajectory calculated by the system as beam interruptions that occurred in the horizontal sensors) and vertical activity (number of rearing episodes breaking the photocell beams of the upper frame) were recorded. At the end of the session, animals were returned to their home cage and arenas were wiped with 1% acetic acid to avoid olfactory cues (Kucerova and Sulcova, 2008, Kucerova et al., 2006).

2.3.6. Forced Swim Test (FST)

A modified FST (Detke et al., 1995, Porsolt et al., 1977) was used to measure immobility of the rats, as described previously (Akinfiresoye and Tizabi, 2013, Tizabi et al., 2012). Briefly, the rats were individually placed into a plexi-glass cylinder filled with 30 cm of

[2]In our collaborating laboratory in Cagliari (Italy) an analogous system Digiscan Animal Activity Analyser (Omnitech Electronics, USA) was used. The dimensions of arenas were 42×30×60 cm.

water (24±1 °C). The sessions were video-taped for later scoring and the water was changed after every animal. A time-sampling scoring technique was used, whereby the predominant behaviour, i.e. immobility, swimming or climbing, in each 5-s period of the 5 minutes test was recorded. OBX rats acquire the depressive-like phenotype by surgery, therefore they should exhibit spontaneous immobility in the forced swim test. Furthermore, the aim of the test was to assess spontaneous behaviour, i.e. not a drug effect. Hence, there is no need to have a pre-test exposure to forced swimming the day before to induce helplessness (Tejani-Butt et al., 2003, Tizabi et al., 2012).

Figure 5: forced swim test procedure

(Source: author's own photo)

2.3.7. Sucrose preference

A two-bottle choice procedure was used to determine the sucrose intake (Chambliss et al., 2004, Matthews et al., 1995, Romeas et al., 2009). During the 24-h training phase, each rat was provided in their home cage with two water bottles on the extreme sides of the cage to adapt rats drinking from two bottles. After training, one bottle was randomly switched to contain sucrose dissolved in drinking water at concentration of 1% or 2%. The side of sucrose presentation in the home cage was counterbalanced across rats. At 4h and 24h time intervals both bottles were removed and the amount of liquid remaining in each bottle was

measured. The sucrose preference score was calculated as the percentage of sucrose solution ingested relative to the total amount of liquid consumed as determined before and after each test, i.e. sucrose preference = sucrose intake / total liquid (sucrose + water) intake x 100.

2.4. Results

2.4.1. The effects of methamphetamine self-administration on behavioural sensitization in the olfactory bulbectomy rat model of depression

This study established the rat model of the dual disorder using olfactory bulbectomy as a model of depression while drug abuse was modelled by intravenous self-administration of methamphetamine. Furthermore, in order to assess the influence of behavioural sensitization (Robinson and Berridge, 1993, Robinson, 1984) to methamphetamine, a chronic exposure to the drug known to induce sensitization (Landa et al., 2005) was employed.

The results showed that olfactory bulbectomy model of depression increases methamphetamine intake in the IV self-administration model. This finding correlates well with the self-medication hypothesis (Hall and Queener, 2007, Khantzian, 1985, Khantzian, 2016) and a previous study with amphetamine (Holmes et al., 2002). Chronic intermittent pre-treatment with methamphetamine was used to evaluate influence of behavioural sensitization on the drug intake of olfactory bulbectomized and sham operated rats. Pre-exposure to methamphetamine decreased the intake of the drug in the self-administration in sham operated animals but not in rats subjected to olfactory bulbectomy. This suggests a differential reactivity to chronic methamphetamine exposure in the OBX model, maybe due to altered expression of behavioural sensitization in this model of depression.

Kucerova J, Pistovcakova J, Vrskova D, Dusek L, Sulcova A. The effects of methamphetamine self-administration on behavioural sensitization in the olfactory bulbectomy rat model of depression. Int J Neuropsychopharmacol. 2012, 15(10): 1503-11. doi: 10.1017/S1461145711001684.

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Erratum

Figure 1(b) presented in this paper contains a typographical error in the names of the groups. The correct version follows.

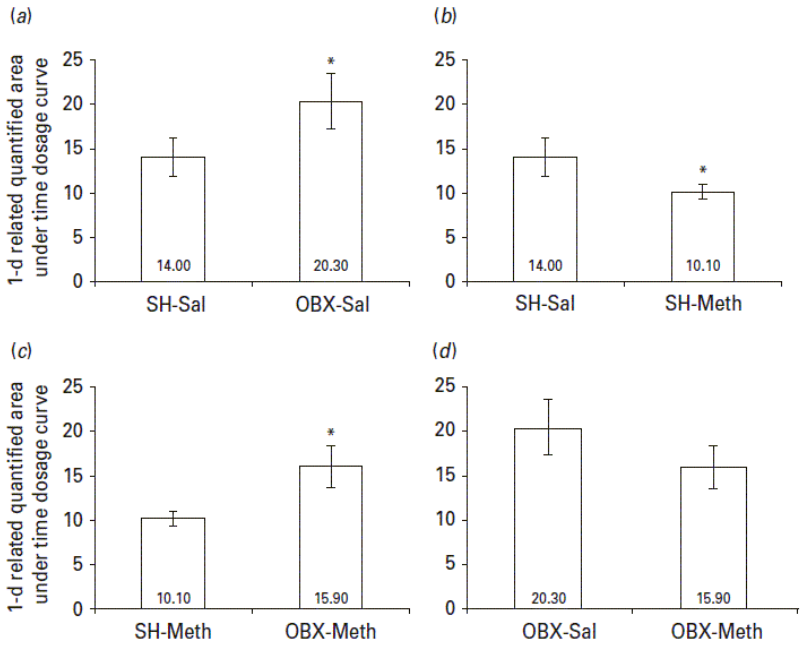

Fig. 1. The differences in methamphetamine (Meth) intake among experimental groups. (*a*) A significant increase ($p = 0.046$) of Meth intake in olfactory bulbectomized (OBX) rats compared to sham-operated (SH) animals, both without Meth pretreatment; (*b*) a significant decrease ($p = 0.047$) of Meth intake in SH animals with a history of Meth administration (sensitized) compared to non-sensitized rats; (*c*) a significant increase ($p = 0.040$) of Meth intake in the OBX rats compared to SH animals, both at conditions of Meth pretreatment; (*d*) statistical comparison of differences in Meth intake of OBX animals with and without history of Meth administration (n.s.). Mean values and corresponding S.E. are shown in the graph. Statistical significance of differences among given groups were evaluated as area under the dosage time curve, calculated per day; tested by Mann–Whitney *U* test. Sal, saline.

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ARTICLE

CINP

The effects of methamphetamine self-administration on behavioural sensitization in the olfactory bulbectomy rat model of depression

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Abstract

Depression is frequently comorbid with a drug addiction and may seriously complicate its treatment. Currently, there is no routinely used animal model to investigate this comorbidity. In this study the effect of repeated administration of methamphetamine on i.v. drug self-administration in an olfactory bulbectomy model of depression in rats was investigated in order to propose and validate a rat model of comorbid depression and addiction. Male Wistar rats were either olfactory-bulbectomized (OBX) or sham-operated. They subsequently underwent a methamphetamine sensitization regime, which consisted of daily i.p. injections of methamphetamine for a 14-d period; controls received Sal injections at the same frequency. The i.v. self-administration of methamphetamine (0.08 mg/kg in one infusion) paradigm on a fixed ratio schedule of reinforcement was performed using operant chambers. A significant decrease of the drug intake was recorded in sham-operated animals pretreated with methamphetamine when compared to the unpretreated group. This was not apparent in the OBX groups. Both groups of OBX animals exhibited a higher intake of methamphetamine compared to the corresponding sham-operated groups, thus confirming the hypothesis of higher drug intake in depressive conditions in this rodent model. The procedure of behavioural sensitization to methamphetamine decreased the number of self-administered drug doses per session in the sham-operated rats. It is hypothesized that this phenomenon resulted from increasing efficacy of the drug after behavioural sensitization caused by repeated methamphetamine intermittent administration.

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Key words: Depression, methamphetamine, olfactory bulbectomy model, IVSA, Wistar rats.

Introduction

Depression and addiction are frequently comorbid, as indicated by a high prevalence of the secondary addictive disorder in patients with a history of major depression and other major psychiatric disorders (Langas *et al.* 2010). This is true for nicotine (Kushnir *et al.* 2010), alcohol (Boschloo *et al.* 2011) and other drugs of abuse. Drug-dependent subjects suffer from comorbid psychiatric disorder, such as depression, in approximately 30–50% of cases (Cottencin, 2009; Davis *et al.* 2008) and the frequency (data from United States) tends to increase over time (Compton *et al.* 2006). The prevalence of depression in drug-addicted individuals in Europe was recorded as approximately 50% in spite of significantly different environmental and social conditions and the type of drugs abused (Reissner *et al.* 2011). This comorbidity was defined in DSM-III (Kessler *et al.* 1996) and is more accurately characterized in DSM-IV (Leventhal *et al.* 2008).

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There is growing evidence that common defective neurobiological mechanisms underlie depression and addiction (Markou *et al.* 1998). Thus, a study of these similarities might stimulate the future development of innovative antidepressants with a new mechanism of action through the reward circuit of the mesocorticolimbic dopaminergic system (Nestler & Carlezon, 2006). However, it is essential to differentiate such comorbid disorders into those patients with a primary diagnosis of depression from those with a primary substance abuse disorder (Nunes & Rounsaville, 2006).

The self-medication hypothesis was developed as a possible explanation of the frequent comorbidity of depression or anxiety with psychostimulant abuse linked to enhanced monoaminergic neurotransmission. Thus, it is hypothesized that symptoms related to a potential monoaminergic deficit in depression may be relieved by the drug of abuse (Hall & Queener, 2007; Khantzian, 1985). Evidence supporting this hypothesis comes from the finding that antidepressant treatment of substance abuse is more effective in depressed than non-depressed individuals (Markou *et al.* 1998; Wohl & Ades, 2009).

Conformable with clinical experience, animal models were employed, finding positive rewarding effects of amphetamine in rodents subjected to chronic mild stress more apparent than in non-stressed rodents. This evidence suggests that stress influences the vulnerability for drug-taking behaviour (Lin *et al.* 2002). There is evidence of an increase of dopamine receptor density in the ventral striatum (Cairncross *et al.* 1975), which correlates with enhanced reinforcing properties of drugs of abuse in the chronic lesion model of depression evoked by bilateral olfactory bulbectomy (OBX) (Kelly *et al.* 1997). Bulbectomized rats were recorded to be hyper-responsive to the locomotor-stimulating properties of cocaine administration. This was explained by the hypersensitivity to the drug induced by OBX and the model was suggested to be appropriate for investigation of comorbid depression and addiction disorder (Chambers *et al.* 2004; Slattery *et al.* 2007). The self-medication hypothesis was further confirmed when bulbectomized rats showed decreased behavioural depressive-like symptoms when they were treated with nicotine either i.p. or by self-administration paradigm (Vieyra-Reyes *et al.* 2008).

Behavioural sensitization to drugs of abuse (Fukushiro & Frussa-Filho, 2010) and the related adaptations in striatal neurotransmission (particularly dopaminergic) are thought to play an important role in certain aspects of addiction, such as a tendency to relapse following abrupt drug withdrawal (Ohmori *et al.* 2000; Shuto *et al.* 2008). Pharmacotherapeutic support in drug addiction is also challenged by heterogeneity of the disorder in humans (Alguacil *et al.* 2011) and, by being dependent on possible distress in early life, is complicated to assess and measure in rodent models (Zellner *et al.* 2011). Moreover, there is a lack of rodent models, taking into account comorbid psychiatric disorders that are common in clinical practice (Wong *et al.* 2010). Therefore, the aim of this study was to utilize the i.v. drug self-administration (IVSA) model, which is known to be reliable for testing dependence potential and abuse liability of drugs (Collins *et al.* 1984), together with the OBX model of depression (Kelly *et al.* 1997) in order to compare methamphetamine (Meth) intake in male rats subjected to repeated drug pretreatment shown to induce behavioural sensitization (Landa *et al.* 2005). From the self-medication hypothesis, the anticipated outcome of this study is: (*a*) a decrease of the total number of Meth doses self-administered by rats per experimental session as indicated by our previous studies (Kucerova *et al.* 2009); (*b*) an enhancement of Meth intake as a reflection of the depressive-like state induced by bilateral OBX.

Method

Animals

Adult male albino Wistar rats weighing 180–220 g at the beginning of the experiment were purchased from Biotest Ltd (Konarovice, Czech Republic). The rats were housed in sections of four (two bulbectomized and two sham-operated) in standardized rat plastic cages. After catheter implantation surgery was performed, the rats were housed individually in standard plastic cages (dimensions: 20.5 cm × 36 cm, height 16.5 cm). Environmental conditions during the whole study were constant: relative humidity 50–60%; temperature 23 ± 1 °C; reversed 12-h light/dark cycle (lights on 17:00 hours). Food and water were available *ad libitum*. All experiments were conducted in accordance with relevant laws and regulations of animal care and welfare. The experimental protocol was approved by the Animal Care Committee of the Masaryk University Faculty of Medicine, Czech Republic and carried out under the European Community guidelines for the use of experimental animals.

Drugs and treatments

Meth from Sigma Chemical, Co., USA was used for both initial drug pretreatment and in the IVSA model. The administration of Meth prior to IVSA was

according to the following dosing regimen, which was successfully used in previous studies carried out at our laboratory (Landa *et al.* 2005, 2008) to induce behavioural sensitization: 0.5 mg/kg.d i.p. for 14 d, administered in home cages. The identical volume and route of administration of saline (Sal) solution was used for all control treatments. The Meth dose available in the operant cage for IVSA was 0.08 mg per infusion. The maximum number of infusions obtainable in one session was set to 50, which was a procedure producing reinforcing effects in the same model of IVSA in our laboratory (Vinklerova *et al.* 2002).

OBX surgery

At the beginning of the study the rats were randomly divided into two groups. The bilateral ablation of the olfactory bulbs was performed in one half of the rats in accordance with the method described by Kelly *et al.* (1997), Leonard & Tuite (1981) and Song & Leonard (2005). The other group received a sham operation consisting of all surgical procedures except olfactory bulb ablation. Animals were anaesthetized with 50 mg/kg ketamine and 8 mg/kg xylazine given i.p. (Narkamon 5%; SPOFA a.s., Czech Republic and Rometar 2%; SPOFA a.s.). The top of the skull was shaved and swabbed with an antiseptic solution, after which a midline frontal incision was made in the skin on the skull and the skin was retracted bilaterally. After exposure of the skull, two burr holes, 2 mm in diameter, were drilled at the points 7 mm anterior to the bregma and 2 mm lateral to the bregma suture. Both olfactory bulbs were removed by aspiration. Care was taken to avoid damage to the frontal cortex. The ablation cavity was filled with a haemostatic sponge. The skin above the lesion was closed with suture and the antibacterial neomycin and bacitracin powder (Framykoin pulv.; Infusia a.s., Czech Republic) was applied. Sham-operated rats underwent the identical anaesthetic and drilling procedures as OBX animals, but their bulbs were left intact. Experiments were carried out 3 wk after the surgery when hyperlocomotion induced by the OBX method was assessed following standard methodology (Pistovcakova *et al.* 2008). During this period, the animals were handled daily to eliminate aggressiveness, which could otherwise arise (Leonard & Tuite, 1981; Song & Leonard, 2005). At the end of the experiment, rats were killed by a lethal overdose of ether and their brains were removed for confirmation of the removal of the olfactory bulbs. Rats with an incomplete bilateral OBX or with damage to other brain structures were excluded from data analysis.

IVSA surgery and procedures

IVSA procedures including surgery were started 3 wk after OBX surgery. The animals were allowed 1 wk recovery and then repeated administration of Meth was performed for 2 wk. Under general anaesthesia with 50 mg/kg ketamine and 8 mg/kg xylazine given i.p. (Narkamon 5%; SPOFA a.s. and Rometar 2%; SPOFA a.s.) in combination with isoflurane inhalation for induction to anaesthesia, a permanent intracardiac silastic catheter was implanted through the external jugular vein to the right atrium. The outer part of the catheter exited the skin in the midscapular area. A small nylon bolt was fixed on the skull with dental acrylic to stainless steel screws embedded in the skull; this served as a tether to prevent the catheter from being pulled out while the rat was in the self-administration chamber. The catheters were flushed daily before all the sessions with heparinized cephalosporine (Vulmizolin 1.0 inj sicc; Biotika a.s., Slovak Republic) solution (0.05 mg/kg cephalosporine dissolved in Sal with 2.5 IU/kg heparin) and finally 0.05 ml heparin (Heparin Leciva inj. sol. 1×10 ml/50 IU) solution (5 IU) to prevent infection and occlusion of the catheter. During this procedure, blood was aspired daily to assess the patency of the catheter and changes in general behaviour, weight and other circumstances were recorded. When a catheter was found to be blocked, the animal was excluded from the analysis.

IVSA protocol

Standard experimental cages with two nose-poke holes located on one side of the cage were programmed by L2T2software (Coulbourn Instruments, USA) and the IVSA sessions were initially conducted under the fixed ratio (FR) schedule of reinforcement, starting at FR1 (each correct response reinforced). FR requirements were raised (e.g. FR2 – two correct responses required, FR3 – three correct responses required, etc.) when the animal fulfilled the following conditions for three consecutive sessions: (*a*) at least 70% preference of the drug-active nose-poke; (*b*) minimum intake of 10 infusions per session; (*c*) stable intake of the drug (maximum 10% deviation). Active nose-pokes led to the activation of the infusion pump and administration of a single infusion followed by 30 s time-out, while the other nose-pokes were recorded but not rewarded. The cage was illuminated by a house light during the session. The light was twinkling when administering infusion and off in the time-out. The IVSA sessions lasted 90 min and took place daily (including weekends) regularly between

07:00 and 16:00 hours during the dark period of the reversed light cycle. After the session the animals were returned to the home cage.

Experimental groups

There were 9–10 rats per experimental group at the beginning of the experiment. However, due to complicated surgical procedures and the nature of the OBX operation, a significant number of the subjects was lost or excluded from analysis for different reasons.

The final groups as statistically analysed were as follows:

(*a*) SH Sal group ($n=7$): sham-operated (not OBX) rats with 14 d of Sal (placebo) pretreatment.
(*b*) SH Meth group ($n=5$): sham-operated rats with 14 d of Meth pretreatment.
(*c*) OBX Sal group ($n=6$): OBX rats with 14 d of Sal (placebo) pretreatment.
(*d*) OBX Meth group ($n=7$): OBX rats with 14 d of Meth pretreatment.

Statistical data analysis

Standard robust descriptive statistics were used for the analysis; categorical variables were described as number of cases and percentage of categories; continuous variables as median and 5th–95th percentile range. Baseline time series in dosage of Meth was summarized as daily number of infusions and as area under the dosage time curve, calculated per day. The area under the curve concept was adopted as a quantitative measure integrating information on how much and how long the intake of the drug remained significant (Dahlquist & Björck, 2008; Mason & Graham, 2002). Non-parametric tests (Kruskal–Wallis test, Mann–Whitney *U* test) were used to compare different experimental groups. A non-parametric approach was used due to proven non-normal sample distribution of analysed datasets.

Special attention was focused on variability in the Meth dosage time series. Day-to-day consecutive differences in injection intake were calculated and summarized as mean absolute difference (MAD). Autocorrelation coefficient of first order (R_1) was estimated as a measure of potential mutual dependence of consecutive dosage points. For each experimental animal, Ljung– Box test statistics (Ljung & Box, 1978) was computed to verify the null hypothesis of independence (overall randomness) in dosage time series; statistical significance was assessed using χ^2 distribution. Individually estimated autocorrelation coefficients and Ljung–Box p values were then summarized for the whole experimental groups as median, minimum and maximum values.

Statistical analyses were computed using SPSS 19.0.1 (IBM Corporation, USA). A value of $p<0.05$ was recognized as the boundary of statistical significance in all applied tests.

Results

Table 1 summarizes all accessible quantitative and qualitative measures reached from the baseline time series of Meth injections. It appears that both the absolute number of infusions per day and 1 d-related quantified area under time dosage curve (Fig. 1) reveal the same trend and reflect similar differences among the experimental groups. The greatest difference occurred between bulbectomized rats and sham-operated rats, in that OBX rats significantly increased Meth intake ($p=0.014$). This was also consistently confirmed within groups pretreated repeatedly by Meth (SH Meth *vs*. OBX Meth, $p=0.040$) as well as within the control group (SH Sal *vs*. OBX Sal, $p=0.046$).

Comparison of animals repeatedly pretreated by Meth to control group (Sal) revealed significantly decreased Meth intake in the Meth group ($p=0.044$). This trend was significantly confirmed in sham-operated animals ($p=0.047$), while no exact statistically significant difference was observed in OBX animals ($p=0.102$).

Preference in taking Meth was recorded as a qualitative measure on each day of baseline experiment; however, with no significant difference among experimental groups (Table 1, Fig. 2).

Table 2 displays the main experimental results from the viewpoint of variability and randomness of Meth intake time series. It appears that sham-operated animals (regardless of type of pre-treatment) showed less variable day-to-day intake than animals in the OBX group (measured as day-to-day MAD, also expressed in % of overall mean intake). The individual intake time series also appeared to be more random in the OBX group as compared to the SH group; none of the first order autocorrelation coefficients and individual tests for randomness were significant in OBX animals. On the other hand, sham-operated rats showed a less random Meth intake with a relatively high first order autocorrelation (SH Sal: 0.298 with maximum 0.594, SH Meth: 0.386 with maximum 0.548). The Ljung–Box test did not demonstrate overall randomness of time of intake in 45% of sham-operated animals, but not in any of the OBX-treated animals.

Table 1. Comparison of experimental groups in baseline characteristics

Group	Time-related profile of infusions[a]		Preference[a] (%)
	No. of infusions per day	Infusions in time: AUC/d	
Pretreatment by Sal			
SH Sal ($n=7$)	13 (10–26)	14.0 (2.2)	79.6 (39.2–94.3)
OBX Sal ($n=6$)	18 (13–34)	20.3 (3.1)	84.8 (46.3–97.0)
Pretreatment by Meth			
SH Meth ($n=5$)	11 (10–12)	10.1 (0.8)	79.4 (61.3–95.0)
OBX Meth ($n=7$)	15 (13–23)	15.9 (2.3)	75.2 (53.4–94.1)
Statistical comparisons[b]			
SH × OBX	$p=0.014$		$p=0.724$
SH Sal × OBX Sal	$p=0.046$		$p=0.946$
SH Meth × OBX Meth	$p=0.040$		$p=0.911$
Sal × Meth	$p=0.044$		$p=0.481$
SH Sal × SH Meth	$p=0.047$		$p=0.933$
OBX Sal × OBX Meth	$p=0.102$		$p=0.705$

Sal, Saline; SH Sal, sham-operated [not olfactory-bulbectomized (OBX)] rats with 14 d of Sal (placebo) pretreatment; OBX Sal, OBX rats with 14 d of Sal (placebo) pretreatment; Meth, methamphetamine; SH Meth, sham-operated rats with 14 d of Meth pretreatment; OBX Meth, OBX rats with 14 d of Meth pretreatment.

[a] No. of infusions and preference: median (5th–95th percentile range); area under curve (curve: cumulative profile of infusions in time) per 1 d (AUC/d). Values are shown as mean (S.E.).

[b] Statistical significance (p value) of differences among given groups in AUC/d and in recorded preference; tested by Mann–Whitney U test.

Discussion

In the present study, the rewarding effect of Meth was apparent in all experimental groups by achieving a high preference (%) of the active nose-poke. However, no significant variability was recorded in nose-poke responding among the groups (Table 1, column Preference). This indicates that there was no difference in rewarding effects of the drug in particular animal groups.

The present study also confirms our previous findings that male Wistar rats repeatedly pre-exposed to Meth (14 daily doses of 0.5 mg/kg) self-administer a significantly lower number of Meth infusions under a FR schedule of reinforcement (0.08 mg/infusion) compared to animals pretreated with Sal (Kucerova *et al.* 2009, 2010). The decreased drug-seeking behaviour in this model can be considered as a sign of behavioural sensitization, suggesting higher rewarding properties of Meth in previously sensitized animals similarly as recorded elsewhere when using amphetamine and cocaine (de Vries *et al.* 1998; Lorrain *et al.* 2000). However, repeated intermittent pretreatment with Meth decreased subsequent drug intake only in the sham-operated animals. In the OBX group the same trend was apparent, but it did not reach statistical significance due to increased behavioural variability. The bulbectomized animals showed a higher day-to-day variability in Meth intake as compared to sham-operated animals. The intake time series were also more frequently random in the OBX group in comparison to the control group. This larger variation could be partially attributed to the cognitive impairment reported in the OBX animals (Kelly *et al.* 1997) and to a reduced number of animals that were lost due to failure of the catheter maintenance combined with surgical complications.

There is a lack of data describing the influence of OBX on self-administration of dependence-producing drugs. In support of the self-medication theory, it has been shown that nicotine (Vieyra-Reyes *et al.* 2008), alcohol (Chiang *et al.* 2008) and cocaine (Slattery *et al.* 2007) are able to decrease the symptoms of depression induced by OBX. However, to date, comorbid drug addiction and OBX-induced depressive conditions was, according to available literature, studied only by Holmes *et al.* (2002). In that study, male Sprague–Dawley rats self-administered significantly more infusions of a low dose of amphetamine (12 μg/infusion of D-amphetamine sulfate) after OBX than sham-operated controls. This difference was only present at

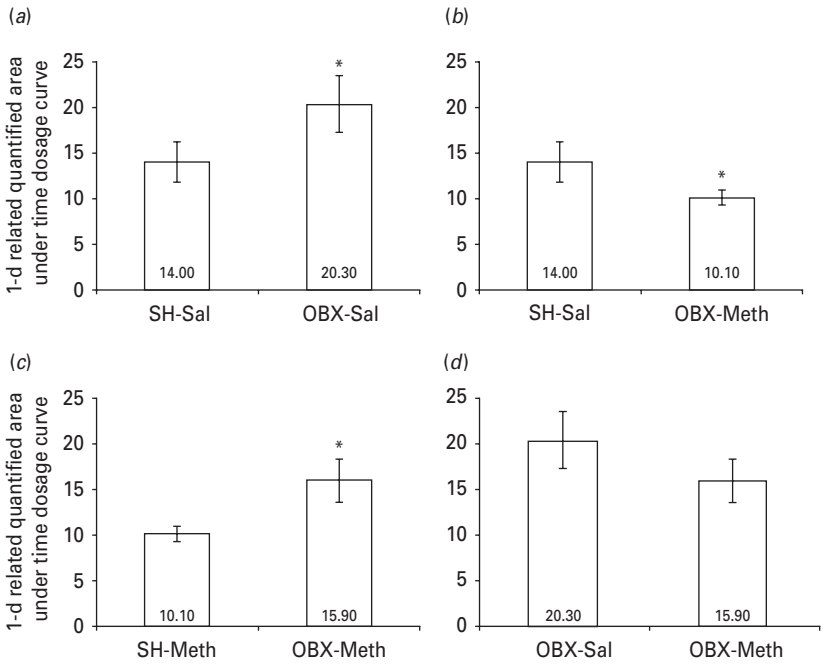

Fig. 1. The differences in methamphetamine (Meth) intake among experimental groups. (*a*) A significant increase ($p = 0.046$) of Meth intake in olfactory bulbectomized (OBX) rats compared to sham-operated (SH) animals, both without Meth pretreatment; (*b*) a significant decrease ($p = 0.047$) of Meth intake in SH animals with a history of Meth administration (sensitized) compared to non-sensitized rats; (*c*) a significant increase ($p = 0.040$) of Meth intake in the OBX rats compared to SH animals, both at conditions of Meth pretreatment; (*d*) statistical comparison of differences in Meth intake of OBX animals with and without history of Meth administration (n.s.). Mean values and corresponding S.E. are shown in the graph. Statistical significance of differences among given groups were evaluated as area under the dosage time curve, calculated per day; tested by Mann–Whitney *U* test. Sal, saline.

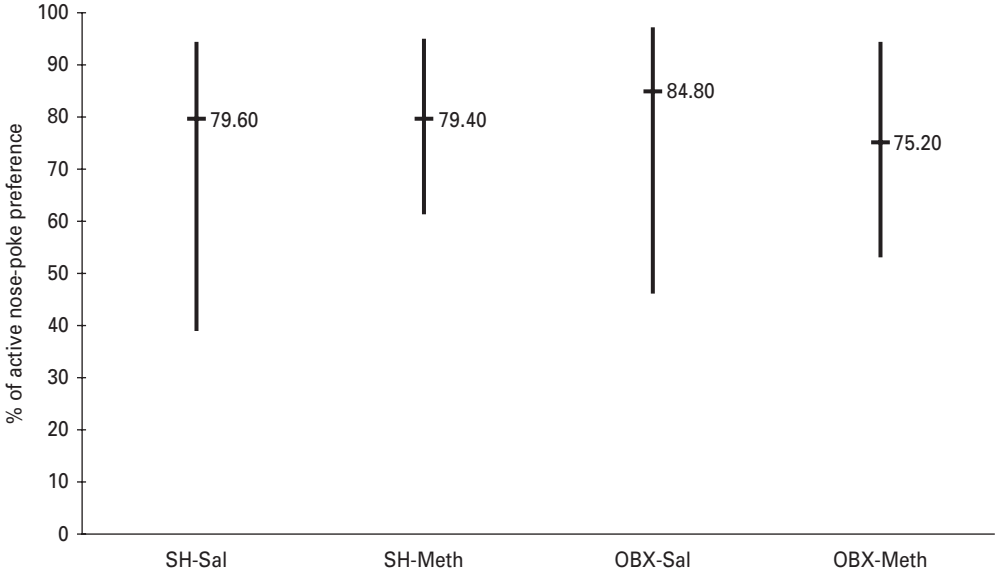

Fig. 2. Minimal, maximal and average (values presented on the graph) percentage of active nose-poke preference among all experimental groups. SH-Sal, sham-operated [not olfactory-bulbectomized (OBX)] rats with 14 d of saline (Sal) (placebo) pretreatment; SH-Meth, sham-operated rats with 14 d of Meth pretreatment; OB-Sal, OBX rats with 14 d of Sal (placebo) pretreatment; OB-Meth, OBX rats with 14 d of Meth pretreatment. Comparison of preferences did not show statistically significant differences. Statistical significance of recorded preference was tested by Mann–Whitney *U* test.

Table 2. Time-related profile of methamphetamine (Meth) intake (number of infusions): analysis of variability in time series

Group	Day-to-day fluctuations		First-order autocorrelation coefficient (R_1)[b]	
	MAD (S.E.)[a]	MAD in % of mean (range)	R_1 median (range)	p value median (range)
Pretreatment by Sal				
SH Sal ($n=7$)	4.29 (1.02)	27.4 (15.0–53.7)	0.298 (0.145 to 0.594)	0.451 (0.004–0.845)
OBX Sal ($n=6$)	5.96 (0.64)	33.8 (18.4–64.5)	0.108 (−0.085 to 0.281)	0.450 (0.245–0.450)
Pretreatment by Meth				
SH Meth ($n=5$)	2.71 (0.91)	24.5 (16.2–48.2)	0.392 (0.189 to 0.548)	0.045 (0.006–0.624)
OBX Meth ($n=7$)	7.15 (2.42)	47.6 (26.6–83.8)	0.144 (0.021 to 0.305)	0.422 (0.101–0.921)

Sal, Saline; SH Sal, sham-operated [not olfactory-bulbectomized (OBX)] rats with 14 d of Sal (placebo) pretreatment; OBX Sal, OBX rats with 14 d of Sal (placebo) pretreatment; SH Meth, sham-operated rats with 14 d of Meth pretreatment; OBX Meth, OBX rats with 14 d of Meth pretreatment.

[a] Mean absolute difference (MAD) of day-to-day Meth intake (measured as number of infusions; supplied with S.E.).

[b] Autocorrelation coefficients and p values in Ljung–Box test for randomness in intake time series: individually based estimates were summarized as median and range within experimental groups.

low doses of amphetamine (Holmes *et al.* 2002) and the experiment did not take into account the influence of behavioural sensitization.

In our study, low-dose Meth had a similar effect to amphetamine (Holmes *et al.* 2002). This also corresponds with self-medication reported in patients with depression (Hall & Queener, 2007; Khantzian, 1985).

Based on these findings, it is suggested that the beneficial effect of various antidepressants, particularly selective serotonin reuptake inhibitors (SSRIs), reduce drug self-administration in animal studies, as shown in the case of alcohol (O'Brien *et al.* 2011), morphine (Raz & Berger, 2010), amphetamine (Yu *et al.* 1986) and Meth (Reichel *et al.* 2009). However, so far there is no strong clinical evidence to suggest the routine use of antidepressants in the treatment of drug dependence (Silva de Lima *et al.* 2010).

According to our knowledge, to date there is no study describing the effects of SSRIs or other antidepressants on spontaneous drug intake in a self-administration model in which OBX animals were used. Behavioural, immunological and neurochemical effects induced by the OBX model can be eliminated by antidepressant treatment (Kelly *et al.* 1997; Song & Leonard, 2005). The results of the present study indicate that the OBX model may be a valid model for the investigation of drugs of abuse.

There is a need to distinguish appetitive and consummatory behaviour in drug administration. Consummatory behaviour is innate and leads to the satisfaction of basic needs, such as eating, drinking, sexual behaviour or 'drug-taking'. Appetitive behaviour is characteristic of exploration of environment, motivation and learning processes – 'drug-seeking' (Craig, 1917). These two types of behaviour can be distinguished pharmacologically. Thus, amphetamine self-administration was shown to suppress consummatory behaviour (eating) at a dose that did not affect appetitive behaviour (Foltin, 2005). These aspects of behaviour are reflected in changes in mesolimbic brain areas that are centres for appetitive and consummatory behaviour (Gan *et al.* 2010).

In summary, this study demonstrates that the OBX model of depression increases Meth intake in the IVSA model of consummatory drug intake. This finding correlates well with the self-medication hypothesis to explain the relationship between depressive and addictive disorders as an attempt to relieve symptoms of monoaminergic deficit in depression by self-administering a psychostimulant drug (Khantzian, 1985). Moreover, in this experiment, chronic intermittent pretreatment with Meth was used to evaluate the influence of behavioural sensitization on the drug intake of OBX and sham-operated rats. Pre-exposure to Meth subsequently decreased the intake of the drug in self-administration in sham-operated animals but not in rats subjected to OBX. Further studies are necessary to describe the specificities of OBX animals in the drug self-administration paradigm and consequently to validate the model of comorbid depression and drug addiction.

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Statement of Interest

None.

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2.4.2. Olfactory bulbectomy increases reinstatement of methamphetamine seeking after a forced abstinence in rats

This experiment was designed to further validate the model by assessing the relapse-like behaviour towards methamphetamine after a short maintenance phase of methamphetamine self-administration. The forced abstinence paradigm with single reinstatement session was chosen to assess methamphetamine seeking behaviour (Fuchs et al., 2006, Reichel and Bevins, 2009, Yahyavi-Firouz-Abadi and See, 2009). This paradigm mimics the human treatment very well, because the patient usually discontinues the drug abuse in the drug rehabilitation centre and for some time does not have access to the drug related environments. Therefore, in the model animals do not have access to the operant box for some time and then they are re-introduced to the box again for one session with no drug availability (Reichel and Bevins, 2009, Fuchs et al., 2006, Yahyavi-Firouz-Abadi and See, 2009). Thus the motivation of drug response behaviour is not influenced by any training procedures and the recorded variable is stimulation of both active and inactive *operandums*. This provides information of the animals' motivation to seek the drug.

Results of this study indicate that the methamphetamine self-administration and forced abstinence in the olfactory bulbectomized rats is a valid model of increased relapse-like behaviour in depressive-like rats. Therefore, we proposed this approach to test drugs intended to suppress the reinstatement to the drug seeking behaviour, as it seems to be relevant for downwards translation of human drug relapse and ultimately for developing innovative treatment strategies.

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Research report

Olfactory bulbectomy increases reinstatement of methamphetamine seeking after a forced abstinence in rats

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HIGHLIGHTS

- Further validation of an animal model of depression-addiction dual disorder.
- Olfactory bulbectomy increases reinstatement of METH seeking behavior.
- Forced abstinence is a valid translational approach to model drug relapse.

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ABSTRACT

Drug addiction is commonly associated with depression and comorbid patients also suffer from higher cravings and increased relapse rate. To address this issue preclinically we combined the olfactory bulbectomy (OBX) model of depression and intravenous methamphetamine self-administration procedure in rats to assess differences in relapse-like behavior.

Male Sprague-Dawley rats were divided randomly into two groups; in one group the bilateral olfactory bulbectomy (OBX) was performed while the other group was sham operated. After recovery, intracardiac catheter was implanted. Intravenous self-administration procedure was conducted in operant boxes using nose-poke operandi (Coulbourn Instruments, Inc., USA) under fixed ratio 1 schedule of reinforcement. Methamphetamine was available at dose 0.08 mg/kg/infusion. After stable methamphetamine intake was maintained, a period of forced abstinence was initiated and rats were kept in their home-cages for 14 days. Finally, one reinstatement session was conducted in operant boxes with no drug delivery.

In the reinstatement session the mean of 138.4 active nose-pokes was performed by the OBX group, while the sham group displayed 41 responses, i.e. 140 % and 48 % of basal nose-poking during maintenance phase in OBX and sham operated group respectively. OBX group also showed significantly more passive nose-pokes indicating hyperactive behavioral traits in bulbectomized rats. However, the % of active operandum preference was equal in both groups.

Olfactory bulbectomy model significantly increased reinstatement of methamphetamine seeking behavior. This paradigm can be used to evaluate potential drugs that are able to suppress the drug-seeking behavior.

1. Introduction

The likelihood of drug addiction and depression to occur together in the same individual is approximately 5 times greater than what would be expected by the prevalence of each disorder alone [1,2] and leads to increased suicide rates among depressive individuals [3]. A widely accepted theory to explain depression and drug addiction comorbidity is the "self-medication hypothesis", arising from common risk factors and similarities in the underlying neurobiology of depression and drug addiction [4,5]. This theory indicates that individuals with depression have deficits in brain reward systems and may turn to drugs that create euphoric feelings to compensate for their anhedonia and motivational inadequacy [6–8] and there is supporting clinical evidence for methamphetamine [9] and other drugs [10]. Consequently,

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individuals with affective disorders suffer from higher cravings and increased relapse rate [11] which is the most demanding problem faced by clinicians [12]. Despite the high prevalence of drug addiction and depression comorbidity, there are only few animal models examining drug abuse behaviors in depression and relapse of drug addiction [13]. Thus, the composite animal model used here should provide a basis for investigation of the mechanisms underlying the interaction between depression and addiction [14].

Bilateral olfactory bulbectomy is a well-established model of depression with high face, construct, and predictive validity which closely mimics neurochemical, neuroanatomical, behavioral and endocrine changes seen in patients with major depression [15]. Our team has developed a rat model of depression and addiction dual disorder where olfactory bulbectomized animals showed a significantly higher vulnerability in methamphetamine intravenous self-administration (IVSA) paradigm [14] and differential dopamine and serotonin release in nucleus accumbens shell after methamphetamine challenge [16]. Similar findings were reported earlier also for self-administration of amphetamine [17] and for self-administration and dopamine release induced by CB1 receptor agonist WIN55,212-2 [18]. Interestingly, this behavioral effect was not replicated in a similar study with cocaine [19].

To mimic relapse in the IVSA paradigm, extinction training is usually employed when the animal still has a regular access to the operant box but the drug delivered by infusion pump is replaced by vehicle. After reaching a specific extinction criteria, one last session is conducted and the reinstatement of the drug seeking behavior is primed by an environmental factor (stress, cue) or a drug dose [20]. In the OBX model of depression combined with drug IVSA Frankowska et al. (2014) and Amchova et al. (2014) proved significantly later extinction of cocaine- and CB1 agonist-seeking behavior in the OBX rats. However, this approach does not mimic the human situation as the patient usually discontinues the drug taking in a different, not drug-related environment. Therefore, a forced abstinence paradigm was suggested as more translational. In this model the animal does not have access to the operant self-administration and is kept in the home cage for certain time period [21,22].

The aim of this study was to assess relapse-like behavior in the OBX model of depression after short maintenance phase of methamphetamine self-administration. We have chosen the highly translational forced abstinence paradigm and we expected higher methamphetamine seeking behavior of the OBX rats in the reinstatement session.

2. Methods

2.1. Animals

Twenty male albino Sprague-Dawley rats (8 weeks old, with weight range of 200-225 g at the beginning of the experiment) were purchased from Charles River (Germany). The rats were housed individually in standard rodent plastic cages. Environmental conditions during the whole study were constant: relative humidity 50-60 %, room temperature 23 °C ± 1 °C, inverted 12-hour light-dark cycle (6 a.m. to 6 p.m. darkness). Food and water were available *ad libitum*. There were two experimental groups: SHAM = sham operated rats (n=8 at the beginning of the study) and OBX = olfactory bulbectomized rats (n=12 at the beginning of the study). All experiments were conducted in accordance with all relevant laws and regulations of animal care and welfare. The experimental protocol was approved by the Animal Care Committee of the Masaryk University, Faculty of Medicine, Czech Republic, and carried out under the European Community guidelines for the use of experimental animals.

2.2. Drugs and treatments

Methamphetamine (METH) from Sigma Chemical, Co., St Louis, MO, USA available in the operant cage for IV self-administration was 0.08 mg/kg per infusion with the maximum number of infusions obtainable in one session set to 50 as was routinely used in our laboratory [14,23].

2.3. Olfactory bulbectomy surgery

At the beginning of the study the rats were randomly divided into two groups and the bilateral ablation of the olfactory bulbs was performed in accordance with the standard method [24] as described earlier [14,18]. In brief, animals were anaesthetized with ketamine 50 mg/kg and xylazine 8 mg/kg given intraperitoneally. The top of the skull was shaved, swabbed with an antiseptic solution, after which a midline frontal incision was made in the skin on the skull. After exposure of the skull, 2 burr holes were drilled at the points 7 mm anterior to the bregma and 2 mm lateral to bregma suture. Both olfactory bulbs were aspirated while paying particular attention not to damage the frontal cortex. Prevention of blood loss was achieved by filling the dead space with a haemostatic sponge. The skin above the lesion was closed with suture and the antibacterial neomycin and bacitracin powder was applied. Sham operated rats underwent the identical anaesthetic and drilling procedures as OBX animals, but their bulbs were left intact. Afterwards animals were treated with non-steroidal anti-inflammatory meloxicam (0.2 ml/kg SC). A period of 14 days was allowed for the recovery from the surgical procedure. During this period, animals were handled daily for few minutes to eliminate aggression, which could otherwise arise [15,25]. At the end of the experiment, rats were euthanized by an anaesthetic overdose and the brains were dissected for confirmation of the successful removal of the olfactory bulbs. Animals with incomplete removal of the olfactory bulbs were eliminated from the analysis.

2.4. Intravenous drug self-administration surgery

The IV self-administration catheter was implanted after recovery from the OBX surgery following standard procedure described earlier [14,18,23]. In brief, animals were deeply anesthetized with IP injections of 50 mg/kg ketamine plus 8 mg/kg xylazine. Catheter was inserted 3.7 cm [26] into the right external jugular vein to the right atrium and securely sutured. A subcutaneous tunnel was made and the catheter exited the skin in the midscapular area. Since the implantation, the catheters were flushed daily by heparinized 0.05 g/kg cefazolin dissolved in saline with 2.5 IU/kg heparin and finally 0.05 ml heparin solution (5 IU/kg) to prevent infection and occlusion of the catheter. When a catheter was found to be blocked or damaged, the animal was excluded from the analysis.

2.4.1. Intravenous self-administration protocol

Methamphetamine self-administration was conducted as previously described [14,23] in 10 standard experimental boxes (30 × 25 × 30 cm, Coulbourn Instruments, USA) using nose-poking as operandum. Each cage was provided with two nose-poke holes allocated on one side and programmed by software Graphic State Notation 3.03 (Coulbourn Instruments, USA). Nose-pokes in the active hole led to the activation of the infusion pump and administration of a single infusion followed by a 10 sec timeout, while nose-poke stimulation was recorded but not rewarded, i.e. fixed ratio (FR) schedule of reinforcement. Specifically, training sessions were initially conducted under a FR-1 schedule of reinforcement. When the animal fulfilled the following acquisition criteria for three consecutive sessions: a) at least 70 % preference of the drug-paired active nose-poke, b) minimum intake of 10 infusions per session, or

c) stable intake of the drug (maximum 10 % deviation) fixed-ratio 1 was then raised to FR-2. Infusions were delivered by a syringe within an automatic infusion pump located outside the chamber. The infusion pumps were connected to liquid swivels which were fixed to the catheters via polyethylene tubing withinside a metal spring tether. The cage was illuminated by a house light during the session. The light was flashing when infusion was being administered (5 sec) and off during the time-out period. Self-administration sessions lasted 90 minutes and took place 7 days/week between 8 a.m. and 3 p.m. during the dark period of the cycle.

After 14 days of stable methamphetamine intake at FR-2 the maintenance phase was terminated and rats were kept in their home cages for the 14 days of the forced abstinence period. As described earlier, on the day of reinstatement, rats were placed into self-administration chambers for the last session taking 90 minutes [27]. The numbers of responses on the active drug paired nose-poke and the inactive nose-poke were recorded but the drug was not delivered. Responses on the active nose-poke are considered to reflect reinstatement of drug seeking behavior, while responses on inactive nose-poke are interpreted to reflect general locomotor and exploratory activity.

2.5. Statistical Data analysis

Primary data were summarized using arithmetic mean and standard error of the mean estimate. IV self-administration data during the 14 days of maintenance were analysed at individual days by t-test and at 5 day intervals by mixed ANOVA model with Greenhouse-Geisser correction. Data from the reinstatement session were analysed by t-test or Mann-Whitney U test for non-parametric data and mixed ANOVA model. Active operandum preferences were compared by unpaired t-test, Welch corrected.

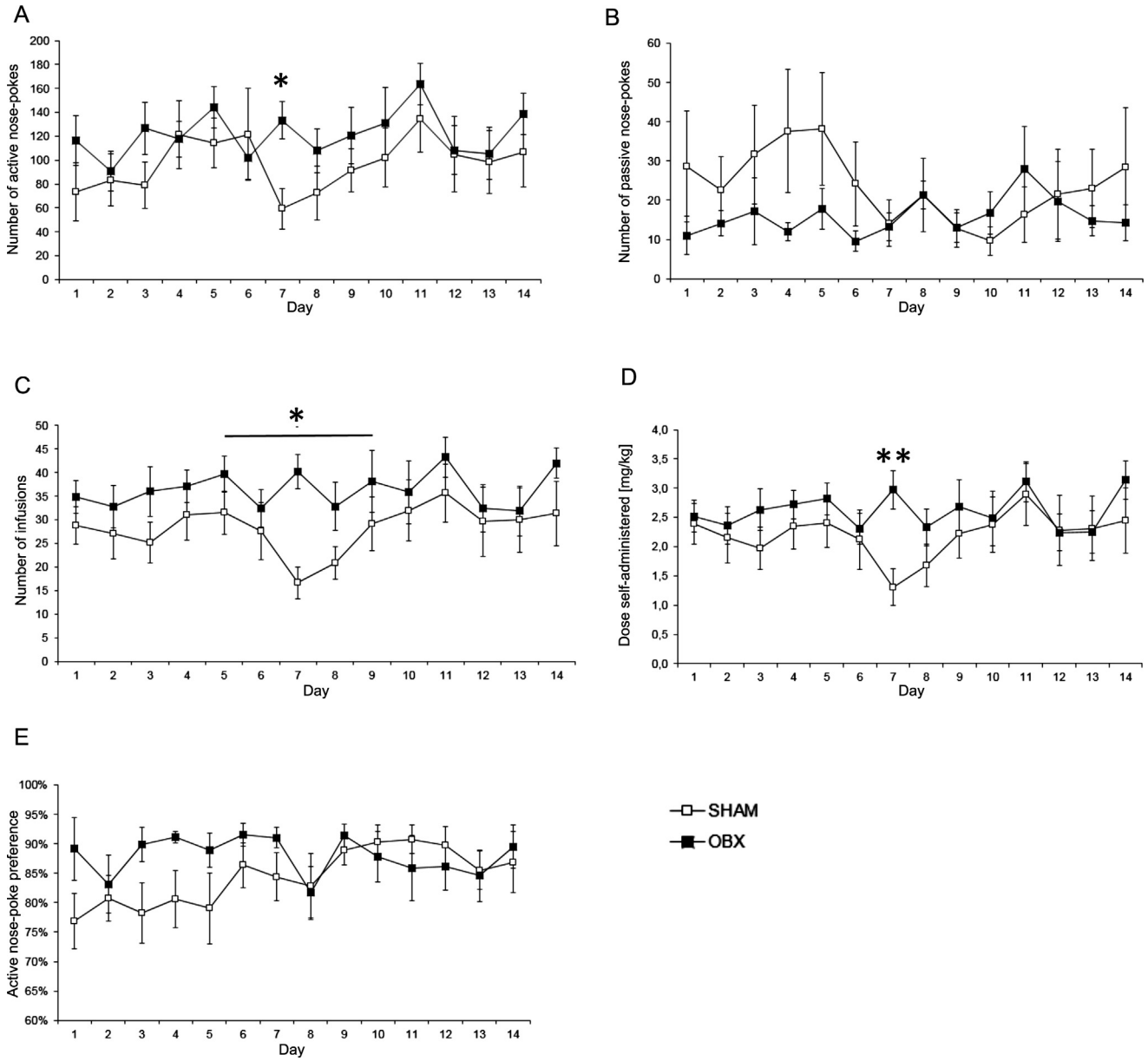

Fig. 1. Maintenance of METH self-administration Fig. 1**A** shows a comparison of SHAM (n=7) and OBX (n=7) groups in mean ±SEM active nose-poking during 14 days of maintenance phase. The groups differ significantly only in the day 7 (*p=0.037). Fig. 1**B** reflects mean ±SEM number of passive nose-pokes during maintenance phase (n.s.). Fig. 1**C** depicts mean ±SEM number of infusions over the whole maintenance phase. The groups differ significantly from the day 5 to day 9 (*p=0.024). Fig. 1D shows mean ±SEM dose of METH self-administered in mg/kg. The groups differ significantly again only in the day 7 (**p=0.006). Fig. 1**E** reflects percent of active nose-poke preference (n.s.).

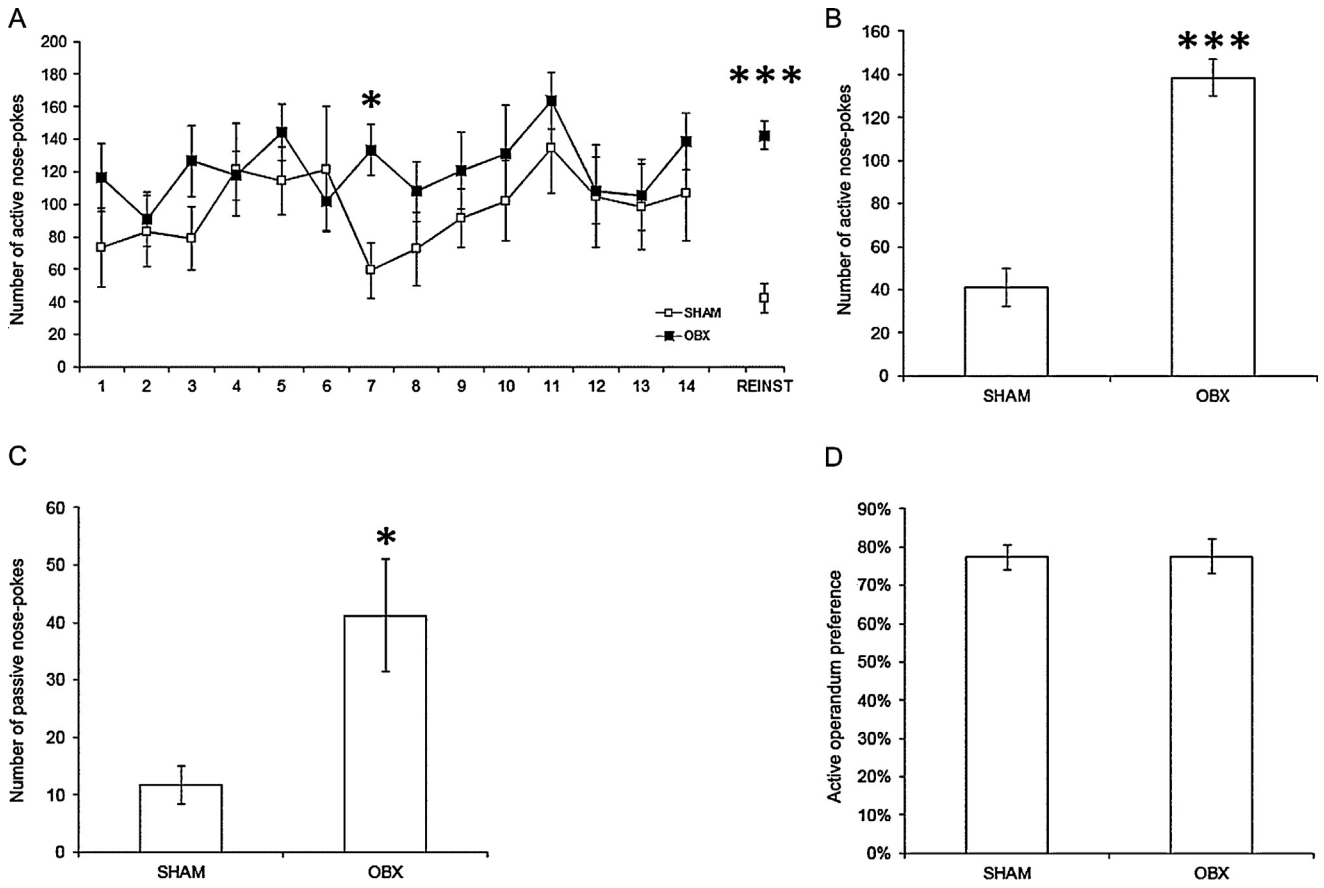

Fig. 2. Reinstatement of methamphetamine self-administration Fig. 2**A** shows mean ±SEM number of active nose-pokes in the reinstatement session in SHAM (n=6) and OBX (n=5) animals, maintenance data are included for comparison. OBX rats performed significantly more nose-pokes in the reinstatement (t-test, ***p=0.0001), specifically Fig. 2**B** depicts mean ±SEM number of active nose-pokes in the reinstatement session. Fig. 2**C** shows mean ±SEM number of passive nose-pokes during reinstatement session in SHAM and OBX rats (Mann-Whitney U test, *p=0.0175). Fig. 2**D** indicates mean ±SEM percent of active nose-poke preference during the reinstatement session (n.s.).

Statistical analyses were computed using SPSS 19.0.1 (IBM Corporation, 2010). A value $p<0.05$ was recognized as boundary of statistical significance in all applied tests.

3. Results

3.1. Maintenance of methamphetamine self-administration in SHAM and OBX rats

The maintenance of METH taking behavior was assessed in terms of mean number of nose-pokes, infusions self-administered per session and by the mean METH dose per session in mg/kg. Fig. 1**A** shows mean number of active nose-pokes obtained per daily session during the maintenance phase in SHAM and OBX rats. ANOVA revealed no significant effects over the whole period of maintenance with an exception of day 7 (p=0.037), where OBX group exhibited more active operant responses. Non drug-paired (passive) nose-poking is a measure of locomotor-exploratory activity of the animal and is generally quite high in animals self-administering METH due to psychostimulant properties of this drug [18]. As expected, mean numbers of passive nose-pokes were variable but there was no difference between the groups recorded, ANOVA, n.s. (Fig. 1**B**). Fig. 1**C** depicts mean number of infusions and ANOVA revealed significantly more infusions in OBX rats in the middle of the maintenance period (days 5 to 9, p=0.024). It should be noted that the number of nose-poke responses does not match the number of infusions delivered in our paradigm. This is always the case when the system uses nose-poke operandi (and in some cases levers which do not retract after infusion delivery). Besides number of infusions we proposed to evaluate also METH dose per kilogram of body weight as more exact measure of actual drug intake. Therefore, Fig. 1**D** indicates mean METH dose in mg/kg showing no difference over the maintenance phase except higher intake in the OBX animals on the day 7 (ANOVA, p=0.006). The fact that the general pattern of METH taking behavior was similar in both groups is further supported by active nose-poke preference data, ANOVA, n.s. (Fig. 1**E**).

3.2. Reinstatement of methamphetamine self-administration in SHAM and OBX rats

After the 2 week-long period of forced abstinence one last reinstatement session was performed with no drug availability. The only measure of the drug-seeking behavior was the number of active operandum responses. Fig. 2**A** and **2B** report the mean number of active nose-pokes obtained during the reinstatement session in SHAM and OBX rats. For easy comparison the maintenance data shown on Fig. 1A are also included in the Figure 2A. SHAM and OBX rats show significant difference in the responding during reinstatement session (138.4 active nose-pokes in OBX group vs 41 in SHAM group, t-test, p=0.0001). Mean ±SEM number of active nose-pokes in the reinstatement session are summarized on the Figure 2**B**. OBX group reached mean 138.4 while SHAM rats 41 active nose-pokes (t-test, $p\leq0.0001$).

To assess the locomotor-exploratory activity we evaluated the differences in the passive nose-pokes between SHAM and OBX group (Figure 2**C**). The comparison revealed significant differences: 41.2 passive nose-pokes in OBX group and 11.7 passive

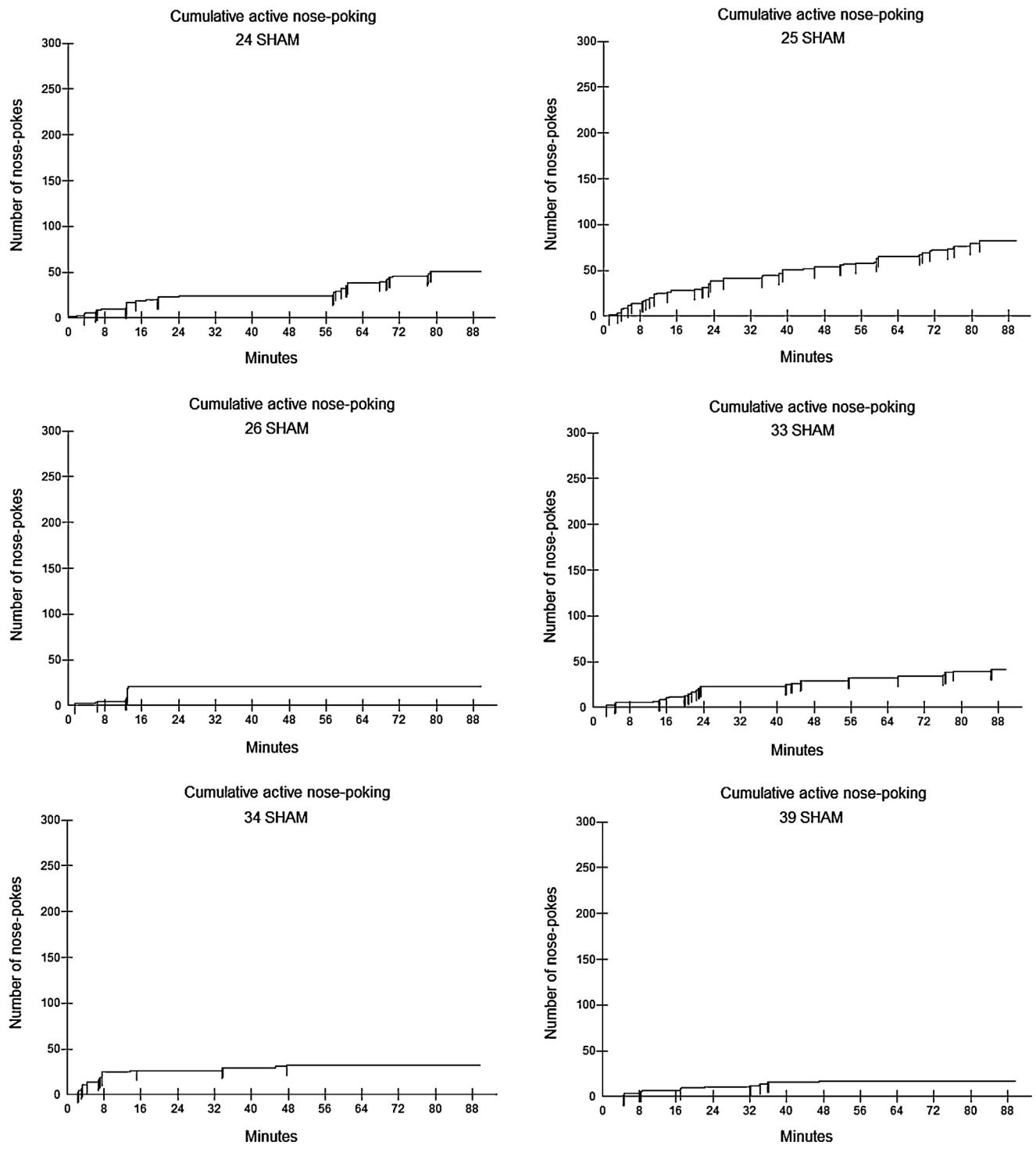

Fig. 3. Responding patterns in the reinstatement session The figure shows temporal responding patterns of all animals in the study. The line indicates the increasing cumulative record of nose-poking while the vertical bars indicate the flashing light as a cue for infusion delivery. Infusions were not delivered in this session but the protocol was kept equal as during maintenance. The caption of each figure refers to the specific number of animal in the SHAM/OBX group. The protocol used in the reinstatement session was the same as for maintenance where the maximum number of infusions is set for safety reasons (prevention of overdose). This limit is not necessary in the reinstatement session but the session was conducted under the same conditions the animals were used to. We have detected significant difference between sham and OBX animals despite this restriction. If we would have not limit the maximum number of infusions in this session the difference would probably be even higher.

nose-pokes in SHAM group (Mann-Whitney U test, p=0.0175). However, Figure 2**D** shows percent of active nose-poke preference in the reinstatement session, revealing no significant differences between the groups (t-test), proving equal active operandum preference among the groups.

In order to evaluate possible qualitative differences in the operant responding between the groups we assessed temporal nose-poking patterns. Whole OBX group finished the reinstatement session prematurely, due to reaching the maximum of operant responding set previously for the maintenance sessions. Responding in the OBX animals was higher during the whole ses-

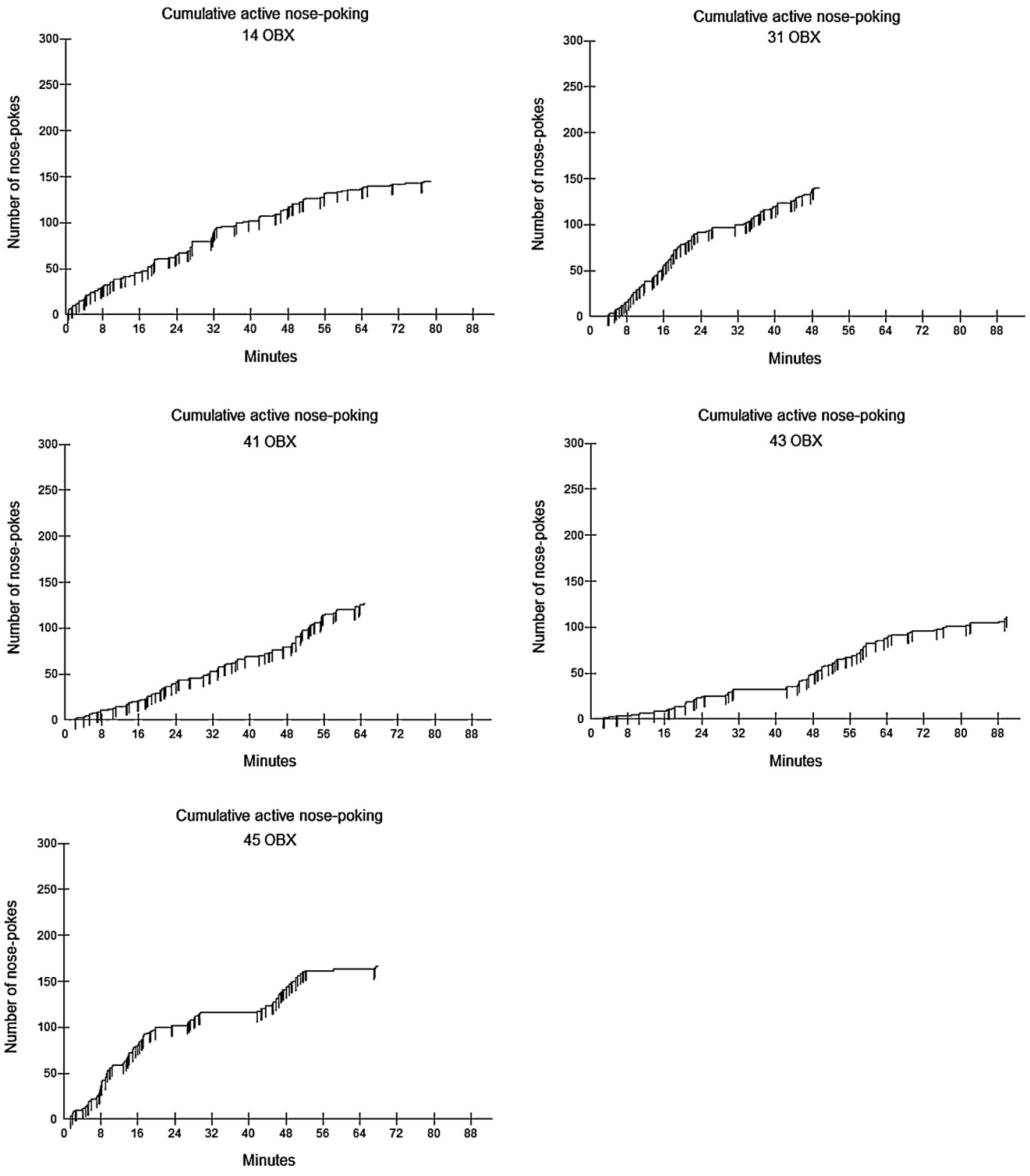

Fig. 3. (*Continued*).

sion. SHAM group showed high responding in the beginning of the session only, followed by long sequences of non-responding in one half and scarce responding in the other half of the group (Fig. 3).

4. Discussion

In the present study we report that bulbectomized rats displayed significantly increased reinstatement of METH seeking behavior indicating higher vulnerability to relapse and trend towards higher drug intake during maintenance phase. We have shown earlier that OBX animals self-administer more METH infusions than SHAM controls [14]. However, as observed in this study, the mean number of active nose-pokes, METH infusions and drug dose in mg/kg were stable with only a trend towards increase in OBX group in the middle of the maintenance phase. Therefore, this study only partially replicates our previous findings. The reason is probably due to the length of the acquisition and maintenance phases together, i.e. total duration of drug exposure. Kucerova et al.

(2012) reported increased METH intake at FR-3 where the self-administration lasted approximately 2 to 3 months due to training and longer maintenance phase. However, the primary goal of this experiment was to evaluate relapse-like behavior, therefore, the allowed period of maintenance was shorter (2 weeks, considered as chronic drug intake in most of preclinical studies [28,29]) and the total length of the self-administration including training was approximately 1 month. Another confounding factor might be the effect of rat strain since Kucerova et al. (2012) employed Wistar rats and this study works with Sprague-Dawley strain. Strain difference is known to be an influential factor in assessing drug taking behavior [30]. Regarding strain differences it has also been proved that Sprague-Dawley rats tend to self-administer more than Wistar rats [31] which might disguise the difference induced by the OBX surgery. Moreover, Frankowska et al. (2014) did not prove higher cocaine intake in OBX Wistar rats in her study using stable dose of cocaine (0.5 mg/kg/infusion) in up to FR-5 and following extinction phase, when drug is replaced by vehicle as opposed to our study using forced abstinence paradigm. The difference in drug intake between the groups vanishes when using higher doses of cocaine or amphetamine [17,19], therefore the specific choice of dose could be an important factor. Low dose amphetamine 0.10 mg/kg/infusion) or methamphetamine (0.08 mg/kg/infusion) resulted in higher drug self-administration in OBX rats [14,17], while a rather high dose of amphetamine (0.25 mg/kg/infusion) led to the same active lever pressing activity in both OBX and SHAM Sprague-Dawley rats [17]. It should be noted that olfactory bulbectomy also increases voluntary 10% [32] and 20% ethanol consumption (manuscript in preparation) and also IV self-administration of CB_1 receptor agonist WIN55,212-2 [18] suggesting that olfactory bulbectomy leads to increased response patterns to a variety of abused substances.

The main finding of this study is the increased vulnerability to reinstatement of METH seeking behavior in the OBX rats in terms of absolute number of active nose-pokes and percent of mean basal nose-poking during the maintenance phase. Interestingly, during the reinstatement session the number of passive nose-pokes was significantly increased in the OBX group. This may be explained mainly by increased motivation to obtain the drug and also by known hyperactive behavioral traits in OBX rats [15,33,34]. In line with this hypothesis, the data on the active nose-poke preference are showing no difference between OBX and SHAM rats. However, the temporal responding patterns show marked differences between the groups. OBX rats made high number of nose-pokes during the whole session while SHAM animals responded either less during the whole session or just at the beginning. This probably reflects the increased motivation to obtain the drug in the OBX group. In line with the present data, other animal models of depression also show higher vulnerability to relapse. The social defeat-induced persistent stress paradigm with depressive-like symptomatology led to increased motivation to obtain alcohol [35]. Moreover, the model of unconditioned foot-shock stress has been proved to reinstate previously extinguished alcohol-seeking behavior to a higher extent than alcohol or stress alone [36]. Additionally chronic restraint stress model of depression during abstinence phase promotes nicotine seeking after extinction of nicotine self-administration [37]. Thus, all these models seem to be at least in partial accordance with the clinical situation.

In summary, reinstatement phenomena induced by drug dose, stress or drug-related cues occurring in rats resembles human relapse [28,38]. On the other hand, there are also important differences between animal and human situation, as the drug discontinuation motive in humans is mostly punishment/incarceration or lack of drug availability in rehabilitation clinic while animal models mostly employ extinction training. Therefore, it is crucial to interpret the data from preclinical studies using the extinction-reinstatement design considering this limitation. This study indicates that the methamphetamine self-administration and forced abstinence is a valid model of increased relapse-like behavior in OBX rats which closely mimics the human situation. Therefore, we propose this approach to test drugs intended to suppress the reinstatement to the drug seeking behavior, as this approach relevant seems to be more relevant for downwards translation of human drug relapse and ultimately for developing innovative treatment strategies.

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2.4.3. Reward related neurotransmitter changes in a model of depression: an *in vivo* microdialysis study

This study was systematically assessed the extracellular levels and turnover of dopamine and serotonin and levels of glutamate and GABA in the OBX rat model of depression in the nucleus accumbens shell, i.e. the main reward-related area (Di Chiara et al., 2004). To this goal, the *in vivo* microdialysis technique was used (Sustkova-Fiserova et al., 2014) and the neurotransmitter levels were assayed both at baseline conditions and after a challenge dose of methamphetamine.

The findings indicated a different baseline condition: significantly decreased basal levels of dopamine, serotonin and their metabolites and increased levels of glutamate and GABA in the OBX rats. Furthermore, dopamine and serotonin turnover was elevated when calculated from the amounts of the neurotransmitters and their respective second-step metabolites. After acute methamphetamine challenge (5 mg/kg, i.p.) a significantly higher release of dopamine, serotonin and their metabolites was detected in OBX rats; however, glutamate levels were lower and GABA was not found to be different from sham control animals. These findings were in accordance with a differential behavioural profile in the OBX rats.

Importantly, a dose-response assessment of dopamine release and changes induced by chronic methamphetamine administration are of high interest. Chronic drug administration data are needed for further elucidation of the underlying pathophysiological processes shared by the depressive-like phenotype and reward.

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ORIGINAL INVESTIGATION

Reward related neurotransmitter changes in a model of depression: An in vivo microdialysis study

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ABSTRACT

Objectives: The self-medication hypothesis assumes that symptoms related to potential monoaminergic deficits in depression may be relieved by drug abuse. The aim of this study was to elucidate the neurotransmitter changes in a rat model of depression by measuring their levels in the nucleus accumbens shell, which is typically involved in the drug of abuse acquisition mechanism. *Methods*: Depression was modelled by the olfactory bulbectomy (OBX) in Wistar male rats. In vivo microdialysis was performed, starting from the baseline and following after a single methamphetamine injection and behaviour was monitored. The determination of neurotransmitters and their metabolites was performed by high-performance liquid chromatography combined with mass spectrometry. *Results*: OBX animals had lower basal levels of dopamine and serotonin and their metabolites. However, γ-aminobutyric acid (GABA) and glutamate levels were increased. The methamphetamine injection induced stronger dopamine and serotonin release in the OBX rats and lower release of glutamate in comparison with sham-operated rats; GABA levels did not differ significantly. *Conclusions*: This study provides an evidence of mesolimbic neurotransmitter changes in the rat model of depression which may elucidate mechanisms underlying intravenous self-administration studies in which OBX rats were demonstrated to have higher drug intake in comparison to intact controls.

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Introduction

Drug abuse is clinically a frequent comorbidity of other psychiatric disorders including mood disorders (Testa et al. 2013). As an explanation of the dual diagnosis of depression and addiction, the self-medication hypothesis is widely accepted (Hall and Queener 2007). It explains drug abuse as an attempt of the patient to relieve the monoaminergic deficits typical for depression. This was clinically confirmed in nicotine (Holma et al. 2013), methamphetamine (McKetin et al. 2011) and other drugs. There is growing evidence that depression and addiction underlie common defective neurobiological regulations shared by major depression and withdrawal syndrome (Markou et al. 1998), namely in dopaminergic (DA; Nestler and Carlezon 2006), serotonergic (5-HT), noradrenergic (NA), cholinergic (Zellner et al. 2011), glutamatergic (Tzschentke 2002) and γ-aminobutyric acid (GABA)-ergic (Koek et al. 2013) systems.

Bilateral olfactory bulbectomy (OBX) is a well-established model of depression (Harkin et al. 2003; Song and Leonard 2005). Our team has developed a rat model of depression and addiction dual disorder where OBX animals showed a significantly higher vulnerability in methamphetamine self-administration paradigm (Kucerova et al. 2012). This finding was reported earlier also for amphetamine (Holmes et al. 2002) and later for CB1 receptor agonist (Amchova et al. 2014) and in a slightly different operant paradigm also partially for cocaine (Frankowska et al. 2014). However, there is only fragmentary knowledge about the neurochemical changes in the reward-related brain areas in the OBX rats which could contribute to the explanation of these behavioural effects.

OBX rats were recorded to have increased basal DA levels in ventral and dorsal striatum but at the same time decreased NA levels (Masini et al. 2004). Regarding serotonin, in vivo microdialysis data demonstrate that

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the OBX procedure decreases serotonin levels but not its turnover in the basolateral amygdala and dorsal hippocampus. DA and NA (van der Stelt et al. 2005), and impaired serotonin functioning was described also in frontal cortex (PFC) and mid-brain (Song and Leonard 2005). The glutamatergic system is known to be strongly influenced by the OBX surgery as density of the NMDA receptors was shown to be elevated in the PFC. This is supposed to contribute to the typical hyperactive response of the OBX animals to novel environment (Song and Leonard 2005) together with enhanced striatal glutamate neurotransmission (Ho et al. 2000). However, the GABA-ergic system was shown to be hyperactive in the amygdaloid cortex and there is an increased turnover and density of GABA-A receptors (Song and Leonard 2005).

Drugs of abuse are consistently reported to strongly influence the reward system of the brain, specifically the nucleus accumbens shell (NACSh) and not the core (Di Chiara et al. 2004). This was shown for cannabinoids, opioids (Tanda et al. 1997), psychostimulants (Pontieri et al. 1995) and nicotine (Pontieri et al. 1996). Regarding glutamate, amphetamine was shown to increase its levels in NAC and PFC, but methamphetamine in the same study failed to do so (Shoblock et al. 2003). Striatal glutamate transmission was increased after a high dose of methamphetamine which could be the cause of neurotoxicity (Nash and Yamamoto 1992). The role of GABA is also substantial in the methamphetamine effect, as GABA-agonistic drugs injected to NAC were reported to suppress reinstatement of the methamphetamine-seeking behaviour (Rocha and Kalivas 2010).

Functioning of neurotransmitter systems was assayed repeatedly in the OBX model, but the reward-related regions have not yet been described. The aim of this study was, therefore, to assess systematically the neurotransmitter levels and turnover in the OBX rat model of depression in the nucleus accumbens shell. To this goal, the in vivo microdialysis technique was used and the neurotransmitter levels were assayed both at baseline conditions and after a dose of methamphetamine. Furthermore, the behavioural profile was established during the in vivo microdialysis session.

Methods and materials

Animals

Thirty adult male albino Wistar rats weighting 180–220 g at the beginning of the experiment were purchased from Velaz (Konarovice, Czech Republic). After the bulbectomy surgery, the rats were housed individually. Environmental conditions were constant: relative humidity 50–60%, temperature 22–24 °C, and a 12-h light–dark cycle, the experiments were carried out during the first half of the light phase. Food and water were available ad libitum. Due to incomplete olfactory bulbectomy, three animals were excluded from analysis, thus the study was completed by 13 sham operated and 14 olfactory bulbectomized rats (OBX). The experimental groups were: SHAM-SAL (sham-operated rats treated with saline as a vehicle (n=5)), SHAM-METH (sham-operated rats treated with 5 mg/kg METH (n=8)), OBX-SAL (olfactory bulbectomized rats treated with saline (n=6)) and OBX-METH (olfactory bulbectomized rats treated with 5 mg/kg METH (n=8)). All experiments were conducted in accordance with relevant laws and regulations of animal care and welfare. The experimental protocol was approved by the Expert Committee for Protection of Experimental Animals of the Third Faculty of Medicine, Charles University, Prague, Czech Republic, and experiments were performed in accordance with the Animal Protection Act of the Czech Republic (No. 246/1992 Sb.) and carried out under the European Community guidelines for the use of experimental animals.

Drugs and treatments

Methamphetamine (METH) from Sigma Chemical Co. (St Louis, MO, USA) was used for acute intraperitoneal (i.p.) administration during in vivo microdialysis session in a dose of 5 mg/kg in 2 ml of sterile saline, and 2 ml/kg i.p. of saline was used as a vehicle.

Olfactory bulbectomy surgery

At the beginning of the study the rats were randomly divided into two groups and the bilateral ablation of the olfactory bulbs was performed in accordance with the standard method (Kucerova et al. 2009,2012; Amchova et al. 2014). In brief, animals were anaesthetized with ketamine 50 mg/kg and xylazine 8 mg/kg i.p., the skull was shaved, swabbed with an antiseptic solution. A midline frontal incision was made in the skin and after exposure of the skull, two burr holes were drilled 7 mm anterior to the bregma and 2 mm lateral. Olfactory bulbs were removed by aspiration and the cavity filled with a haemostatic sponge. The skin was sutured and the antibacterial neomycin/bacitracin powder was applied. Sham operated rats underwent the identical procedures as OBX animals, but their bulbs were left intact. Microdialysis experiments were carried out 3 weeks after the surgery.

In vivo *microdialysis*

Surgery. As described in detail earlier (Sustkova-Fiserova et al. 2014), under ketamine–xylazine anaesthesia

(ketamine 100 mg/kg i.p., xylazine 10 mg/kg i.p.), rats were implanted with a disposable dialysis guide cannula (MAB4 probes, Agnthos, Sweden) using a stereotaxic instrument (StoeltingCo). After taking the co-ordinates with a guide mounted on the stereotaxic holder (NACSh: A: +2.0 mm and L: ±1.2 mm from bregma and V: 6.2 mm from occipital bone) (Paxinos and Watson 2007), the guide was slowly lowered into the brain and secured to the skull with dental cement and an anchoring screw. The guide was randomly alternated on the left and right side. After completion of the microdialysis experiments, the successful bulbectomy was confirmed and placement of the dialysis probe was verified histologically (Figure 1).

Microdialysis and chemical analysis assay. Forty-eight hours after implantation, the probe (MAB4, 2 mm active cuprophane membrane, Agnthos, Sweden) was inserted into the guide cannula and artificial cerebrospinal fluid (Ringer's solution; 147 mM NaCl, 2.2 mM $CaCl_2$ and 4.0 mM KCl; adjusted to pH 7.0) was flushed through the probe at a constant rate of 2.0 μl/min (Univentor 864 Syringe Pump, Agnthos, Sweden). After 80 min of habituation to the microdialysis set-up (when dialysate was discarded), 40-μl samples were collected at 20-min intervals in small ice-cooled polyethylene test tubes containing 12 μl HCl 0.1 mM, to prevent catecholamine hydrolysis. After three consecutive baseline samples, methamphetamine (5 mg/kg in 2 ml) or vehicle (saline 2 ml/kg) was administered intraperitoneally (at 60 min) and dialysates were collected every 20 min. Total duration of the sampled session was 240 min in animals with administration of METH and 180 min in animals after saline injection. This is because administration of the vehicle was not expected to induce any changes so the session was shortened. Immediately following collection, the samples were frozen at −70 °C. The amount of dopamine, serotonin and their metabolites 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA), respectively, as well as glutamate and GABA in the dialysates were quantified using high-performance liquid chromatography combined with mass spectrometry (HPLC-MS). The appropriate HPLC-MS determination methods were described in detail earlier (Syslova et al. 2011). In brief, the microdialysates were first lyophilised (to concentrate the inherent substances), the lyophylisation residue was dissolved in methanol and thus induced suspension of precipitated salts was briefly centrifuged and the supernatant was immediately analysed using LC-ESI–MS/MS (following specific procedure). The LC–MS system consisted of a chromatograph Accela 1250 autosampler and a TSQ Vantage mass spectrometer (all Thermo Scientific, USA). The analytes were separated on a Gemini® NX-C18 (5 μm at 110 Å) LC Column (150 × 2 mm) using a mobile phase (solvent A: aqueous solution of acetic acid (pH 2); solvent B: methanol) with a gradient elution flow rate of 150 μl/min. The conditions on the mass spectrometer were optimised and were as follows: spray voltage 3000 V, temperature of ion transfer tube 350 °C, temperature of H-ESI vaporiser 350 °C, sheath gas pressure (nitrogen) 35 psi, flow of auxiliary gas (nitrogen) 10 arbitrary units. The data were acquired and processed using Xcalibur 2.1.0 software (Thermo Scientific, USA).

Behavioural assay

Behaviour was studied simultaneously, while microdialysis measurements were performed as described earlier (Fiserova et al. 1999; Sustkova-Fiserova et al. 2014). Three behavioural categories were distinguished: immobility (sedation, eyes closed, akinesia, and reduced responsiveness to environmental cues), locomotion (non-stereotyped activity, sniffing, grooming, rearing, and walking) and stereotyped activity (stereotypical head movements, confined gnawing, licking and stereotypical sniffing). Behavioural categories were scored every 20 min (at each microdialysis interval) by an observer who was unaware of the manipulation and treatment that each rat had received. The percentage of time spent by the animal in each behavioural category was calculated for each 20-min interval. Behavioural changes were monitored during the entire dialysis period (60 min baseline and 3 h following methamphetamine or 2 h following saline injection).

Statistical data analysis

For comparison of basal neurotransmitter levels between OBX versus SHAM rats (in the *in vivo* microdialysis study), one-way analysis of variance (ANOVA) followed by Bonferroni *t*-test was used. The metabolic turnover was evaluated by *t*-test in parametric data and Mann–Whitney *U*-test in non-parametric data. Raw values for neurotransmitters and their metabolites were further transformed into percentage of baseline levels (mean of the three values prior to treatment). For statistical analysis of differences between OBX versus SHAM rats in time-related changes in the course of the in vivo microdialysis study, the two-way analysis of variance for repeated measures (ANOVA) followed by Bonferroni *t*-test was used. In this ANOVA analysis, the group of rats (SHAM vs. OBX) was entered as the between-group factor and the time-points as repeated within-subject measure (to compare all treatments to baseline).

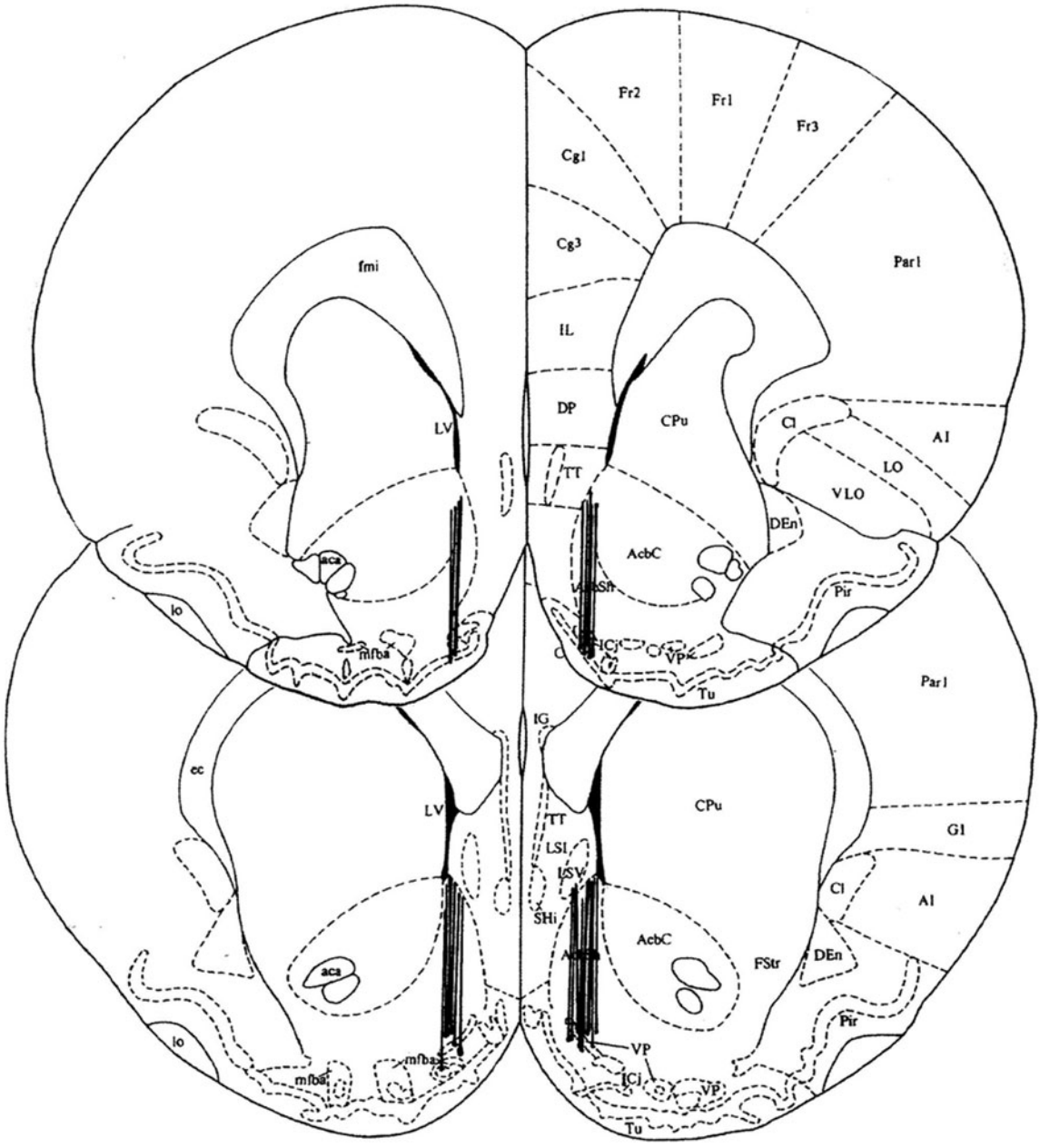

Figure 1. Location of dialysis probes within the nucleus accumbens shell (NACSh). Schematic locations of probe tips in rats, which were included in analyses of accumbens neurotransmitter concentrations (the solid lines indicate the dialysing portions) as described in the atlas of Paxinos and Watson (2007). On the left, for each section, the distance from bregma (in mm) is indicated.

For evaluation of the behavioural data, a *t*-test was used for each time point and a mixed ANOVA model for paired data was also used in order to assess the within subject effects with rat group regarding interaction differences and SHAM/OBX between subject effects.

The data were analysed using SigmaStat 3.5 (Systat Software, Inc., USA) (neurochemical data) and SPSS, version 2.0 (behavioural data). Results are presented as the arithmetic mean and standard error of the mean estimate (±SEM). A value $P<0.05$ was recognised as boundary of statistical significance in all applied tests.

Results

Basal NACSh neurotransmitter/metabolite levels

There were recorded highly significant differences in the basal levels of all assayed neurotransmitters and their metabolites. As shown on the Figure 2, extracellular levels of dopamine, serotonin and their metabolites in NACSh were decreased in the olfactory bulbectomy model of depression. On the other hand, glutamate and GABA levels were significantly increased. The exact concentration values are summarised in Table I.

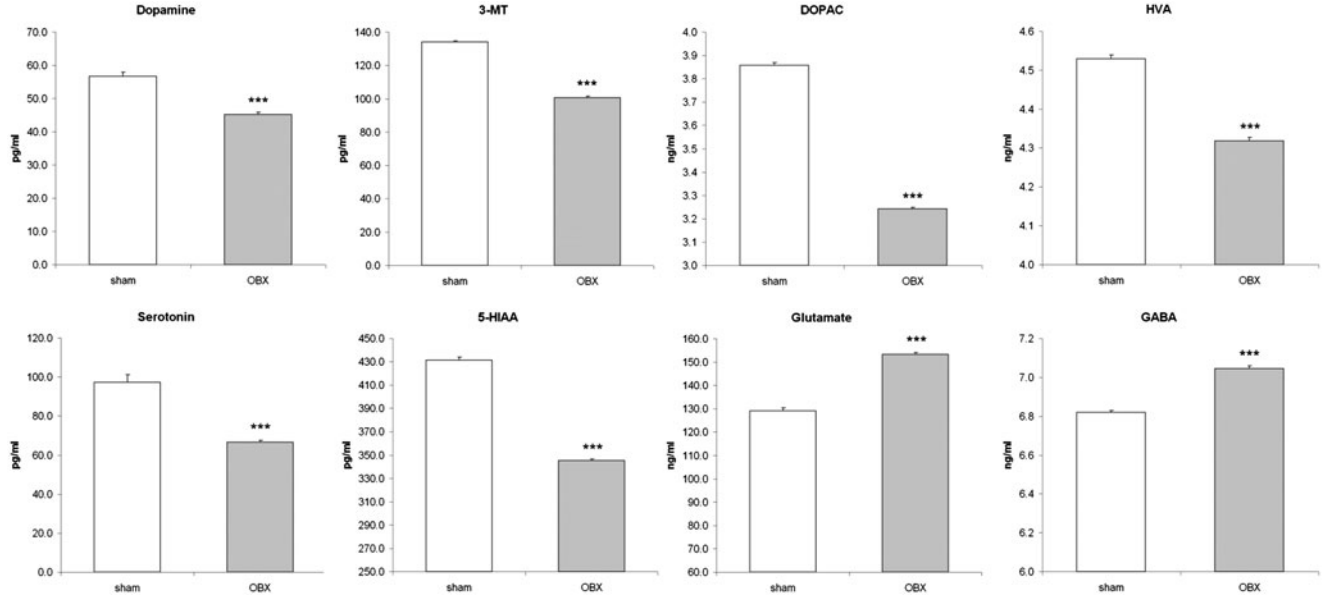

Figure 2. Baseline levels of the specific neurotransmitters/metabolites in the NACSh. The bars depict the mean±SEM values of extracellular concentrations (pg/ml or ng/ml) of the specific neurotransmitters and their metabolites in the NACSh in the SHAM (n=12) and OBX (n=14) rats during all three baseline measurements (at 20-, 40- and 60-min intervals). One-way ANOVA followed by Bonferroni t-test: ***P<0.001 in all measures, F values are presented in Table 1. Note that in order to show the SEM error bars clearly the y-axis in the graphs does not always begin at zero value.

Table I. Baseline neurotransmitter/metabolite levels in baseline measurements at sampling intervals 20, 40 and 60 min.

		Mean baseline values			OBX effect		
		20 min	40 min	60 min	P value	F(1,79) value	
DA	Sham	56.92±1.71	55.54±2.32	57.69±2.11	<0.001	71.251	↓
(pg/ml)	OBX	45.79±0.98	44.64±1.39	45.07±1.07			
3-MT	Sham	134.00±1.29	135.46±0.68	132.08±0.67	<0.001	985.739	↓
(pg/ml)	OBX	100.79±1.46	103.07±1.34	98.07±1.30			
DOPAC	Sham	3.87±0.02	3.87±0.01	3.84±0.02	<0.001	2231.631	↓
(ng/ml)	OBX	3.24±0.01	3.25±0.01	3.23±0.01			
HVA	Sham	4.52±0.02	4.53±0.01	4.53±0.02	<0.001	260.768	↓
(ng/ml)	OBX	4.29±0.01	4.34±0.01	4.33±0.02			
5-HT	Sham	98.54±5.81	97.31±6.17	98.23±6.55	<0.001	76.177	↓
(pg/ml)	OBX	66.64±0.99	66.64±1.37	66.64±1.89			
5-HIAA	Sham	430.31±4.82	432.54±4.38	430.77±3.48	<0.001	961.212	↓
(pg/ml)	OBX	344.79±2.06	345.86±2.18	345.36±2.51			
GLU	Sham	130.85±2.31	127.31±2.28	129.77±2.04	<0.001	241.426	↑
(ng/ml)	OBX	153.50±1.67	153.93±1.32	152.57±1.32			
GABA	Sham	6.81±0.01	6.83±0.02	6.82±0.02	<0.001	146.549	↑
(ng/ml)	OBX	7.06±0.02	7.03±0.03	7.05±0.03			

Extracellular turnover of dopamine and serotonin in the NACSh

For analysis of dopamine and serotonin turnover metabolic ratios were calculated as follows: for each animal baseline value of the metabolite concentration (mean of 20-, 40- and 60-min interval values) was divided by mean baseline concentration value (analogously mean of 20-, 40- and 60-min values) of the neurotransmitter. Before the calculation the concentration data were all passed to pg/ml units. In this way relative data were obtained (expressed as a mean±SEM). As shown on the Figure 3 in DA metabolism there was found a statistical difference between SHAM and OBX animals only in the increased HVA/DA ratio – which could suggest an increased turnover of DA (P<0.01). Production of 3-MT and DOPAC was not found to be significantly different. As for the 5-HT metabolism, 5-HIAA (similarly to HVA in DA pathway, a second step metabolite of 5-HT) production was found to be significantly increased (P<0.05).

NACSh neurotransmitter/metabolite changes induced by methamphetamine administration

The changes of the neurotransmitter/metabolite levels are expressed as percentage of the mean baseline values

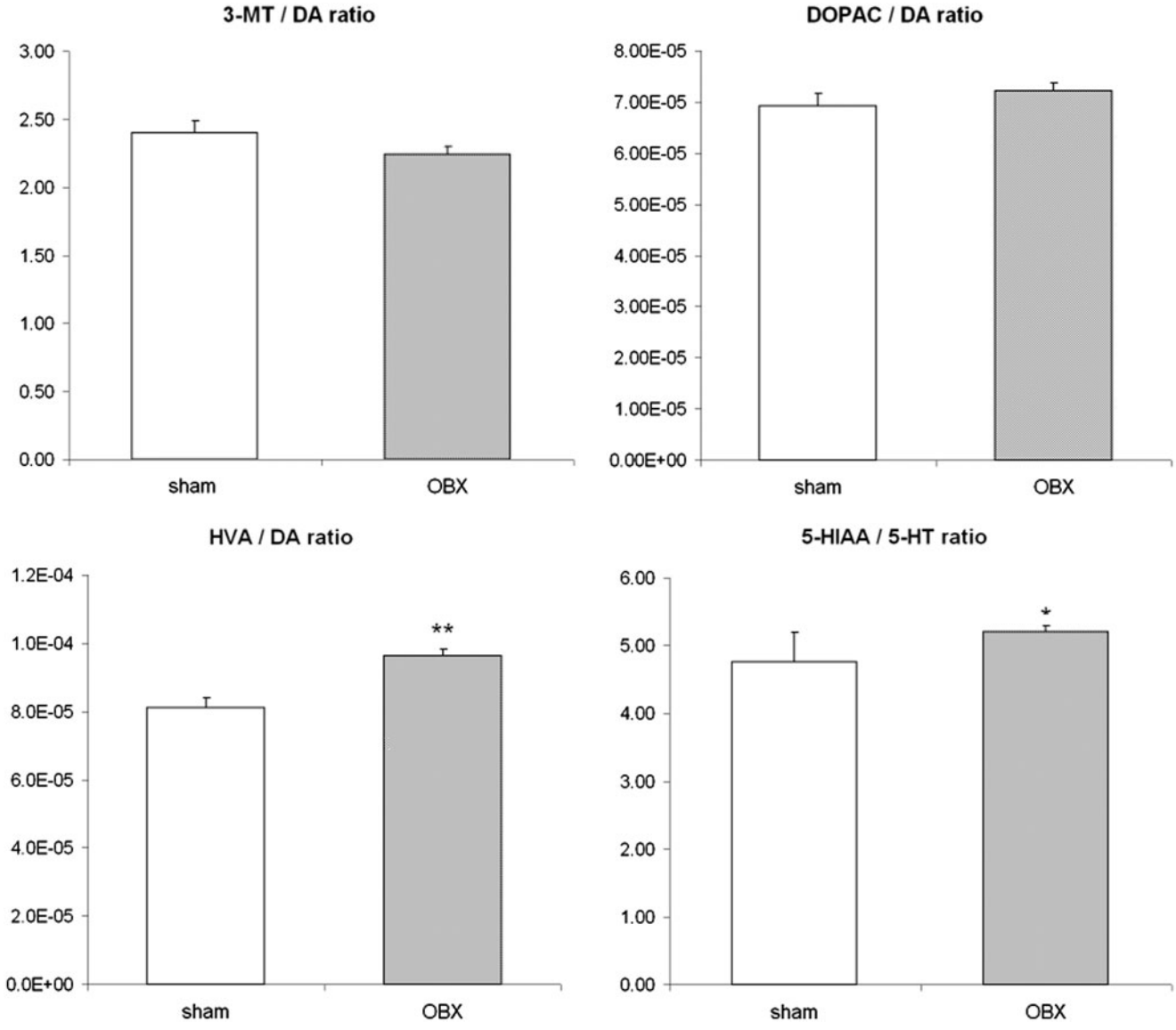

Figure 3. Dopamine and serotonin turnover in the NACSh. The graphs show mean baseline concentration value of the metabolite divided by mean baseline concentration value of the neurotransmitter. In the DA metabolism a statistically significant difference between SHAM and OBX animals was found in the increased HVA/DA ratio what indicates that OBX rats produce more HVA than SHAM controls. Similarly, in the 5-HT metabolism, 5-HIAA production was found to be significantly higher which indicates that OBX rats produce more 5-HIAA than SHAM controls. The statistical significance was determined by *t*-test in dopamine (parametric data) and Mann–Whitney *U*-test in 5-HT (non-parametric data): $^{**}P<0.01$, $^{*}P<0.05$.

(at 20-, 40- and 60-min intervals) at the specific time-point (from 80-min interval on). Figure 4 pools graphs showing the accumbens release of **dopamine, 3-MT, DOPAC** and **HVA** after an acute dose of both vehicle and 5 mg/kg METH in SHAM and OBX rats. As expected, acute METH induced an immediate strong release of dopamine in both sham-operated and OBX animals as compared to their respective baselines. However, a significantly higher percent of release was recorded in OBX rats when the relative values were compared between SHAM-METH and OBX-METH groups (at peaks approximately 400 vs. 500%, respectively). Following the dopamine release, levels of its metabolites were also changed. HVA values show a delayed moderate increase, as this metabolite follows the formation of 3-MT and DOPAC. There were no significant changes after saline dose.

Figure 5 pools graphs showing accumbens release of **serotonin**, its metabolite **5-HIAA**, **glutamate** and **GABA** after treatments. Acute dose of METH induced a release of serotonin and 5-HIAA in both sham-operated and OBX animals ($P<0.001$). Similarly, as in the case of dopamine,

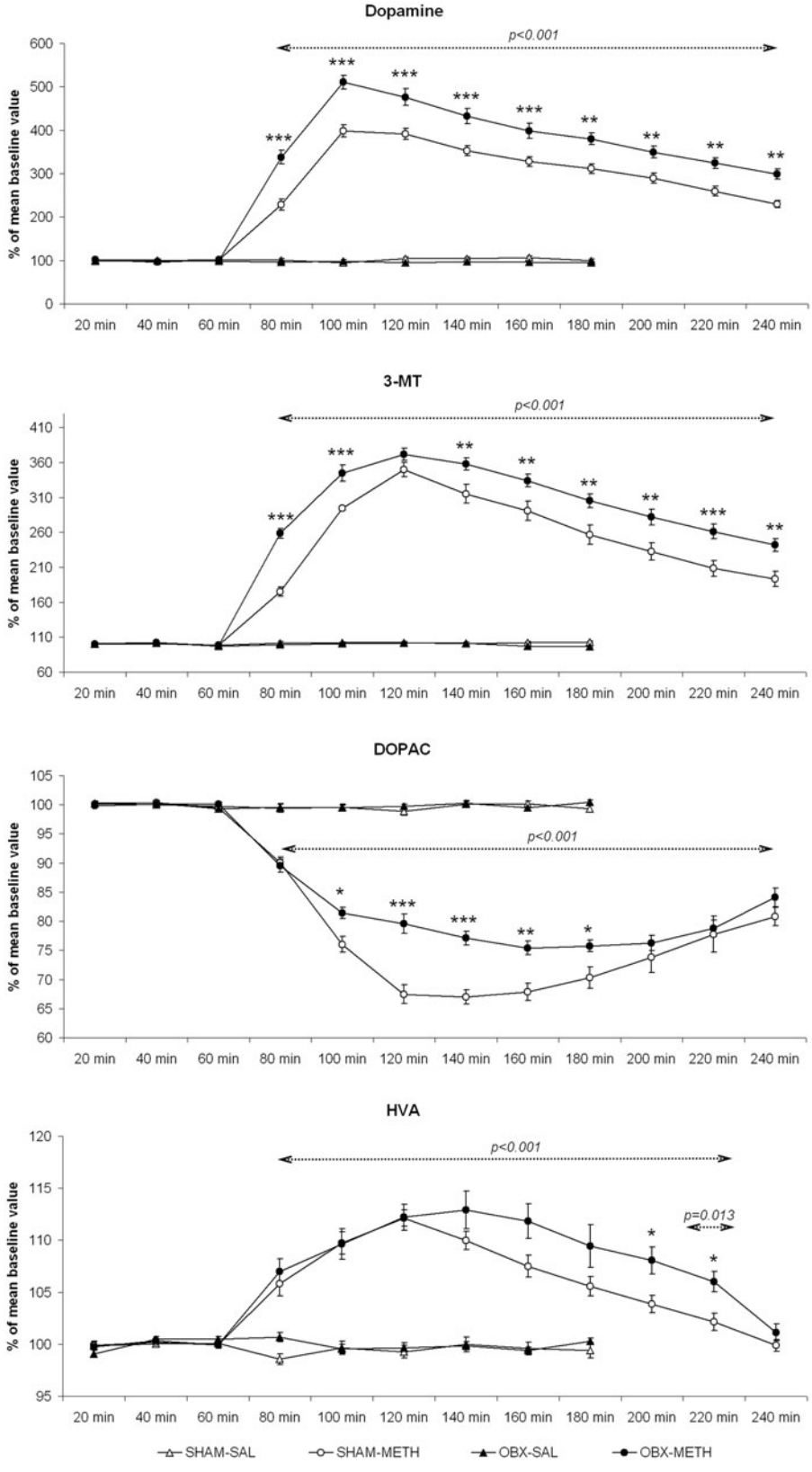

Figure 4. Methamphetamine-induced release of dopamine and its metabolites in the NACSh. The graphs show the mean±SEM of relative values of concentration (% of mean baseline value) of dopamine and its metabolites in the NACSh in all groups and treatments. The horizontal line shows significant change in the neurotransmitter level induced by the METH treatment versus baseline in both SHAM-METH and OBX-METH groups in the time points below it with indication of significance level. (In case of HVA the short line indicates lower *P* value in the SHAM-METH group in the 220-min time point.) Asterisks indicate significant differences between groups, respectively in SHAM-METH versus OBX-METH groups, ***$P<0.001$, **$P<0.01$, *$P<0.05$, two-way ANOVA for repeated measures followed by Bonferroni *t*-test. Note that the *y*-axis scales differ among the graphs.

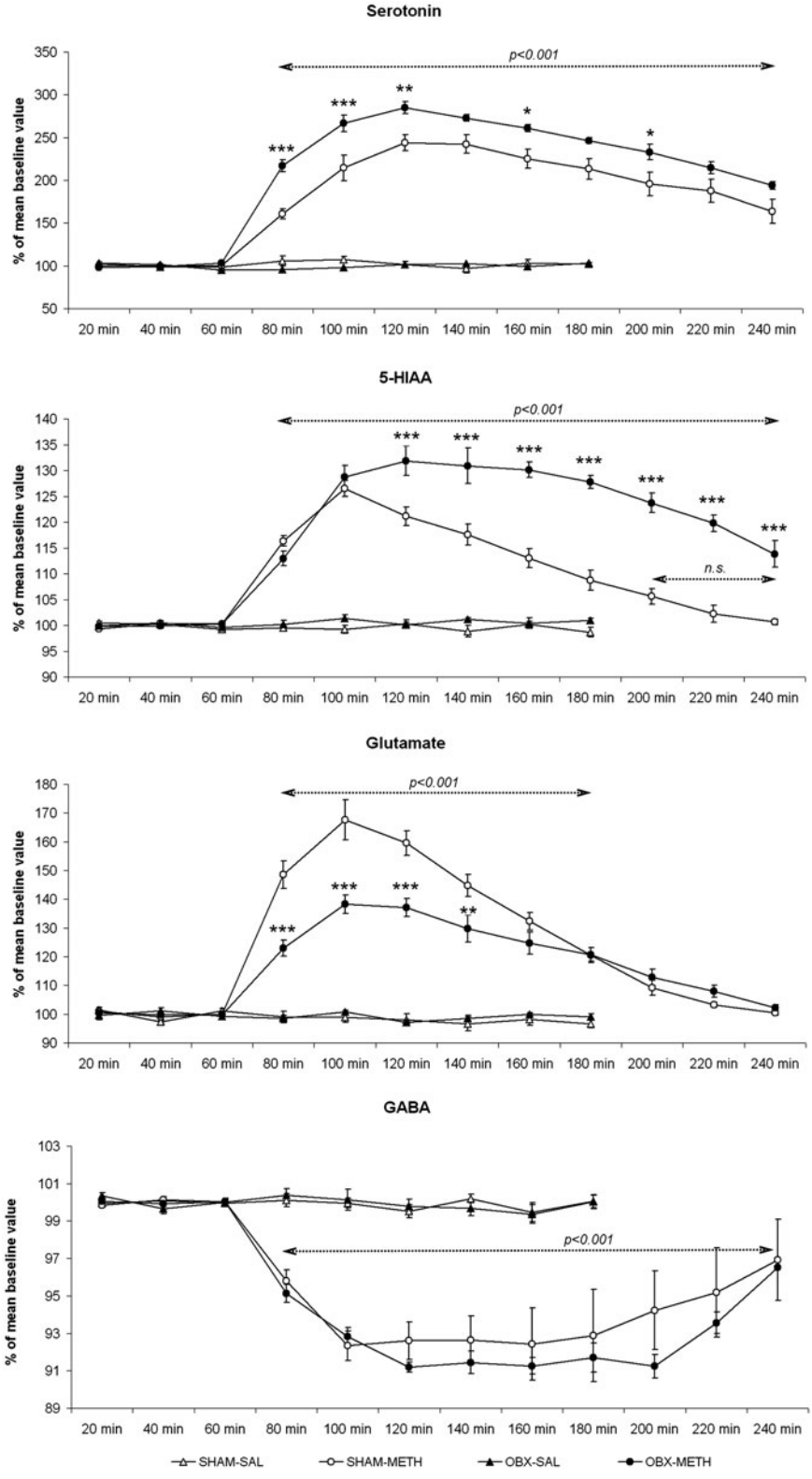

Figure 5. Methamphetamine-induced release of serotonin, its metabolite, glutamate and GABA in the NACSh. The graphs show the mean±SEM of relative values of concentrations (% of mean baseline value) of serotonin, its metabolite, glutamate and GABA in the NACSh in all groups and treatments. The horizontal line shows significant change in the neurotransmitter level induced by the METH treatment versus baseline in both SHAM-METH and OBX-METH groups in the time points below it with indication of significance level. (In case of 5-HIAA the short line indicates a non-significant effect of METH in the SHAM-METH group starting from the 200-min time point.) Asterisks indicate significant differences between groups, respectively in SHAM-METH versus OBX-METH groups, ***$P<0.001$, **$P<0.01$, *$P<0.05$, two-way ANOVA for repeated measures followed by Bonferroni t-test. Note that the y-axis scales differ among the graphs.

a significantly higher percent of release was recorded in OBX rats (at peaks approximately 240 vs. 280%, respectively). Formation of 5-HIAA was enhanced in the OBX-METH as compared to the SHAM-METH group 1 h after methamphetamine injection and remained significantly higher till the end of the microdialysis session. 5-HIAA levels in the SHAM-METH group fell down to the baseline levels in the last three measurements. Acute dose of METH led also to a strong accumbens release of glutamate in both groups ($P<0.001$) and normalised at the same time before the end of the microdialysis session. A significant difference in percentage of glutamate release was recorded in OBX rats when compared to the control group. In OBX-METH group the glutamate peak was lower than in the SHAM-METH animals (at peaks approximately 140 vs. 160%, respectively).

Accumbens GABA levels were significantly decreased by the METH treatment in both groups with no difference between OBX and SHAM rats. There were no significant changes after saline administration in any neurotransmitter.

Behavioural assessment

We have recorded significantly altered behavioural profile in the OBX rats over the course of the microdialysis session, including both basal differences (increased locomotion) and response to methamphetamine dose (in comparison to SHAM rats), depicted in Figure 6. As expected, methamphetamine induced significant stereotyped behaviour and proportionally decreased immobility in both groups. However, OBX rats were recorded to show more stereotypies and lower immobility than SHAM controls (expressed in percentage of total behaviour). Furthermore, locomotion in OBX was higher at the beginning and lower at the end of the session as compared to SHAM which corresponds to higher METH effects in this group in terms of increased locomotion and longer stereotyped behaviour. Saline, as vehicle, did not induce any significant behavioural changes in either SHAM or OBX animals and did not provoke any stereotyped behaviour.

Discussion

As one of the core symptoms of depression is anhedonia, the mesolimbic dopaminergic reward circuit of the brain is believed to be dysregulated and contributes to the high incidence of comorbid drug abuse (Nestler and Carlezon 2006). Furthermore, clinically it is known that moderate enhancement of DA function is at least partially responsible for antidepressant activity of some drugs, e.g., bupropione (Quesseveur et al. 2013). These findings are supported by some preclinical studies which showed that similarly, as in our present study, DISC1-Q31L (disrupted-in-schizophrenia-1 protein) mutant mice which have depression-like behaviours were found to have reduced levels of dopamine, serotonin and norepinephrine in the NAC (Lipina et al. 2013). This could indicate that lower monoamine levels in the NAC are linked to the depressive-like phenotype. However, the baseline DA level varies greatly in different brain regions, because in another in vivo microdialysis study, OBX rats unexpectedly exhibited significantly higher basal DA levels and lower NE levels in both ventral and dorsal striatum (Masini et al. 2004). In our earlier study with Lister-hooded rats we did not record any basal difference in NACSh DA levels in OBX and sham rats which was probably caused by low sample size – four animals per group (Amchova et al. 2014), while the present study provides robust baseline data on 14 OBX and 13 sham-operated rats.

D1 and D2 receptor densities in the ventral striatum of the OBX animals, as another measure of DA-ergic system functioning, were found to be increased (Holmes 1999), which is in accordance with lower basal levels of DA. However, the matter remains inconclusive: one study found a decreased expression of the D1 receptor in the striatum (nucleus accumbens, caudate/putamen and olfactory tubercle) in the OBX, with an increase after chronic antidepressant but not cocaine treatment (Taoka et al. 2006). However, an autoradiographic study showed no difference (Sato et al. 2010). There could be some strain- or gender-specific issues that could shed light into the discrepancy, but these have not yet been evaluated.

Furthermore, DA metabolic turnover evaluated in this study was found to be significantly increased in the second step metabolite – HVA and a trend to decrease the production of 3-MT and increase DOPAC was found. These latter ones are the first-step DA metabolites, forming two alternative pathways using two different enzymes: catechol-O-methyl transferase (COMT) and monoaminooxidase (MAO). This could indicate certain adjustments in the DA metabolism induced by OBX surgery in terms of higher MAO activity. However, there is high interindividual variability in both SHAM and OBX groups and we have not included in this study any more measures to assess the situation more specifically. Resultantly, this hypothesis does not go beyond a speculation. Previously, a mouse OBX model exhibited a decrease of the dopamine turnover in the hypothalamus which was not apparent in the striatum, PFC and hippocampus. However, the validity of this finding may be compromised by the fact that an ex vivo assay was

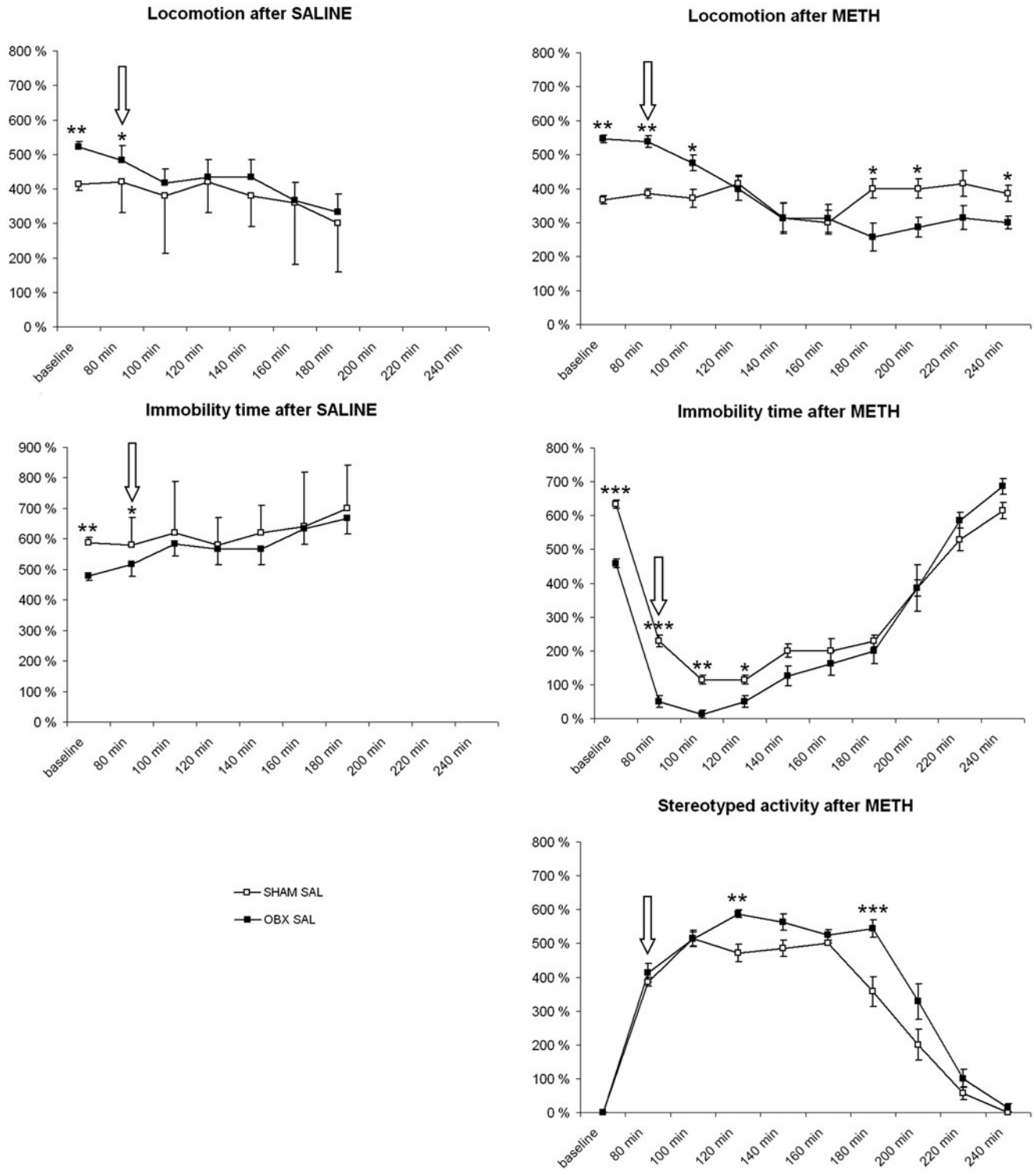

Figure 6. Behavioural scores during microdialysis session. The graphs indicate the percentage of time spent in each behavioural category (lomomotion, immobility or stereotyped behaviour) during the appropriate 20-min sampling intervals in SHAM and OBX animals after saline and methamphetamine treatment. The baseline point is a mean of all three measurements before the injection of SAL/METH, injection indicated by arrows). The SAL-treated animals finished the microdialysis session earlier which is reflected in the behavioural record as well. Asterisks indicate significant differences between the SHAM and OBX groups (a mixed ANOVA model), ***$P<0.001$, **$P<0.01$, * $P<0.05$.

used to evaluate this finding (Hellweg et al. 2007). Hence, further elucidation by more sensitive methodological means (e.g., evaluation of MAO and COMT activity, number of DA transporters, etc.) need to be used in order to explain the mechanisms underlying lower DA levels and higher turnover.

The self-medication hypothesis was repeatedly confirmed in drug self-administration experiments where OBX animals self-administer higher doses of amphetamine (Holmes et al. 2002), methamphetamine (Kucerova et al. 2012) and CB1 agonist (Amchova et al. 2014). This study shows higher methamphetamine-induced DA release and metabolism in OBX animals. However, earlier we recorded an abolished DA release in OBX animals after a challenge dose of the CB1 agonist WIN55,212-2 (WIN) (Amchova et al. 2014). The explanation could lie in the challenge dose of the respective drug. Amchova et al. (2014) reported a DA release after 0.3 mg/kg of WIN, which is a dose typically self-administered by sham-operated rats. The WIN dose recorded to be self-administered by OBX rats was approximately 0.5 mg/kg. Therefore, it could be concluded that the dose used in the microdialysis study simply is not high enough to produce DA release in OBX rats, which compensates the lower DA tonus in the reward pathway by self-administering a higher dose. This study employed 5 mg/kg of methamphetamine in order to produce a robust neurotransmitter release. However, the usual METH dose self-administered by sham/OBX animals is approximately 1.8/3 mg/kg, respectively (Amchova et al. 2014), so the possible explanation is a ceiling effect of this dose. The differences presented in this paper are shown as relative values (% of baseline in each animal). When absolute (concentration) values are compared, strong baseline dependence is apparent and the peak concentration values are similar in sham and OBX groups. Notably, self-administration studies have a chronic nature, in contrast to cited microdialysis experiments. METH is known to induce DA release in the NACSh after acute administration, but the repeated treatment leads to a decrease of DA release (Broom and Yamamoto 2005). The neuroplastic adjustments induced by OBX surgery were never investigated in this correlation, thus, the lowered reactivity induced by chronic administration can be more pronounced in the OBX model and lead to the increase of the self-administered dose.

The reduced amount of serotonin throughout the brain is a well-known symptom of major depression and impaired serotonergic signalling after OBX surgery is one of the key aspects of validity of this model (Song and Leonard 2005). In both OBX mice and rats, a decrease of serotonin content has been consistently reported in many depression-related brain regions: frontal cortex, nucleus accumbens, hippocampus, corpus striatum and basolateral amygdala (van der Stelt et al. 2005; Hellweg et al. 2007; Pudell et al. 2014). These changes can be reversed by chronic antidepressant treatment (Harkin et al. 2003; Song and Leonard 2005). The evidence of hypo-serotonergic depressive-like symptoms following OBX surgery is further supported by the compensatory 5-HT hyperinnervation of the PFC and increased numbers of 5-HT transporters (Harkin et al. 2003; Song and Leonard 2005).

Brain serotonin turnover was found to be significantly elevated in depressed patients before treatment (Barton et al. 2008). However, these data might be misleading as the 5-HT turnover is not easy to measure in human subjects and possible seasonal effects may have contributed to these results (Luykx et al. 2013). Furthermore, the interpretation is complicated due to different turnover definitions used by the authors either as a metabolic ratio (Barton et al. 2008) or directly as a 5-HIAA level (Luykx et al. 2013). Apart from that, numerous other factors may influence levels of 5HT and 5-HIIA, such as availability of tryptophan, activity of tryptophan hydroxylase, formation of melatonin, activity of other retrograde signalling systems, etc. In the same ex vivo study mentioned above in DA, a mouse OBX model showed a decrease of the serotonin turnover (calculated as a ratio) in the hippocampus, frontal cortex and hypothalamus (Hellweg et al. 2007). Similar findings were found in hippocampus of OBX Wistar rats (Pudell et al. 2014). However, in our study we recorded an increase of serotonin turnover in the NACSh and currently there are no other NAC-specific data to compare available. Furthermore, OBX rats showed a lower rate of 5-HT synthesis under basal conditions without impairment of the synthetic capacity in the hippocampus and basolateral amygdala (van der Stelt et al. 2005) which is in accordance with our data on the basal serotonin level. However, in some limbic areas the synthesis was reported to be enhanced in this model: the cingulate, the medial forebrain bundle, the hippocampus and the thalamus (Watanabe et al. 2003).

Serotonin levels are known to be enhanced especially by MDMA (ecstasy) to a higher extent than dopamine levels, but other amphetamines (as well as other drugs) induce strong serotonin release in multiple brain regions as shown in numerous studies (Schenk 2009; Matsumoto et al. 2014). As expected, we have recorded this effect in both SHAM and OBX rats. However, similar to case of dopamine, a higher 5-HT and 5-HIAA relative release was found after a challenge dose of methamphetamine in the OBX group. When absolute (concentration) values are compared, strong baseline dependence is apparent

and the peak concentration values are actually higher in the SHAM than the OBX group.

It has been sufficiently confirmed that the concentrations of monoamine neurotransmitters (including dopamine and serotonin) in the microdialysates reflecting their presence in the extracellular compartment represent an overflow of neuronally released transmitters from the synapses. For example, infusion of tetrodotoxin or calcium antagonist (Cd^{2+}) results in substantial reduction of these monoamine levels in the dialysates (Westerink and de Vries 1989; Sharp et al. 1990; Morari et al. 1993). However, in case of GABA and glutamate the situation is more complicated. Therefore, in our previous experiments (Sustkova et al., in preparation) we tested the sensitivity of basal GABA and glutamate levels in the NACSh to the Ca^{2+} presence in the perfusion solution. Temporary replacement (1 h 20 min) of Ca^{2+} in Ringer's solution with Cd^{2+} resulted in an immediate and steep reduction of GABA in the NACSh dialysates (drop to maximum 8 % of basal levels). This indicates that GABA release in the NACSh in our experiment is Ca^{2+} dependent. In the case of glutamate, we did not observe any decrease; on the contrary, glutamate concentrations showed a dramatic increase during Cd^{2+} perfusion (increase to maximum 284 % of basal levels) which began to decrease as soon as perfusion with normal Ringer's solution was resumed, although glutamate levels did not recover to basal values. These results are in accordance with similar data from the striatum (Miele et al. 1996) and indicate that in our experiment glutamate is not derived from exocytotic release. Therefore, similar to Miele et al. (1996) we assume that the glial glutamate release might be considered.

Excitatory and inhibitory systems including glutamate and GABA are imbalanced in OBX animals (Song and Leonard 2005). The condition of the glutamatergic system in the model is usually reported as hypoactive in different brain regions (Harkin et al. 2003; Song and Leonard 2005), but the typical hyperactive response to a novel environment seen in OBX rats has been hypothesised to result from increased glutamate levels in the striatum recorded in OBX but not in sham-operated rats while performing the microdialysis study during open field test (Ho et al. 2000). In other microdialysis studies, glutamate basal levels were found to be no different from SHAM control in the PFC, but acute riluzole (a drug with multiple indirect glutamate antagonistic mechanisms) decreased glutamate levels in OBX rats only (Takahashi et al. 2011) suggesting higher sensitivity of the glutamate system in the OBX model. Our data on the higher extracellular levels of glutamate in the NACSh in the OBX rats contrast with these findings; however, direct comparison is not possible for the lack of available data.

The GABA-ergic system is reported mostly as hyperactive in the OBX model, specifically in the amygdaloid cortex an increased GABA turnover and density of GABA-A receptors were found together with decreased density of GABA-B receptors (Song and Leonard 2005). This evidence is supported by our present data at basal conditions. Moreover, GABA-B-positive allosteric modulators have been proposed as a promising treatment for drug addiction (Filip et al. 2014), probably via inhibition of the mesolimbic DA pathway.

As repeatedly demonstrated, glutamatergic (Tzschentke 2002) and GABA-ergic systems are involved in the reward processes and their role is especially important in repeated drug intake contributing to the biological basis of sensitisation. We have shown here a lower glutamate release after METH challenge in OBX rats in comparison to their SHAM counterparts. There is an apparent mutual modulation mechanism between dopamine, glutamate and GABA in the NAC (Schoffelmeer et al. 2000). Therefore, the neurotransmitter changes seen after METH challenge dose in this study are probably interdependent. This is supported by a microdialysis study during cocaine self-administration (Wydra et al. 2013). However, our acute dose-related data show similar level of decrease in GABA levels in both SHAM and OBX group.

As expected, in this study we have recorded a significantly altered behavioural profile in the OBX rats over the course of the microdialysis session. Hyperactive response of the OBX rats to novel environment is a widely established measure of success of OBX surgery validated in numerous laboratories (Harkin et al. 2003; Song and Leonard 2005). Also, the locomotor response to acute dose was repeatedly found to be increased in the OBX model after cocaine (Slattery et al. 2007; Eisenstein et al. 2009) or amphetamine (Romeas et al. 2009). Increased locomotion correlates with dopaminergic signalling in the striatum and NAC (Do et al. 2012), so the presented behavioural and neurochemical data in this study are in full accordance with previous reports. Furthermore, we have observed an increase of stereotypical behaviour after METH dose in the OBX group compared to the SHAM control. Methamphetamine-induced stereotypies have been shown to correlate also with the DA firing in the NAC – interestingly only NAC core and not shell (Morra et al. 2010).

In summary, this study provides comprehensive data on extracellular levels of four neurotransmitter systems in the nucleus accumbens shell, the main reward-related area (Di Chiara et al. 2004) in the OBX rat model of depression. The most important finding is the different baseline condition: significantly decreased basal levels of dopamine, serotonin and their metabolites and

increased levels of glutamate and GABA in OBX rats. After acute methamphetamine administration we detected a significantly higher release of dopamine, serotonin and their metabolites in OBX rats; however, glutamate levels were lower and GABA was not found to be different from SHAM control. These findings are further supported by a differential behavioural profile in the OBX rats. This study provides comprehensive data on extracellular levels of four neurotransmitter systems in the nucleus accumbens shell, the main reward-related area. However, a dose–response assessment of DA release and changes induced by chronic methamphetamine administration are of high interest. Furthermore, chronic drug administration data are needed to further elucidate the underlying pathophysiological processes shared by the depressive-like phenotype and reward.

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Statement of interest

None to declare.

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2.4.4. Enhanced self-administration of the CB1 receptor agonist WIN55,212-2 in olfactory bulbectomized rats: evaluation of possible serotonergic and dopaminergic underlying mechanisms

The aim of this study was to extend the rat model of the dual disorder to intravenous self-administration of synthetic CB1 receptor agonist WIN55-212,2, (WIN) in the olfactory bulbectomy model of depression. Since cannabinoid self-administration was shown to be associated to an increased dopaminergic transmission in the shell of the *nucleus accumbens* (Fadda et al., 2006), the *in vivo* microdialysis technique was used in order to test whether OBX and SHAM rats displayed similar increase in dopamine levels within the *nucleus accumbens* shell in response to a challenge dose of WIN (0.3 mg/kg). This dose is equivalent to the mean daily amount of the drug typically self-administered by trained rats (Fattore et al., 2001, Fattore et al., 2007). Furthermore, the 5-HT1B receptor is greatly involved in the modulation of both depression and drug intake (Miszkiel et al., 2012, Murrough et al., 2011), therefore we tested the effect of the 5-HT1B agonist CGS-12066B (CGS) on WIN self-administration in OBX and SHAM Lister Hooded rats and Sprague Dawley rats self-administering methamphetamine.

Findings of this study showed that olfactory bulbectomy markedly increases self-administration of WIN, possibly through a reduction of its rewarding effects to which animals compensate by increasing WIN intake. A decreased dopamine neurotransmission in the nucleus accumbens shell might contribute to this compensatory behaviour. CGS did not show any influence on drug taking behaviour in any strain and drug.

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Enhanced self-administration of the CB_1 receptor agonist WIN55,212-2 in olfactory bulbectomized rats: evaluation of possible serotonergic and dopaminergic underlying mechanisms

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Depression has been associated with drug consumption, including heavy or problematic cannabis use. According to an animal model of depression and substance use disorder comorbidity, we combined the olfactory bulbectomy (OBX) model of depression with intravenous drug self-administration procedure to verify whether depressive-like rats displayed altered voluntary intake of the CB_1 receptor agonist WIN55,212-2 (WIN, 12.5 μg/kg/infusion). To this aim, olfactory-bulbectomized (OBX) and sham-operated (SHAM) Lister Hooded rats were allowed to self-administer WIN by lever-pressing under a continuous [fixed ratio 1 (FR-1)] schedule of reinforcement in 2 h daily sessions. Data showed that both OBX and SHAM rats developed stable WIN intake; yet, responses in OBX were constantly higher than in SHAM rats soon after the first week of training. In addition, OBX rats took significantly longer to extinguish the drug-seeking behavior after vehicle substitution. Acute pre-treatment with serotonin $5HT_{1B}$ receptor agonist, CGS-12066B (2.5–10 mg/kg), did not significantly modify WIN intake in OBX and SHAM Lister Hooded rats. Furthermore, acute pre-treatment with CGS-12066B (10 and 15 mg/kg) did not alter responses in parallel groups of OBX and SHAM Sprague Dawley rats self-administering methamphetamine under higher (FR-2) reinforcement schedule with nose-poking as *operandum*. Finally, dopamine levels in the nucleus accumbens (NAc) of OBX rats did not increase in response to a WIN challenge, as in SHAM rats, indicating a dopaminergic dysfunction in bulbectomized rats. Altogether, our findings suggest that a depressive-like state may alter cannabinoid CB_1 receptor agonist-induced brain reward function and that a dopaminergic rather than a $5\text{-}HT_{1B}$ mechanism is likely to underlie enhanced WIN self-administration in OBX rats.

Keywords: WIN55212-2, cannabinoid, methamphetamine, olfactory bulbectomy, depression, drug dependence, serotonin, dopamine

INTRODUCTION

Many psychiatric disorders including depression, schizophrenia, and anxiety are frequently associated to drug addiction (Langas et al., 2010; Testa et al., 2013). Recently, clinical associations between depression and marijuana smoking have been reported (Horwood et al., 2012; Lev-Ran et al., 2013). Yet, whether cannabis abuse in depressed patients antedates the disorder onset or is a consequence of its course is still to be determined. Several hypotheses have been offered to explain the high rates of marihuana smoking in people with depression including genetic factors, environmental influences, and self-medication. Recently, a genetically conditioned hypersensitivity to elicit cannabis dependence was evaluated in a depressive population, and although the outcome was not fully conclusive authors suggested that the links between cannabis use and depressive symptoms are conditional on the individual's genetic makeup (Otten and Engels, 2013). Social difficulties such as limited economical resources, impaired interpersonal skills, social isolation, or stressful events may also trigger both depression and cannabis abuse (Baker et al., 2010). On the other hand, cannabis use has been proposed to serve as a self-medication in depressed patients (Degenhardt et al., 2003), although some studies excluded that

subjects with prior depression experience symptom relief after smoking cannabis (Arendt et al., 2007).

The self-medication theory was developed on the basis of the monoaminergic hypothesis of depression, according to which depression is associated with a reduced monoaminergic transmission, in particular noradrenaline and serotonin (5-HT) (Rotenberg, 1994; Prins et al., 2011). In fact, symptoms related to monoaminergic deficits (as in depression) may be relieved by a variety of abused drugs (Khantzian, 1985; Hall and Queener, 2007; Becker et al., 2012; Holma et al., 2013). Accordingly, stimulant-dependent patients with depressive disorders reduce their abuse of stimulants when treated with antidepressants to a greater extent than non-depressed (stimulant-dependent) individuals (Markou et al., 1998; Wohl and Ades, 2009). Similar to other abused drugs, cannabis induces release of dopamine (DA) in the mesolimbic reward pathway (Oleson and Cheer, 2012), thus elevating mood and improving wellbeing. However, it also significantly affects bioavailability of serotonin. Notably, genetic deletion of the cannabinoid CB_1 receptor reduces the functionality of the brain serotonin system (Mato et al., 2007), while chronic CB_1 receptor antagonism induces a depression-like phenotype (Beyer et al., 2010).

Majority of available studies aimed at investigating the role of endocannabinoid system in animal models of depression reported a decreased activity of the endocannabinoid system (Micale et al., 2013a,b). Pharmacological and genetic blockade of cannabinoid CB_1 receptors result in symptoms that mimic those seen in depression (Ashton and Moore, 2011). In keeping with this, stimulation of CB_1 receptors exerts an antidepressant-like effect similar to that induced by the antidepressants desipramine and fluoxetine in the rat forced-swim test (Hill and Gorzalka, 2005) and the olfactory bulbectomy (OBX) rat model of depression (Rodriguez-Gaztelumendi et al., 2009), respectively. These antidepressant effects are antagonized by administration of CB_1 receptor antagonists leading back to depression-like phenotypes (Hill and Gorzalka, 2005). All these and other preclinical evidence strengthen the involvement of the endocannabinoid system in depressive-like states. However, no data are available on the voluntary consumption of cannabinoid receptor agonists in animal models of depression.

The multi-faceted effects of 5-HT are mediated by at least 14 receptor subtypes (Hoyer et al., 1994). Among them, the 5-HT_{1B} receptor subtype has recently attracted scientific attention for its potential role in modulating addictive behaviors (Pentkowski et al., 2012; Neisewander et al., 2013). Serotonin 5-HT_{1B} receptors are widely distributed in the brain where function as both autoreceptors and heteroreceptors, that mediate release of serotonin and other neurotransmitters (Barnes and Sharp, 1999; Moret and Briley, 2000; Pytliak et al., 2011; Cai et al., 2013). A number of human and animal studies demonstrated a causal link between altered 5-HT_{1B} receptor activity and development of neuropsychiatric conditions, including depression and drug addiction. For example, lower functioning of 5-HT_{1B} receptors was found in patients suffering major depressive disorders (Murrough et al., 2011), while a polymorphism at the 5-HT_{1B} receptor gene (*HTR1B*) was reported to be associated significantly with alcoholism (Lappalainen et al., 1998). In animal models of drug addiction, stimulation of the 5-HT_{1B} receptor was shown to induce antidepressant effects (Tatarczynska et al., 2004) and to decrease the number of behavioral responses for alcohol (Grant et al., 1997; Maurel et al., 1999; Tomkins and O'Neill, 2000), d-amphetamine (Fletcher and Korth, 1999), intracranial self-stimulation (Hayes et al., 2009) and the positive reinforcing effects of cocaine in rats (Harrison et al., 1999). More importantly, administration of the 5-HT_{1B} receptor agonist CGS-12066B into the nucleus accumbens (NAc) core was shown to decrease operant responses for ethanol but not sucrose solution in rats (Czachowski, 2005) indicating a selective effect of this compound on drug-induced responses. However, systemic administration of CGS-12066B did not reduce cocaine self-administration in rats (Parsons et al., 1996), which implies a certain drug selectivity of this compound in attenuating self-administration behavior.

The main aim of this study was to investigate the intravenous self-administration of the CB_1 receptor agonist WIN55-212,2, (WIN), using lever-pressing as *operandum* under a continuous [fixed ratio 1 (FR-1)] schedule of reinforcement, in a well-established rat model of depression, the bilateral OBX. Given the significant differences observed in WIN self-administration between OBX and SHAM rats, we decided to perform pilot experiments in an attempt to shed some light on possible underlying mechanisms. Therefore, since the 5-HT_{1B} receptor has been recently involved in the modulation of both depression and drug intake, we tested the effect of the 5-HT_{1B} agonist CGS-12066B (CGS) on WIN self-administration in OBX and sham-operated (SHAM) Lister Hooded rats displaying depressive-like phenotypes. To further investigate the role of 5-HT_{1B} receptor in drug-taking behavior and verify its effect on the self-administration of a different drug, in different strains of rats and under dissimilar experimental conditions, we tested the CGS compound in OBX and SHAM Sprague Dawley rats self-administering methamphetamine (METH) as previously reported (Kucerova et al., 2012). CGS was chosen because of its high selectivity to 5-HT_{1B} receptors (Neale et al., 1987) and its reducing effects on amphetamine (Fletcher and Korth, 1999) and alcohol self-administration (Tomkins and O'Neill, 2000; Czachowski, 2005). Finally, since cannabinoid self-administration was shown to be associated to an increased DA transmission in the shell of the NAc (Fadda et al., 2006), we used the *in vivo* microdialysis technique to test whether OBX and SHAM rats displayed similar increase in DA levels within the NAc shell in response to a challenge of WIN at a dose (0.3 mg/kg) mimicking daily mean amount of the drug typically self-administer by trained rats.

MATERIALS AND METHODS

ANIMALS

Adult male Lister Hooded rats weighting 250–270 g at the beginning of the experiment (9 weeks old) were purchased from Harlan-Nossan (Italy) and housed four per cage at the Animal Facility of the Department of Biomedical Sciences, University of Cagliari, Italy. Rats were provided with free access to water and food and maintained on a reversed 12/12 h

light/dark cycle (lights on 7 p.m.) with constant room temperature (22 ± 2°C) and humidity (60%). The experimental protocols were approved by the local Animal Care Committee at the Department of Biomedical Sciences, University of Cagliari, Italy.

Adult male albino Sprague Dawley rats weighting 220–240 g at the beginning of the experiment (8 weeks old) were purchased from Charles River (Germany) and housed individually at the Animal Facility of the Department of Pharmacology, Masaryk University in Brno, Czech Republic. Animals were maintained on a reversed 12/12 h light/dark cycle (lights on 5 p.m.) with constant relative humidity of 50–60% and temperature of 23 ± 1°C, and food and water available *ad-libitum*. The experimental protocols were approved by the Animal Care Committee of the Faculty of Medicine, Masaryk University, Czech Republic.

All experiments were carried out in strict accordance with the E.C. Regulations for Animal Use in Research (CEE No. 86/609) and local acts.

DRUGS AND TREATMENTS

For self-administration training, WIN55-212,2 (R-[2,3-dihydro-5-methyl-3-[(morpholinyl) methyl]-pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)-methanone mesylate), (WIN, RBI, USA) was freshly dissolved in one drop of Tween 80, diluted in heparinized (1%) saline solution and made available at the dose of 12.5 μg/kg/infusion (volume of infusion: 100 μl), as previously described (Fattore et al., 2001). To ensure sterility, fresh drug solutions were filtered by 0.22 μm syringe filters prior to use. For microdialysis testing, WIN solution was prepared as described above and administered intravenously at the dose of 0.3 mg/kg (volume of injection: 1 ml/kg). This drug dose was selected on the basis of the daily amount of WIN typically self-administered by male Lister Hooded rats under the same experimental conditions (Deiana et al., 2007; Fattore et al., 2007; Spano et al., 2010). Importantly, this dose of WIN was also shown to significantly increase DA release in the shell part of the NAc of rats (Tanda et al., 1997).

Methamphetamine (METH, Sigma Chemical Co., St Louis, MO, USA) was dissolved in saline sterile solution and made available at dose of 0.08 mg/infusion as previously described (Vinklerova et al., 2002).

CGS-12066B, 7-trifluoromethyl-4(4-methyl-1-piperazinyl)-pyrrolo[1,2-a]-quinoxaline dimaleate (CGS, R&D systems, Abingdon, Oxon, UK) was dissolved in saline and administered intraperitoneally (i.p.) at doses ranging from 2.5 to 15 mg/kg (volume of injection: 2 ml/kg), and administered 20 min before starting the session. These drug doses were selected on the basis of their ability to acutely reduce self-administration behavior in rats in a dose-dependent manner (Parsons et al., 1996). Treatments were assigned on the basis of a Latin square design whereby at least three training sessions separated two consecutive testing sessions to allow for assessment of carryover effects. Each animal was tested once with each drug dose and once with saline in a counterbalanced manner, i.e., the order of presentation of different treatments was varied between animals.

All antibiotics and anesthetics were purchased as sterile solutions from local distributors.

OLFACTORY BULBECTOMY (OBX) SURGERY

At the beginning of the behavioral and neurochemical experiments, rats were randomly divided into two groups: OBX and SHAM rats. The bilateral ablation of the olfactory bulbs was performed as previously described (Kucerova et al., 2012). Animals were anaesthetized with isofluran 2% (Italy) or i.p. injections of 50 mg/kg ketamine plus 8 mg/kg xylazine (Czech Republic). The top of the skull was shaved and swabbed with an antiseptic solution. Then, midline frontal incision was made on the skull and the skin was retracted bilaterally. Two burr holes, 2 mm in diameter, were drilled in the frontal bone 7 and 7.5 mm anterior from the bregma, 1.5 and 2 mm lateral to bregma suture for rats weighing 230 ± 10 g and 260 ± 10 g, respectively. Both olfactory bulbs were removed by aspiration paying particular attention to not damage the frontal cortex. Prevention of blood loss from the ablation cavity was achieved by filling the dead space with a hemostatic sponge. The skin above the lesion was closed with suture. Finally, bacitracin plus neomycin powder was applied to prevent bacterial infection. SHAM rats underwent identical anesthetic and drilling procedures but their bulbs were left intact.

A period of at least 20 days was allowed for the recovery from the surgical procedure and the development of the depressive-like syndrome. During this period, animals were handled daily for few minutes to eliminate aggressiveness, which could otherwise arise (Leonard and Tuite, 1981; Song and Leonard, 2005). Before starting either drug self-administration training or microdialysis experiments, animals were tested in the sucrose preference and motor activity test for anhedonia and hyperactive locomotor response to a novel environment, respectively, (Song and Leonard, 2005).

SUCROSE PREFERENCE TEST

After 20 days of recovery from the OBX surgery, Lister-Hooded animals were transferred into single housing with free access to food. A two-bottle choice procedure was used to determine baseline sucrose intake. During the 24-h training phase, all rats were provided in their home cage with two water bottles on the extreme sides of the cage to adapt for drinking from two bottles. After training, one bottle was randomly switched to contain 2% sucrose solution, a concentration known to provide a robust sucrose preference (Muscat and Willner, 1989). The side of sucrose presentation in the home cage was counterbalanced across rats. At 4 and 24 h time intervals both bottles were removed and the amount of liquid remaining in each bottle was measured. After 4 h, the relative position of the bottles was inverted, i.e., they were switched from one side of the cage to the other to avoid perseveration effects. The sucrose preference score was calculated as the percentage of sucrose solution ingested relative to the total amount of liquid consumed as determined before and after each test, i.e., sucrose preference = sucrose intake/total liquid (sucrose + water) intake × 100.

LOCOMOTOR ACTIVITY TEST

A day after conclusion of the sucrose preference test, the validity of OBX lesions was further confirmed by assessing increased activity in a brightly lit novel environment. Rats were individually

tested for locomotor activity using the Digiscan Animal Activity Analyser (Omnitech Electronics, USA) as previously described (Castelli et al., 2013). Each operant cage (42 × 30× 60 cm) was equipped with two sets of 16 photocells located at right angles to each other projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor. The outside of the four walls was covered with aluminium foil and two 90-W light bulbs were located at diagonally opposed corners to provide bright illumination. Rats were brought into the testing room individually, placed in the center of the box, and allowed to move freely for 10 min. Locomotor activity was defined from measurement of sequential infrared beam breaks recorded at every 5 min after placing the animals individually in the cage. During the 10-min test the following behavioral parameters were measured:

- Horizontal activity: The total number of beam interruptions that occurred in the horizontal sensors;
- Vertical activity: The total number of beam interruptions that occurred in the vertical sensors; that is the number of times the animal rose onto its hind legs with the front limbs either against the wall or freely in the air (number of rearing episodes);
- Total distance (cm): The horizontal distance travelled by the animal (dependent on animal's trajectory).

At the end of the session, animals were gently removed from the Plexiglas boxes and returned to their home cage. Boxes were wiped with H_2O_2 between sessions to prevent olfactory cues.

INTRAVENOUS DRUG SELF-ADMINISTRATION SURGERY

At the end of the motility test, OBX and SHAM animals were deeply anesthetized with isofluran 2% (in Italy) or i.p. injections of 50 mg/kg ketamine plus 8 mg/kg xylazine (in the Czech Republic). Under aseptic conditions, a permanent intracardiac silastic catheter was implanted through the external jugular vein to the right atrium. The outer part of the catheter exited the skin in the midscapular area. After surgery, each animal was allowed for recovery, individually, in its home cage with food and water freely available. On the following 6–7 days, each rat received an intravenous infusion of gentamicin (0.16 mg/kg, Italy) or heparinized cephazoline (Vulmizolin 1.0 g, Czech Republic) solution followed by 0.1 ml of a heparinized (1%) sterile saline solution to prevent infection and occlusion of the catheter. During recovery, changes in general behavior and body weight were monitored. When a catheter was found to be blocked or damaged, the animal was excluded from the analysis. Once completely recovered from surgery, food-restriction condition was applied, where rats were fed with 20 g/day of standard rat chow given in the home cage immediately after each session.

INTRAVENOUS SELF-ADMINISTRATION

WIN55-212,2 self-administration was conducted in 12 operant chambers (29.5 × 32.5 × 23.5 cm, Med Associates, Vermont, USA) using lever-pressing as *operandum* under a continuous (FR-1) schedule of reinforcement, i.e., each active response was reinforced. Each chamber was encased in a sound and light attenuating cube. In addition, chambers had a ventilation fan, and a front panel equipped with two retractable levers (each 4 cm wide) positioned 12 cm apart, 8 cm from the grid and extending 1.5 cm into the box. A white stimulus light was placed above each lever and a red house light was located on the opposite wall. Intravenous infusions of WIN were delivered by a software-operated infusion pump (Med Associates, Vermont, USA) through a counterbalanced single-channel swivel and an extra length of plastic tubing enclosed in a metal spring connecting the swivel to the catheter fitting on the animal's back. Pressure on the lever, defined as active, resulted in: (i) extinction of the house light and illumination of the stimulus light above the active lever for 15 s; (ii) retraction of both levers; and (iii) activation of the infusion pump for 5.8 s delivering 0.1 ml intravenous infusion of drug solution. On completion of the 15 s timeout period, levers were re-extended into the chamber, stimulus light went out and the house light was switched on. Pressure on the other lever, defined as inactive, was not coupled to any successive event, but was always recorded to provide an index of basal activity levels. Assessment of schedules and data collections was programmed by means of a computer using the MED Associates MED-PC software package. Throughout each phase of the study, locomotor activity was monitored within the operant chambers, which were equipped with a series of photocells located 3.5 cm above the cage floor. The number of photocell beam breaks was recorded and served as a measure of general horizontal locomotor activity. Self-administration sessions lasted 120 min and took place 7 days/week between 9 a.m. and 1 p.m. during the dark period of the cycle.

Acquisition training was carried out until steady baseline of drug intake was reached. Response was considered stable when animals displayed accurate discrimination between the active and inactive lever. Acquisition was defined as the number of active lever-presses >15 and not differing by more than 20% for three consecutive days. Rats not meeting the acquisition criterion were excluded from the subsequent phases of the study. Only rats developing a stable pattern of WIN intake were allowed to continue daily self-administration sessions until day 30. Then, extinction condition was introduced by replacing WIN with sterile vehicle solution (1% Tween 80 in saline solution) which allowed responses to be recorded without drug consequences. All other experimental parameters were left unchanged; therefore pressure on the active lever resulted in an infusion of 0.1 ml of vehicle accompanied by the presentation of the stimulus light previously paired with WIN delivery. Drug-reinforced behavior was considered extinguished when the maximum number of responses on the active lever was ≤ 10 and the total number of lever presses (i.e., active + inactive) in a single test session was ≤ 20.

Methamphetamine self-administration was conducted as previously described (Kucerova et al., 2009, 2012) in 10 standard experimental (30 × 25× 30 cm, Coulbourn Instruments, USA) boxes using nose-poking as *operandum* under a final FR-2 schedule of reinforcement, i.e., animal had to make two consecutive nose-pokes on the active hole to obtain a single drug infusion. Each cage was provided with two nose-poke holes allocated on one side and programmed by software Graphic State Notation 3.03 (Coulbourn Instruments, USA). Specifically, training sessions were initially conducted under a FR-1 schedule of reinforcement. Fixed-ratio requirement was then raised to FR-2 when the

animal fulfilled the following conditions for three consecutive sessions: (a) at least 70% preference of the drug-active nose-poke, (b) minimum intake of 10 infusions per session, or (c) stable intake of the drug (maximum 10% deviation). Nose-pokes in the active hole led to the activation of the infusion pump and administration of a single infusion followed by a 10 s timeout, while nose-pokes in the inactive hole were recorded but not rewarded and reset the count of active nose pokes back to zero. The cage was illuminated by a house light during the session. The light was flashing when administering infusion and off during the time-out period. Self-administration sessions lasted 90 min and took place 7 days/week between 7 a.m. and 2 p.m. during the dark period of the cycle. Acquisition criteria were the same as for WIN self-administration behavior.

In both apparatuses, assignment of the active (drug-paired) and the inactive (not drug-paired) levers/holes was counterbalanced between rats and remained constant for each subject throughout all the experiments. CGS testing was performed on Lister Hooded and Sprague Dawley rats self-administering WIN and METH, respectively, during the maintenance phase of the self-administration training, i.e., once animals stabilized their drug intake.

MICRODIALYSIS SURGERY AND PROCEDURE

A separate batch of drug-naïve Lister Hooded male rats was used for the *in vivo* microdialysis study**.** Rats were anesthetized with 2% isoflurane and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). The skull was exposed and a small hole was drilled on the right side. A concentric self-made microdialysis probe with a 2 mm dialyzing surface length (AN 69AF; Hospal-Dasco, Bologna, Italy; cut-off 40,000 Da, *in vitro* recovery about 30%) was inserted vertically into the shell of the NAc (coordinates from bregma, AP: +1.7, L: ±0.7, V: −8.2) according to the Paxinos anatomical atlas (Paxinos and Watson, 1998) and then fixed to the skull using dental acrylic cement. During the same surgery session, rats were implanted with intravenous catheters as previously described, which allowed intravenous administration of WIN. Starting 24 h from implantation of the dialysis probe, artificial cerebrospinal fluid (147 mM NaCl, 4 mM KCl, 1.5 mM CaCl2, pH 6–6.5) was pumped through the dialysis membrane at a constant rate of 2.5 μL/min with a CMA/100 microinjection pump (Carnegie Medicine, Sweden). Dialysate samples (50 μL) were collected every 20 min and directly injected into a high performance liquid chromatography system in order to quantify DA. The system consisted of an isocratic pump (ESA model 580; ESA, Chelmsford, Massachusetts), a 7125 Rheodyne injector connected to a Hewlett Packard (Waldbronn, Germany) series 1100 column thermostat with a reverse phase column (LC18 DBSupelco, 5 μm, 4.6 × 150 mm), and an ESA Coulochem II detector. The first electrode of the detector analytical cell was set at 400 mV and the second at −180 mV; column temperature was set at 30°C. The mobile phase, delivered at 1.0 mL/min, consisted of 50 mM/L sodium acetate, 0.073 mM/L Na2 ethylenediaminetetraacetic acid, 0.35 mM/L 1-octanesulfonic acid, 12% methanol, pH 4.21, with acetic acid. In this condition the sensitivity of the assay for DA was 2 fM/sample. Only results deriving from rats with correctly positioned dialysis probes were included in statistical analysis of data. The location of the probe was determined histologically at the end of each experiment by examining coronal brain sections (50 μm) stained with cresyl violet.

STATISTICAL DATA ANALYSIS

At the end of the study, rats were anesthetized by isofluran inhalation and decapitated. Their brains were removed for confirmation of the ablation of the olfactory bulbs. Only rats with a complete removal of both olfactory bulbs with no damages to the frontal cortex were included for data analysis.

Primary data were summarized using arithmetic mean and standard error of the mean estimate. Statistical analysis of differences between OBX and SHAM rats in time-related differences used One-Way and Two-Way analysis of variance (ANOVA) with repeated measures model. In Two-Way repeated measures ANOVA, the group of rats was entered as the between-group factor and the time-points as repeated within-subject measure. One-Way ANOVA was used when comparing the time-points within given experimental group of rats.

Time-related changes and differences between OBX and SHAM groups during drug self-administration after acute pretreatment with the 5-HT1B receptor agonist CGS-12066B were analyzed using Two-Way repeated measures ANOVA.

Microdialysis data were analyzed using One-Way or Two-Way ANOVA (treatment × time), followed by Tukey's or Bonferroni's *post-hoc* test comparison procedures, respectively. The level of statistical significance was set at $p < 0.05$.

Statistics were calculated using the statistical package SPSS (version 2.0).

RESULTS

BEHAVIORAL PROFILE AFTER OBX SURGERY (LISTER HOODED RATS)

To verify the development of a depressive-like phenotype in OBX lesioned animals, basal behavioral differences between SHAM and OBX rats were established by measuring sucrose preference and locomotor activity. Anhedonia and hyperactive response to a novel brightly lit environment are two major features of OBX rats consistently described by previous studies (Kelly et al., 1997; Song and Leonard, 2005; Romeas et al., 2009).

As shown in **Figure 1**, OBX rats consumed significantly lower proportion of sucrose than SHAM rats ($p < 0.001$) after both 4 and 24 h from sucrose solution presentation, which confirmed a reduced hedonic response as a consequence of ablation of the olfactory bulbs. Differences between the two dependent variables were tested using independent Student *t*-test.

Figure 2 illustrates results from the motor activity test conducted in brightly lit conditions. In all locomotor measures (i.e., horizontal activity, vertical activity, and total distance travelled), statistically significant differences were detected at 5 min of measurement, a time-point representing response to novel environment (horizontal activity, $p < 0.001$; vertical activity, $p < 0.05$; total distance travelled, $p < 0.01$). At the 10 min time-point (little effect of novelty), a significant difference was only present in vertical activity measure ($p = 0.032$). Differences between the two independent variables were tested using independent Student *t*-test.

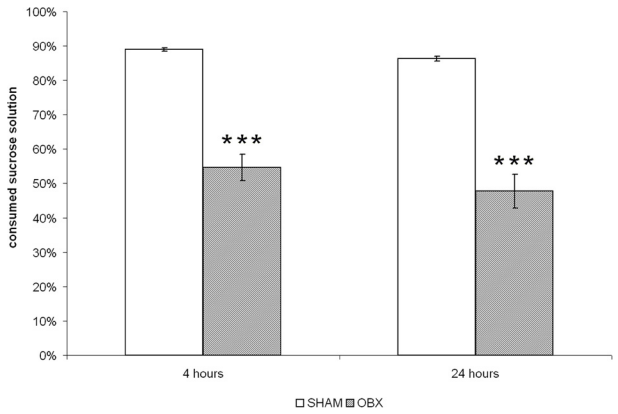

FIGURE 1 | OBX rats display anhedonia. The sucrose preference test in SHAM ($n = 6$) and OBX ($n = 7$) Lister Hooded rats. Data are percentages (±s.e.m.) of sucrose solution consumed at two time-points, i.e., after 4 and 24 h from the beginning of the test. Student *t*-test, $^{***}p < 0.001$.

WIN 55-212,2 SELF-ADMINISTRATION IN LISTER HOODED RATS

Figure 3A shows responses of SHAM and OBX rats on the active lever during the acquisition phase of WIN self-administration training. Repeated measures ANOVA revealed no significant effects over the first 8 days of training, whereas from day 9 onward a significantly higher active lever-pressing rate was observed in OBX compared with SHAM rats (repeated measures ANOVA: $\alpha < 0.001$). In contrast, inactive lever-pressing rates of OBX and SHAM rats were statistically indistinguishable throughout the 30 days of training and remained constantly below 6 responses per session starting from the first week of training, with the sole exception of the initial 4 days of training (data available as Supplementary Figure 1A). This indicates that the increase in the rate of responding observed in OBX rats was not due to an unspecific effect, as further confirmed by the finding of no significant differences between OBX and SHAM animals in the basal motor activity during the self-administration daily sessions, as measured by the mean number of interruptions of the photocell beams located inside the boxes (mean activity over the maintenance phase: 989 ± 41 and 1005 ± 27 for OBX and SHAM rats, respectively).

In accordance with this, the total amount of WIN consumed by OBX rats during the maintenance phase, i.e., once animals stabilized drug intake, was significantly higher than that consumed by SHAM rats. More specifically, mean WIN intake during the last 7 days of training before extinction was significantly higher (+105%) in OBX than in SHAM rats (repeated measures ANOVA: $\alpha < 0.001$) (**Figure 3B**). However, the percentages of rats meeting acquisition criteria for WIN self-administration in OBX and SHAM groups were similar, being 85.5 and 86.8%, respectively.

Furthermore, OBX and SHAM rats displayed clear cut differences in the time course of operant behavior even when saline was substituted for WIN, i.e., during extinction training (**Figure 3C**). Analysis of response on the active lever by repeated measures ANOVA showed significant differences between OBX and SHAM animals ($\alpha = 0.012$ from day 1 to 7; $\alpha = 0.004$ from day 8 to 14, $\alpha < 0.001$ from day 15 to 28). Specifically, on the 1st day of extinction, OBX and SHAM rats reacted to saline substitution by increasing their mean active responding from 39 to 78.5 and from 16.86 to 45.17, respectively, which corresponds to

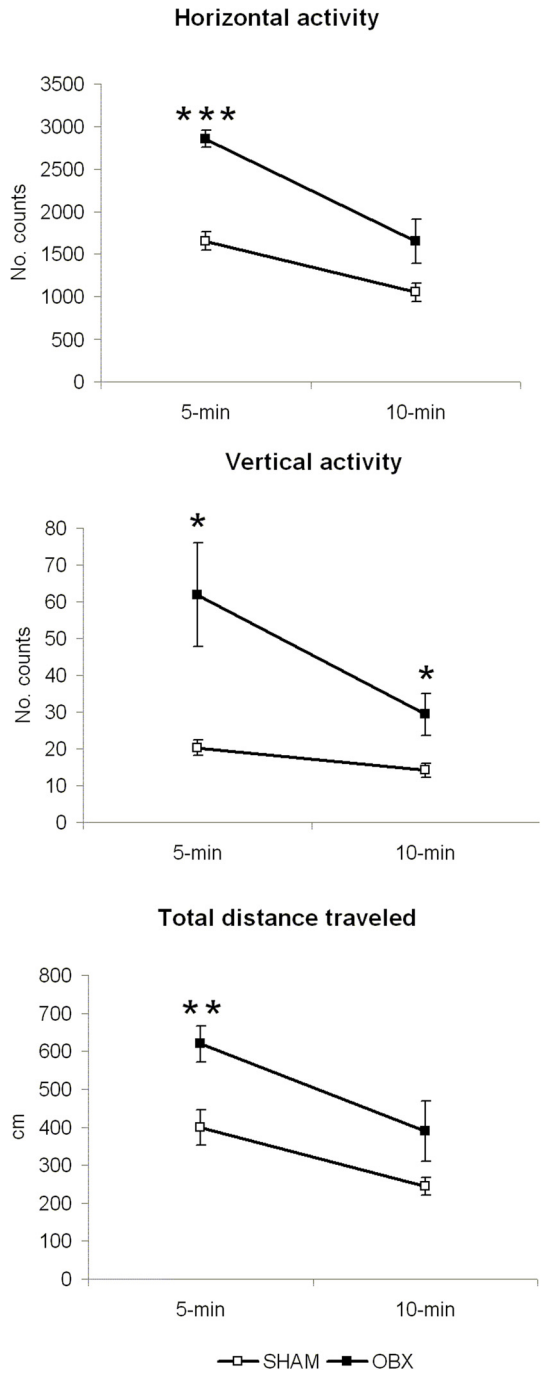

FIGURE 2 | OBX rats display hyperactivity in a novel aversive environment. Spontaneous horizontal and vertical activity, expressed as mean counts of photobeam interruptions, and total distance travelled (in centimeters) in SHAM ($n = 6$) and OBX ($n = 7$) Lister Hooded rats. Data are shown as means (±s.e.m.) at two time-points (5 and 10 min of measurement). Student *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

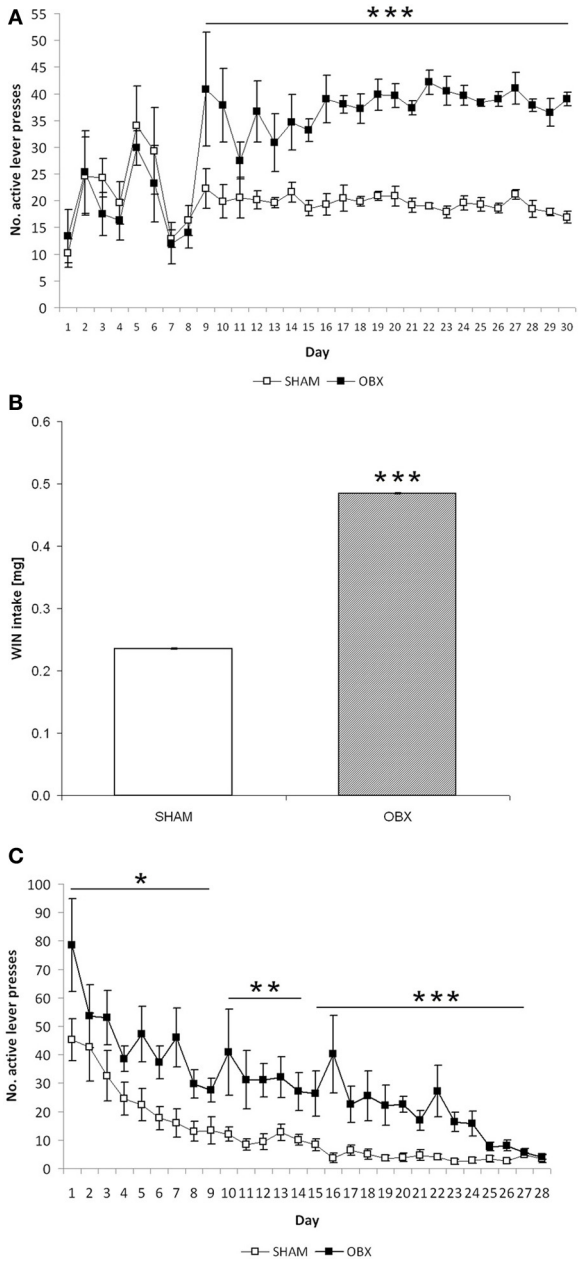

FIGURE 3 | Cannabinoid self-administration training in OBX and SHAM rats. Data are shown as means (±s.e.m.). **(A)** Acquisition phase: mean active lever presses in SHAM ($n = 6$) and OBX ($n = 7$) Lister Hooded rats during WIN55,212-2 self-administration. ***$\alpha < 0.001$, repeated measures ANOVA. **(B)** Maintenance phase: mean WIN intake over the last seven training sessions in SHAM ($n = 6$) and OBX ($n = 7$) Lister Hooded rats during WIN self-administration. ***$\alpha < 0.001$, repeated measures ANOVA. **(C)** Extinction phase: mean active lever presses in SHAM ($n = 6$) and OBX ($n = 7$) Lister Hooded rats during extinction of WIN self-administration. *$\alpha < 0.05$, **$\alpha < 0.01$, and ***$\alpha < 0.001$, repeated measures ANOVA.

+101 and +168% with respect to the last (30th) day of WIN self-administration training. Following 1-week extinction, OBX and SHAM rats reduced their active responding of −41 and −65%, respectively, with respect to day 1 extinction. Differences between OBX and SHAM groups in the responding on the active lever slightly reduced after 2 and 3 weeks of extinction training (OBX: −65 and −79%, SHAM: −78 and −90%, respectively).

Finally, response latency (defined as time elapsed from commencement of the experimental session until the first active lever press) was significantly different between the two groups. More specifically, response latency was shorter in OBX than SHAM rats from day 9 onward (repeated measures ANOVA: $\alpha = 0.029$ from day 9 to 16; $\alpha < 0.001$ from day 17 to 30) (**Figure 4A**), which suggests that after initial exposure to the CB_1 receptor agonist the bulbectomized rats may be more motivated than control animals to obtain it. Moreover, analysis of temporal patterns of responses revealed quantitative but not qualitative differences between OBX and SHAM rats during self-administration training since the response rate was typically slow and evenly distributed throughout the 2 h test session in both groups (**Figure 4B**).

EFFECT OF ACUTE PRE-TREATMENT OF CGS-12066B ON DRUG SELF-ADMINISTRATION

The effect of an acute administration of the serotonin 5-HT_{1B} receptor agonist CGS-12066B (CGS) was tested only after acquiring reliable WIN self-administration, i.e., once rats stabilized daily drug intake. Overall, we did not find changes in WIN self-administration after acute pre-treatment with the CGS in Lister Hooded OBX or SHAM rats. **Figure 5** illustrates the percentage changes of active lever presses from the baseline after an acute challenge with CGS (2.5, 5, and 10 mg/kg) and saline control as compared to previous 6-day mean responding (i.e., baseline). The repeated measures ANOVA with OBX/SHAM group as cofactor did not detect a significant effect of drug treatment within each group nor between groups. The *post-hoc* *p*-values for each drug dose were as follows for SHAM: 2.5 mg/kg: $p = 0.241$; 5 mg/kg: $p = 0.071$; 10 mg/kg: $p = 0.128$, and for OBX: 2.5 mg/kg: $p = 0.963$; 5 mg/kg: $p = 0.652$; 10 mg/kg: $p = 0.523$.

It was reported that the effects of serotonergic drugs may differ significantly depending on the animal strain and the experimental conditions used (Horowitz et al., 1997; Uphouse et al., 2002; Miryala et al., 2013), and that not all rat strains do self-administer WIN spontaneously (Deiana et al., 2007). Moreover, the CGS compound was found to reduce amphetamine (Fletcher and Korth, 1999) and alcohol (Tomkins and O'Neill, 2000; Czachowski, 2005), but not cocaine (Parsons et al., 1996), self-administration. Thus, we decided to test the CGS compound on the self-administration of a pharmacologically different drug, such as METH, which is known to be strongly self-administered by OBX rats. We therefore tested CGS in OBX and SHAM Sprague Dawley rats self-administering METH using different response-like *operandum*, i.e., nose-poking instead of lever-pressing, and a slightly higher schedule of reinforcement (FR-2). **Figure 6A** illustrates responses of SHAM and OBX rats on the active hole during the acquisition and maintenance phases of METH self-administration. In line with previous findings (Kucerova et al., 2009, 2012), rats stabilized METH self-administration behavior within 14 days of training intake with a mean daily drug intake of 1.8 mg in SHAM and 3 mg in OBX animals (METH intake data shown in Supplementary Figure 2). Repeated measures ANOVA revealed no significant effects over the first 6 days of training,

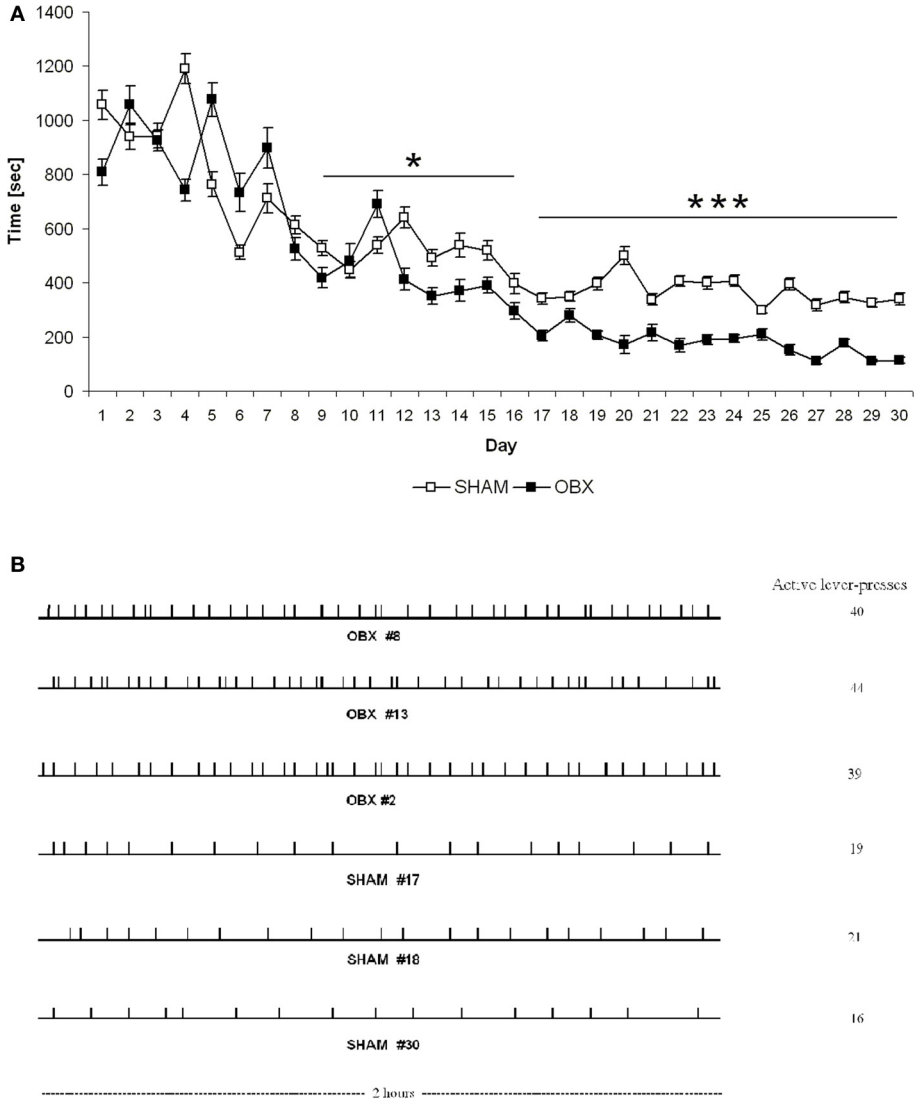

FIGURE 4 | Latency to the first active response (A) and patterns of responding (B) in SHAM and OBX Lister Hooded rats during WIN self-administration. (A) Latency (seconds) to the first active lever presses in SHAM ($n = 6$) and OBX ($n = 7$) rats. Data are shown as means (±s.e.m.). *$\alpha < 0.05$ and ***$\alpha < 0.001$, repeated measures ANOVA. **(B)** Quantitative, but not qualitative, differences in the active responding patterns between OBX and SHAM rats. Individual representative records illustrating responding patterns of OBX (first three patterns) and SHAM (last three patterns) rats on the active lever on the last day of the self-administration training (day 30). Each tick denotes the time of every response on the active lever. Cumulative numbers of active responses made over the 2 h test sessions are illustrated on the right side of each pattern.

whereas from day 7 onward a significantly higher active nose-poking rate was observed in OBX compared with SHAM rats (repeated measures ANOVA: $\alpha < 0.05$). However, while during WIN self-administration the numbers of inactive lever-presses was constantly below 5 during the maintenance phase, inactive nose-pokes during METH self-administration were higher in both OBX and SHAM rats (see Supplementary Figure 1B), an effect likely due to the activational motor effects of METH. Yet, the mean number of active nose-pokes was substantially higher than the inactive ones, which supports the specificity of animal responding for METH (preference of the active operandum during the maintenance phase was higher than 70% in all animals).

Figure 6B reports the percentages of active nose pokes for METH after acute pre-treatment with saline control, 10 and 15 mg/kg of CGS-12066B compared to previous 6-day mean responding (i.e., baseline) in SHAM and OBX Sprague Dawley rats. The repeated measures ANOVA with OBX/SHAM group as cofactor did not detect a significant effect of drug treatment within each group nor between groups. The *post-hoc p*-values for each drug dose were as follows for SHAM: 10 mg/kg: $p = 0.508$; 15 mg/kg: $p = 0.550$, and for OBX: 10 mg/kg: $p = 0.232$; 15 mg/kg: $p = 0.319$. These findings indicate that, as for WIN self-administration, acute pre-treatment with the 5-HT1B receptor agonist did not modify the voluntary intake of METH.

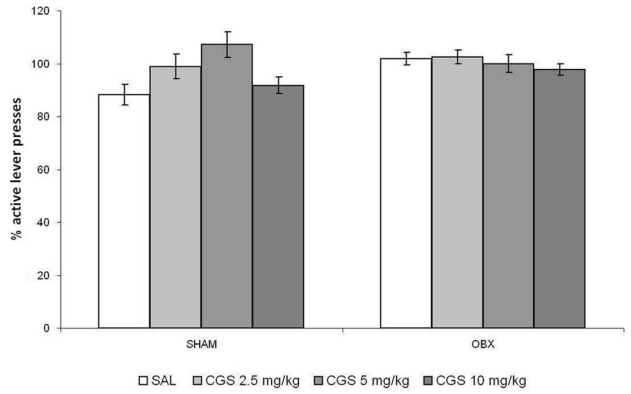

FIGURE 5 | Acute pre-treatment with CGS does not affect WIN self-administration. Effect of acute pre-treatment with CGS-12066B on WIN self-administration in SHAM ($n = 6$) and OBX ($n = 7$) Lister Hooded rats. Data are expressed as percentage changes of active lever pressing compared to 6-day baseline (assumed as 100%). The repeated measures ANOVA did not detect a significant effect of drug treatment.

IN VIVO MICRODIALYSIS OF DOPAMINE LEVEL IN THE NUCLEUS ACCUMBENS SHELL OF LISTER HOODED RATS

Figure 7 shows results from microdialysis experiment aimed at measuring the release of DA in the NAc shell of Lister Hooded SHAM and OBX rats following an intravenous injection of 0.3 mg/kg of WIN, a dose mimicking the mean amount of drug typically self-administered by naive Lister Hooded rats (Deiana et al., 2007; Fattore et al., 2007; Spano et al., 2010), and known to increase DA level in the rat NAc shell (Tanda et al., 1997). During the pre-treatment period, basal extracellular values of DA in the NAc shell did not differ significantly between the two groups (**Figure 7A**). As shown in **Figure 7B**, after WIN administration, we found a significantly increased (about +40%) extracellular DA level in SHAM rats compared to their basal level during the first 40 min after drug injection [One-Way ANOVA $F_{(8,\ 24)} = 4.997$, $p = 0.0010$]. However, the WIN challenge did not increase DA levels in OBX rats, in which DA levels did not significantly differ from to their previous baseline during the 2-h measurement [One-Way ANOVA $F_{(8,\ 24)} = 0.3730$, p = n.s.]. Data are expressed as mean ± s.e.m. percentage variation of basal levels. Two-Way ANOVA revealed a significant effect of treatment × time interaction [$F_{(8,\ 48)} = 3.07$, $p = 0.0071$; $^{**}p < 0.01$ and $^{*}p < 0.05$, Bonferroni post-test].

DISCUSSION

Findings of the present study demonstrated that bulbectomized rats: (i) do self-administer higher amount of the cannabinoid CB_1 receptor agonist WIN55,212-2 than SHAM control rats, (ii) do not alter voluntary intake of the CB_1 receptor agonist after acute pre-treatment with the serotonergic 5-HT_{1B} receptor agonist CGS12066B, and (iii) do not increase DA level in the NAc shell in response to an acute challenge with a dose of WIN (0.3 mg/kg), as SHAM rats do.

WIN SELF-ADMINISTRATION IN OBX AND SHAM RATS

Bulbectomized rats have been previously reported to self-administer more nicotine (Vieyra-Reyes et al., 2008), amphetamine (Holmes et al., 2002), and METH (Kucerova et al., 2012) than SHAM control rats. Yet, despite clinical evidence for a significant association between smoking cannabis and major depression (Horwood et al., 2012; Lev-Ran et al., 2013), cannabimimetic drug-taking behavior was never investigated in an animal model of depression. Cannabinoid CB_1 receptor agonists were shown to be readily self-administered by mice, rats, and monkeys (Martellotta et al., 1998; Fattore et al., 2001; Justinova et al., 2003). Notably, rate of responses was critically dependent on a variety of experimental conditions including drug unitary dose (Martellotta et al., 1998), food restriction regimen (Fattore et al., 2001), and type of *operandum* (Deiana et al., 2007). In this study, we adopted all parameters and experimental conditions that support a robust cannabinoid drug-taking behavior in the Lister Hooded rat strain (Deiana et al., 2007).

Before starting self-administration training and microdialysis experiments, we verified the development of a depressive-like phenotype in OBX lesioned animals by assessing the presence of anhedonia and hyperactive locomotor response to novel environment, which are two of the major hallmarks of this animal model of depression (Kelly et al., 1997; Song and Leonard, 2005; Romeas et al., 2009).

WIN self-administration by OBX rats significantly differed from SHAM controls as OBX animals showed higher rates of drug-associated operant responses during the maintenance phase, i.e., after initial acquisition. Indeed, although both OBX and SHAM rats needed a similar number of training sessions to acquire self-administration behavior, rates of active lever-pressing during the maintenance sessions were remarkably higher in OBX than SHAM rats. Therefore, the amount of WIN consumed by OBX rats over the 30 test sessions resulted significantly higher than in SHAM rats (mean cumulative amount of WIN over the 30-day training: 12.71 vs. 7.9 mg for OBX and SHAM, respectively). Similar rates of acquisition indicate that OBX rats required the same time of SHAM rats to stabilize their drug intake and suggest that development of a depressive-like phenotype is not associated with learning or memory deficits able to affect the acquisition of the operant task, although OBX animals have been reported to display impaired spatial learning (Song and Leonard, 2005). On the other hand, higher drug intake in OBX rats during the maintenance phase suggests that bulbectomized animals are differently responsive than SHAM controls to the cannabinoid, which might lend some support to the proposed self-medication theory of smoking cannabis to alleviate symptoms of depression (Gruber et al., 1996; Ogborne et al., 2000).

Our results are in line with the notion that OBX rats differ in the behavioral responses to acute and repeated exposure to other addictive drugs including METH (Kucerova et al., 2012), alcohol (Chiang et al., 2008), nicotine (Vieyra-Reyes et al., 2008), cocaine (Calcagnetti et al., 1996; Chambers et al., 2004), and amphetamine (Holmes et al., 2002). Notably, enhanced WIN intake by OBX rats recorded in this study was unlikely due to changes in locomotor activity since motor activity during the

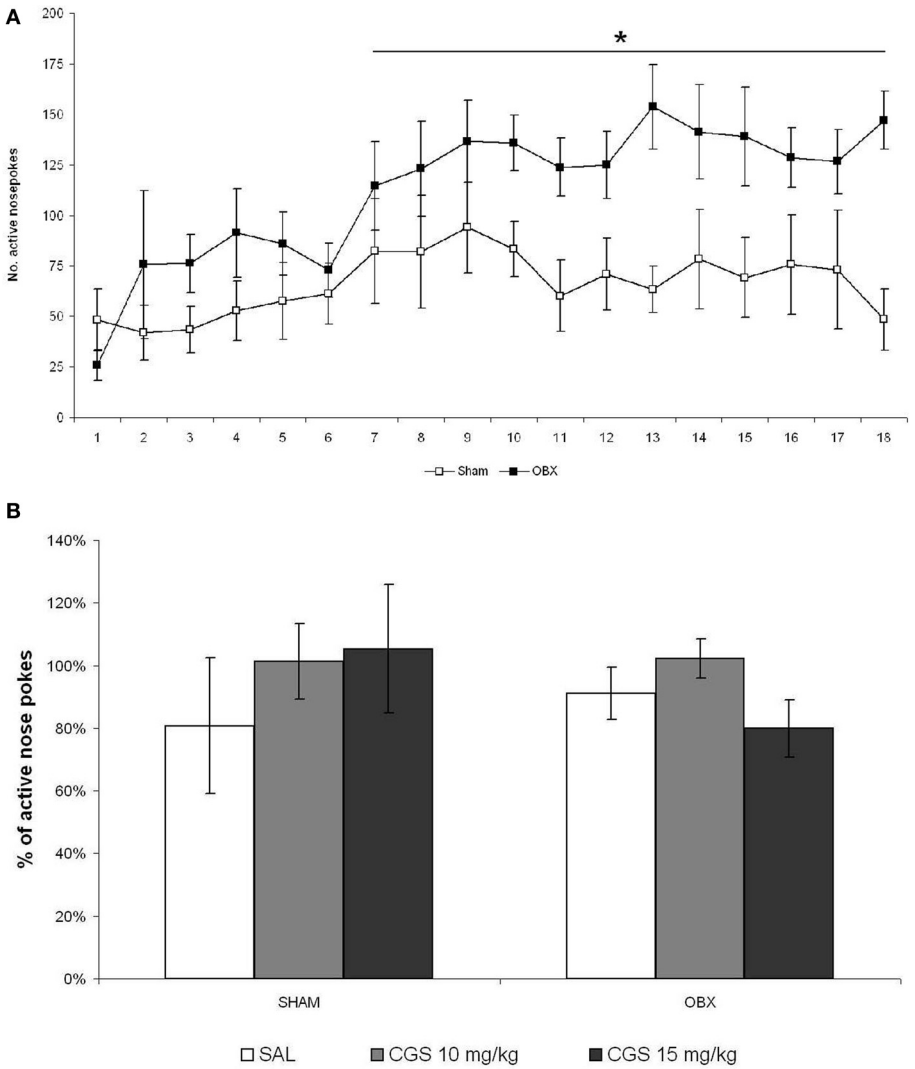

FIGURE 6 | Acute pre-treatment with CGS does not affect METH self-administration. (A) METH self-administration in OBX and SHAM rats. Data expressed as daily means (±s.e.m.) of active nose-pokes in SHAM ($n = 7$) and OBX ($n = 7$) Sprague Dawley rats during methamphetamine self-administration training. Significant difference was recorded from the day 7 onwards, *$\alpha < 0.05$ (day 7–12: *$\alpha = 0.041$, day 13–18: *$\alpha = 0.027$, repeated measures ANOVA). **(B)** Effect of acute pre-treatment with CGS-12066B on METH self-administration. Data are expressed as percentage changes of active lever pressing compared to six-day baseline (assumed as 100%). The repeated measures ANOVA did not detect a significant effect of drug treatment.

daily training session was not dissimilar between OBX and SHAM animals as confirmed by their similar numbers of photocell beams breaks measured within the operant boxes during daily training sessions.

Differences in the response rates on the active lever were also observed when vehicle was substituted for the CB_1 receptor agonist. In fact, rates of responses in OBX rats were consistently higher than in SHAM rats not only when WIN was contingently available but also when it was absent, as during extinction training. A neurobiological mechanism that may contribute to the resilience of OBX rats to extinguish not-rewarded operant responses is a dysfunction of the front-cortical neuronal circuits critically involved in the inhibition of on-going activity upon withdrawal of the reinforcers (Jentsch and Taylor, 1999). This hypothesis is corroborated by the finding that OBX animals (i) are unable to adapt to environmental changes and show hyperemotional responses (Van Riezen and Leonard, 1990), (ii) exhibit impulsive-like traits (Kamei et al., 2007), and (iii) display significant increases in both CB_1 receptor density and functionality in the prefrontal cortex (Rodriguez-Gaztelumendi et al., 2009).

EFFECT OF 5-HT_{1B} RECEPTOR ACUTE STIMULATION ON DRUG SELF-ADMINISTRATION

In an attempt to evaluate possible mechanisms underlying the observed differences in WIN self-administration between OBX and SHAM rats, we tested the effect of a serotonin 5-HT_{1B} receptor agonist on the cannabinoid agonist intake. This choice was based on the finding that cortical and hippocampal 5-HT_{1B}

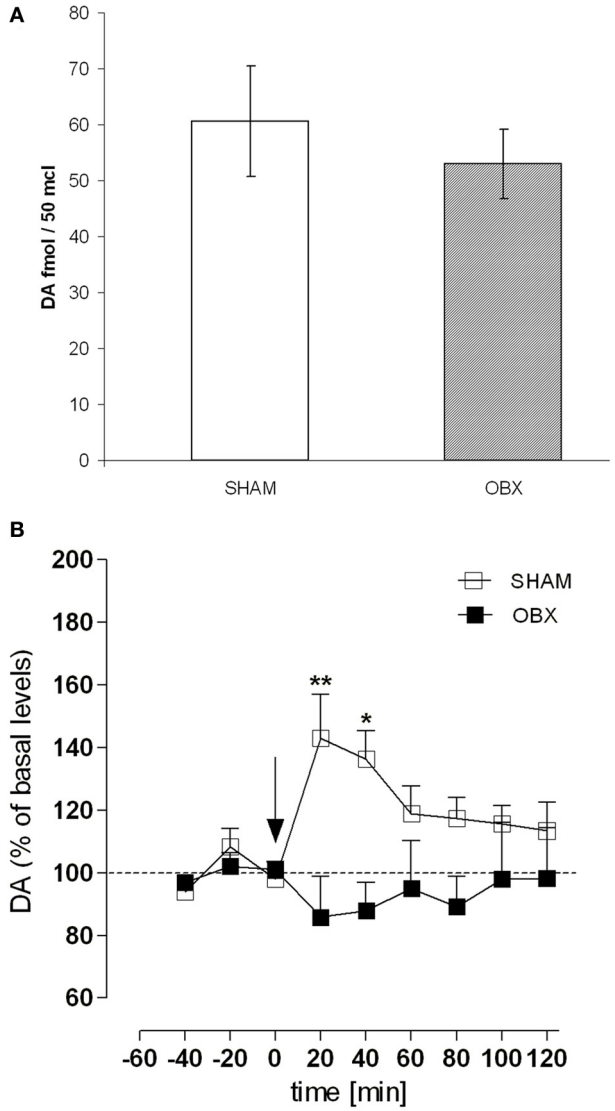

FIGURE 7 | OBX rats do not show enhanced dopamine level following acute WIN challenge. (A) Basal extracellular levels (fmol/μl of dialysate, mean ±s.e.m.) of DA in the NAc shell of SHAM ($n = 4$) and OBX ($n = 4$) Lister Hooded rats. No significant difference was found between the two groups (One-Way ANOVA, $p = 0.45$). **(B)** Effect of an intravenous administration of WIN 0.3 mg/kg on DA release in the nucleus accumbens shell of SHAM ($n = 4$) and OBX ($n = 4$) Lister Hooded rats. $^*p < 0.05$ and $^*p < 0.01$, Two-Way ANOVA followed by Bonferroni's *post-hoc* test.

receptors are critically involved in ethanol dependence and that their activation in limbic areas may attenuate amphetamine self-administration (Miszkiel et al., 2012). Moreover, a hypofunctionality of 5-HT_{1B} receptors was observed in depressed patients (Murrough et al., 2011), and a polymorphism at the 5-HT_{1B} receptor gene was found to be associated with alcoholism (Lappalainen et al., 1998). The 5-HT_{1B} receptor agonist CGS-12066B was shown to selectively decrease operant responses for ethanol (Czachowski, 2005). This compound is a full agonist with high selectivity to the 5-HT_{1B} receptor (Neale et al., 1987) and, to minor extend, to the 5-HT_{1A} receptors. The range of doses of the 5-HT_{1B} receptor agonist CGS-12066B used in this study was shown to be effective in acute in altering aggressive (De Boer and Koolhaas, 2005) and sexual behavior (Maciag et al., 2006) as well as some reward-related behaviors, such as DA-mediated reinforcement (Parsons et al., 1996). However, it did not affect cocaine self-administration in rats (Parsons et al., 1996), similarly to our findings on WIN self-administration.

Importantly, both the effects of serotonergic drugs (Horowitz et al., 1997; Uphouse et al., 2002; Miryala et al., 2013) and WIN self-administration behavior (Deiana et al., 2007) have been reported to greatly vary depending on rat strain and/or experimental parameters and procedures adopted. Moreover, the CGS compound was found to affect drug self-administration selectively, as it decreases alcohol (Grant et al., 1997; Maurel et al., 1999; Tomkins and O'Neill, 2000; Czachowski, 2005) and d-amphetamine (Fletcher and Korth, 1999), but not cocaine (Parsons et al., 1996) intake. Thus, we decided to test CGS-12066B on the self-administration of METH for which OBX rats are known to display higher responding level than SHAM rats (Kucerova et al., 2012), as for WIN. Yet, acute pre-treatment with the 5-HT_{1B} receptor agonist CGS-12066B did not significantly alter the intake of METH neither in OBX and SHAM Sprague Dawley rats nor in intact Wistar rats as recorded in our earlier unpublished pilot experiment (data available as Supplementary Figure 3).

Thus, the present findings indicate that WIN and METH, like cocaine (Parsons et al., 1996) self-administration, are not affected by acute stimulation of the 5-HT_{1B} receptor. Serotonin 5-HT_{1B} receptors are expressed throughout the brain of rodents. They are located in the axon terminals of both 5-HTergic and non-5-HTergic neurons where they act as inhibitory autoreceptors or heteroreceptors, respectively, (Barnes and Sharp, 1999; Moret and Briley, 2000; Pytliak et al., 2011; Cai et al., 2013), and have been difficult to study because of the diversity of their localization and the absence of highly selective receptor antagonists. Findings from the present study do not allow excluding the possibility that a chronic rather than an acute stimulation of 5-HT_{1B} receptors might alter WIN and METH self-administration. Thus, future studies will evaluate the effects of chronic stimulation of 5-HT_{1B} receptor by CGS-12066B, administered systemically or locally, on WIN and METH self-administrations.

REDUCED SENSITIVITY OF OBX RATS TO THE WIN STIMULATION EFFECT ON DOPAMINE RELEASE IN THE NUCLEUS ACCUMBENS SHELL

Enhanced drug self-administration can be linked to a dysfunction in the reward system, which is very likely to occur in OBX animals given the chemical and molecular changes that bulbectomy induces in several neurotransmitter systems, including the dopaminergic one (Masini et al., 2004; Sato et al., 2010), a major component of the brain reward system.

In intact animals, acute administration of WIN55,212-2 is known to increase extracellular DA levels in the NAc of freely moving rats (Gardner and Lowinson, 1991; Cheer et al., 2004; Polissidis et al., 2013). Moreover, DA content in the rat NAc shell was shown to increase appreciably in respect to basal values during WIN self-administration (Fadda et al., 2006). According to

this, the SHAM rats in this study significantly increased accumbal DA levels in response to an intravenous administration of a dose of WIN, 0.3 mg/kg, very similar to that daily self-administered by rats. Notably, the same dose of WIN enhances DA levels in the NAc shell of drug-naïve Sprague Dawley rats (Tanda et al., 1997). However, in our experiment WIN did not enhance DA levels in bulbectomized rats.

To explain this finding it could be of help to consider the multiple dysregulations that OBX induce in the endocannabinoid brain system. Cannabinoid CB_1 receptor density is significantly increased in OBX rats in the medial prefrontal cortex (mPFC) and amygdala while it does not change in the caudate-putamen, hippocampus, and dorsal raphe nucleus (Rodriguez-Gaztelumendi et al., 2009). CB_1 receptor function is significantly enhanced in the mPFC of OBX animals with respect to SHAM controls, but not in other brain regions with the exception of a slight, not significant, increase in the amygdala (Rodriguez-Gaztelumendi et al., 2009). Moreover, OBX does not affect DA D1- and D2-like receptors in the NAc (Sato et al., 2010). Thus, potential changes in the number or function of either the CB_1 or the dopaminergic receptors following OBX are unlikely to account for the absence of WIN-induced effect on DA release in the NAc of OBX rats. On the other hand, there is no clear evidence that DA release in the reward pathway depends on DA receptors. Instead, it is known that midbrain DA neurons produce endocannabinoids which retrogradely influence the glutamate and GABA projections and thus regulate the inhibitory and excitatory inputs to the reward circuit (Melis and Pistis, 2007). Glutamatergic and GABAergic systems are both dysregulated in the OBX model leading, among others, to a hyperactive response to novel environment (Song and Leonard, 2005). These dysregulations may contribute to the differential reactivity of the mesolimbic dopaminergic system in OBX rats. Thus, future studies will be performed to assess whether chronic treatment with this dose of WIN (0.3 mg/kg) as well as acute challenges with higher WIN doses may elicit an increase in DA levels in the NAc shell of OBX rats, and to evaluate CB_1 and DA receptor densities.

To summarize, this study demonstrated that OBX rats self-administer more cannabinoid agonist than SHAM control rats, and that WIN taking behavior is not significantly affected by acute stimulation of 5-HT_{1B} receptors. The close anatomical and functional association between the olfactory bulbs and the limbic system may help to understand why OBX rats differ from SHAM rats in drug self-administration behavior. The neurons of the olfactory bulbs are widely interconnected with other brain regions including cortical areas and limbic nuclei (Song and Leonard, 2005). The projections to these nuclei may be particularly relevant to changes in emotional and reward-related behavior in bulbectomized rats. Removal of the olfactory bulbs may alter, if not disrupt, the activity in brain circuits, particularly those influencing the dopaminergic system which is critical for processing drug taking and seeking behaviors. As OBX rats, contrary to SHAM, did not display a significant increase of DA levels in the NAc shell after an acute WIN challenge, we hypothesize that a depressive-like state may alter the rewarding effects of the drugs.

In conclusion, our findings showed that OBX markedly affects self-administration of cannabinoid CB_1 receptor agonist, possibly through a reduction of its rewarding effects to which animals compensate by increasing WIN intake. A decreased DA neurotransmission in the NAc shell might contribute to this compensatory behavior. Thus, a follow-up study will evaluate (i) a dose-response effect of acute and chronic WIN and METH administration on NAc shell DA levels in OBX and SHAM rats, and (ii) DA levels after immediate (24 h), short-term (1 and 2 weeks), and long-term (4-weeks) cessation from chronic drug exposure. Future studies will also evaluate whether OBX and SHAM rats also differ in the reinstatement of cannabinoid-seeking behavior trigger by drug, cue, or stress primings.

AUTHOR CONTRIBUTIONS

Petra Amchova was responsible for the induction of OBX model in the Czech Republic and its transfer to the Italian laboratory; she collected the data in the Czech Republic and processed them for analysis, and wrote a substantial part of the introduction and methods sections of the manuscript. Jana Kucerova developed the original idea and organized the experimental work in the Czech Republic, and wrote a substantial part of the introduction, methods, results and discussion sections of the manuscript. Valentina Giugliano was responsible for the behavioral testing and performance of the OBX surgery in Italy and collection of data, and she cross-checked the materials and methods section of the manuscript. Zuzana Babinska was responsible for the behavioral testing in the Czech Republic and contributed to microdialysis experiments in Italy, and she cross-checked the materials and methods section of the manuscript. Mary Tresa Zanda contributed substantially to behavioral testing and OBX surgery in Italy and collection of data, and she cross-checked the materials and methods section of the manuscript. Maria Scherma was responsible for the microdialysis experiment in Italy and for the related statistical data analysis and graphical presentation of data. Ladislav Dusek performed the statistical data analysis and contributed substantially to the results section and graphical presentation of the results. Paola Fadda organized and supervised the microdialysis experiment in Italy and was involved in the analysis and discussion of the data, and contributed to the final version of the manuscript. Vincenzo Micale established the collaboration of the two departments, was involved in discussion of the data and contributed to the final version of the manuscript. Alexandra Sulcova was involved in the design of the study and discussion of the data, and contributed to the final version of the manuscript. Walter Fratta was involved in the analysis and discussion of the data, and contributed to the final version of the manuscript. Liana Fattore organized and supervised the experimental work in Italy and enabled the transfer of the OBX technique; she wrote a substantial part of the introduction, methods, results and discussion sections of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/journal/10.3389/fphar.2014.00044/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL FOR PAPER:

Enhanced self-administration of the CB1 receptor agonist WIN55,212-2 in olfactory bulbectomized rats: evaluation of possible serotonergic and dopaminergic underlying mechanisms

Authors: Petra Amchova, Jana Kucerova, Valentina Giugliano, Zuzana Babinska, Mary Tresa Zanda, Maria Scherma, Ladislav Dusek, Paola Fadda, Vincenzo Micale, Alexandra Sulcova, Walter Fratta, Liana Fattore

Frontiers in Pharmacology

Category: Original research

Research Topic: Addictive drugs targeting GPCRs: new cross-talk mechanisms

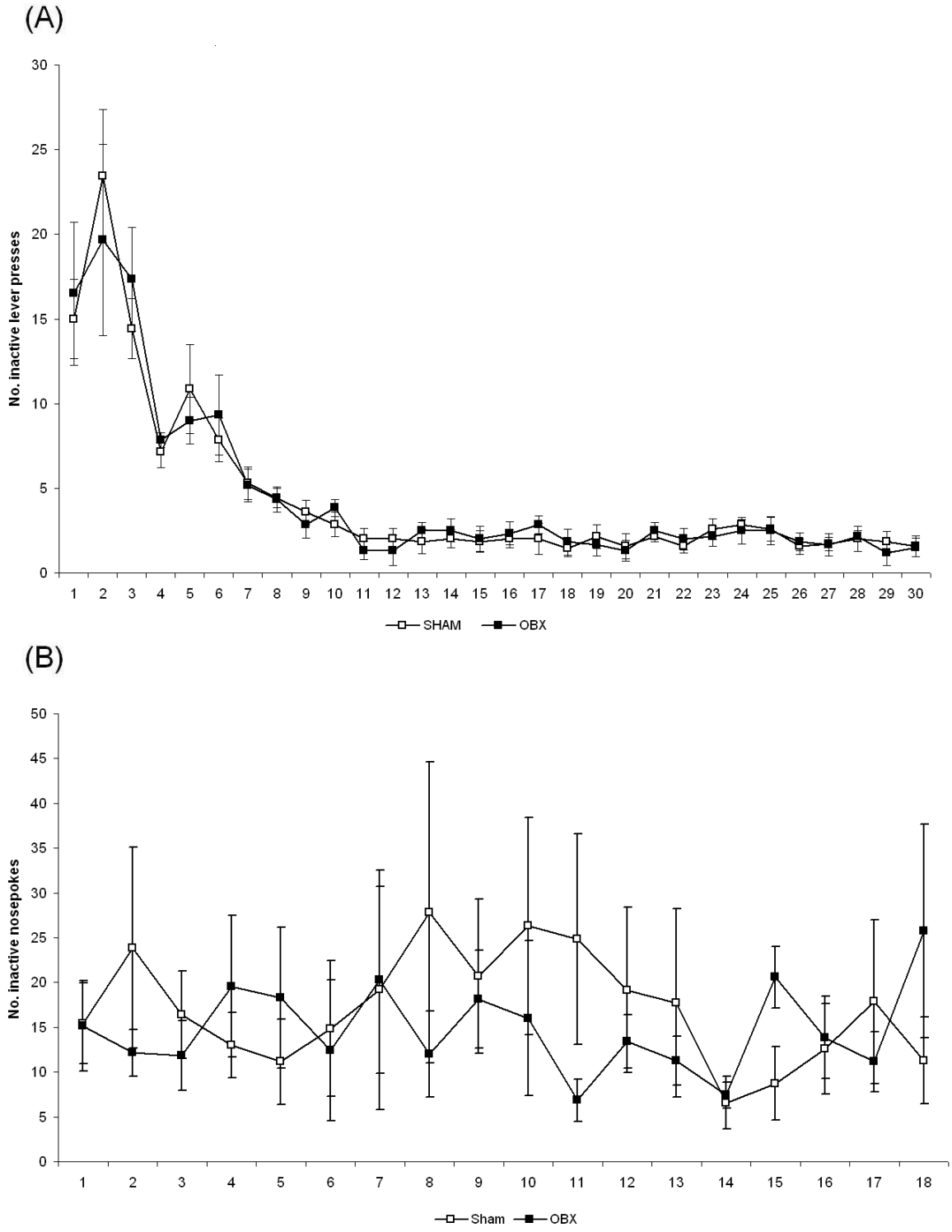
SUPPLEMENTARY FIGURE LEGENDS:

Supplementary Figure 1. OBX and SHAM rats do not differ in inactive *operandum* responding. A) Mean number of inactive lever presses over the acquisition and maintenance period (30 days) in SHAM (n=7) and OBX (n=7) Lister Hooded rats during WIN self-administration. Data are shown as means (±SEM). Not significant differences, repeated measures ANOVA, $\alpha = 0.768$. **B)** Mean number of inactive nosepokes over the acquisition and maintenance period (18 days) in SHAM (n=7) and OBX (n=7) Sprague Dawley rats during METH self-administration. Data are shown as means (±SEM). The repeated measures ANOVA did not detect a significant effect of drug treatment.

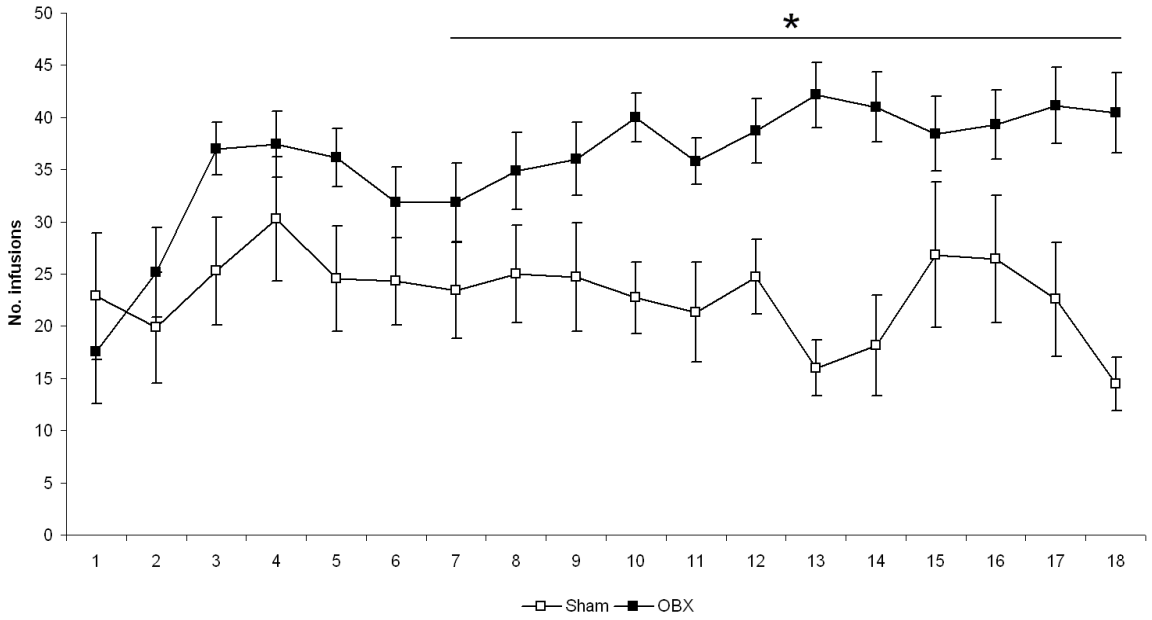
Supplementary Figure 2. OBX rats display enhanced METH self-administration behaviour. Mean number of infusions in SHAM (n=6) and OBX (n=7) Sprague Dawley rats during METH self-administration. Each infusion contains 0.08 mg/kg METH. The mean number of infusions during the whole self-administration training was 23 in SHAM and 35.8 in OBX (approx. 1.8 and 3 mg/kg METH respectively). Data are shown as daily means (±SEM) and start to differ significantly from day 7 onwards, * $\alpha < 0.05$, repeated measures ANOVA.

Supplementary Figure 3. Acute pre-treatment with CGS does not affect METH self-administration in intact Wistar rats. Effect of acute pre-treatment with CGS-12066B on methamphetamine self-administration in intact Wistar rats (n=5). Data are expressed as percent of active lever pressing compared to six-day baseline (assumed as 100%). The repeated measures ANOVA did not detect a significant effect of drug treatment.

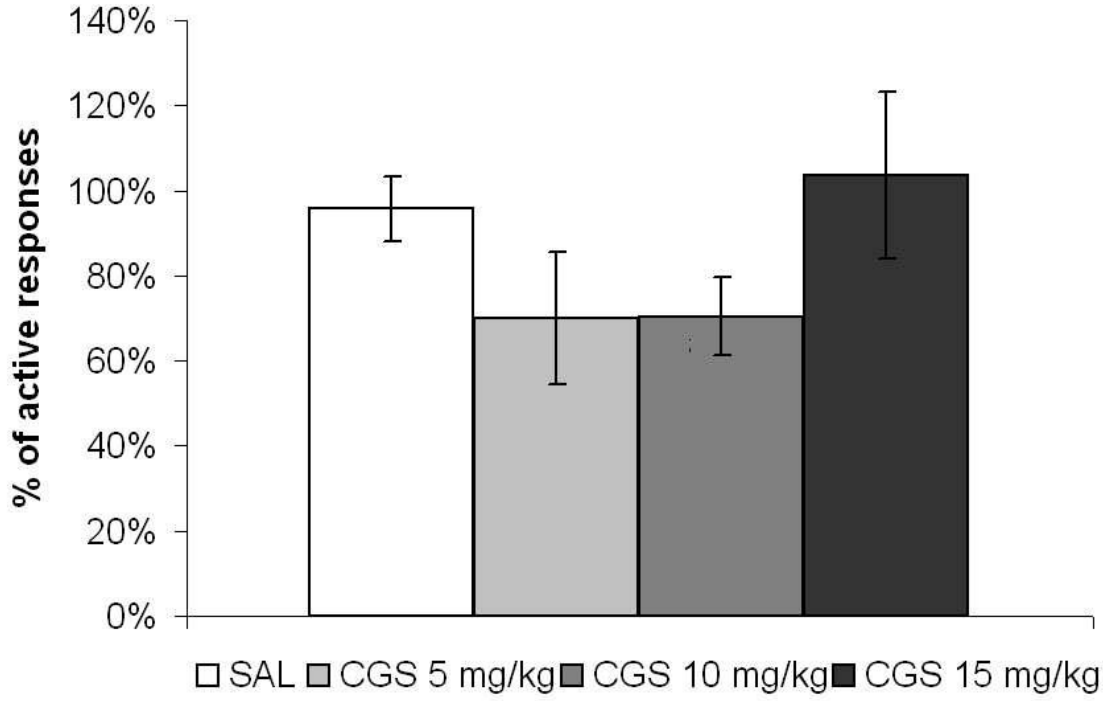
Supplementary Figure 1: OBX and SHAM rats do not differ in inactive operandum responding.



Supplementary Figure 2: OBX rats display enhanced METH self-administration behaviour.



Supplementary Figure 3: Acute pre-treatment with CGS does not affect METH self-administration in intact Wistar rats.



2.4.5. Differential characteristics of ketamine self-administration in the olfactory bulbectomy model of depression in rats

Ketamine is studied for its rapid antidepressant effect with promising results in both preclinical experiments (Scheuing et al., 2015) and clinical studies have yield promising results demonstrating the antidepressant potential of ketamine (Newport et al., 2015, Xu et al., 2015). Therefore, this study assessed the characteristics of operant ketamine self-administration and relapse-like behaviour in the OBX model of depression following a previously validated approaches (De Luca and Badiani, 2011, Caffino et al., 2016)coll. We hypothesized increased ketamine taking behaviour in the OBX model and increased relapse-like behaviour in the self-administration and reinstatement paradigms as in analogous studies on methamphetamine (Babinska et al., 2016, Kucerova et al., 2012).

This study supports the validity of the animal model of dual disorder of depression and drug abuse. Chronic ketamine intake reversed the depressive-like phenotype. In accordance with previous studies, OBX animals showed increased operant intake of the drug. However, ketamine-seeking behaviour in the model of relapse was lower in the OBX animals compared to SHAM animals. This finding contradicts previous studies reporting increased methamphetamine (Babinska et al., 2016) and cocaine (Frankowska et al., 2014) seeking behaviour in the reinstatement paradigm. This indicates substantially different underlying neuroadaptation changes between chronic ketamine vs. psychostimulant exposure.

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Twenty-five male Wistar rats were divided randomly into two groups; in one group the bilateral olfactory bulbectomy was performed while the other group was sham operated. Intravenous self-administration procedure was conducted under a fixed ratio 1 schedule of reinforcement, ketamine was available at 0.5 mg/kg/infusion. After stable drug intake was established, rats underwent a 14-day period of forced abstinence. A drug-free reinstatement session was then conducted in operant boxes. Forced swim test took place before the self-administration protocol and on the first day of abstinence.
Consistent with previous studies with other substances, OBX animals showed increased operant IV ketamine self-administration. In contrast ketamine-seeking behaviour in the OBX group was no different from SHAM animals during the reinstatement session; whereas, previous studies on other psychostimulants like methamphetamine and cocaine reported increases. Our findings suggests substantially different underlying neuroadaptations between chronic ketamine vs. psychostimulant exposure.

Differential characteristics of ketamine self-administration in the olfactory bulbectomy model of depression in rats

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Short Title: Ketamine abuse in the OBX model

Abstract

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Consistent with previous studies with other substances, OBX animals showed increased operant IV ketamine self-administration. In contrast ketamine-seeking behaviour in the OBX group was no different from SHAM animals during the reinstatement session; whereas, previous studies on other psychostimulants like methamphetamine and cocaine reported increases. Our findings suggests substantially different underlying neuroadaptations between chronic ketamine vs. psychostimulant exposure.

Keywords: ketamine; self-administration; reinstatement; olfactory bulbectomy; depression; Wistar rats

1. Introduction

Ketamine at sub-anaesthetic doses is now extensively studied for its antidepressant potential in both preclinical experiments (Scheuing et al., 2015) and clinical studies with promising results (Xu et al., 2015). However, potential clinical use of ketamine raises questions of its abuse potential. Intake of the drug leads to psychological addiction but probably not physical dependence (Bokor and Anderson, 2014). Notably, there is clear evidence suggesting a higher ketamine abuse rate in patients with clinical depressive symptoms (Fan et al., 2016) in accordance with the self-medication hypothesis (Hall and Queener, 2007). Notably, increased availability of ketamine on the market may also lead to increase in its abuse rate (Arunogiri et al., 2016). The fact that incidence of fatal poisoning from ketamine dramatically increased in the recent years (Hamani and Nobrega, 2012) demonstrates the need of prudence when using ketamine clinically.

In preclinical setting ketamine was shown to possess strong reinforcing potential in both conditioned place preference and self-administration paradigms (Mutti et al., 2016; van der Kam et al., 2009). A self-administration study in rats described a clear relationship between environmental conditions and development of ketamine addiction (De Luca and Badiani, 2011). This relation has also been confirmed in humans (De Luca et al., 2012). A recent report aimed to distinguish the effect of one dose and chronic ketamine self-administration and reported differences in brain derived neurotrophic factor (BDNF) levels, as a key variable distinguishing the antidepressant and reinforcing properties of the drug. Acute ketamine dose increased BDNF in hippocampus while chronic operant intake has an opposite effect (Caffino et al., 2016). This finding is in accordance with a clinical study showing increased serum BDNF levels in ketamine abusers (Ricci et al., 2011). Ketamine was also shown to dose-dependently increase dopamine levels in the nucleus

accumbens, which is a shared mechanism of all abused substances (Masuzawa et al., 2003).

Currently, there is no study evaluating operant ketamine intake in animals with depressive-like phenotype. Such study would benefit future development of new antidepressants with glutamatergic mechanism of actions. For this study, we used the bilateral olfactory bulbectomy model of depression. This model closely mimics neurochemical, neuroanatomical, behavioural and endocrine changes in patients with major depression (Song and Leonard, 2005). Our team has developed a rat model of depression and addiction dual disorder where olfactory bulbectomized animals showed higher self-administration of methamphetamine (Kucerova et al., 2012), increased relapse-like behaviour (Babinska et al., 2016) and differential dopamine and serotonin release in nucleus accumbens shell after a methamphetamine challenge (Ruda-Kucerova et al., 2015b). Similar findings were also reported earlier in self-administration of amphetamine (Holmes et al., 2002) and of CB1 receptor agonist WIN55,212-2 (Amchova et al., 2014). The overall aim of this study is to assess the characteristics of operant ketamine self-administration and relapse-like behaviour in the OBX model of depression. We hypothesized increased ketamine taking behaviour in the OBX model and increased relapse-like behaviour in the self-administration and reinstatement paradigms as in analogous studies on methamphetamine (Babinska et al., 2016; Kucerova et al., 2012).

2. Material and methods

2.1. Animals

Twenty five male albino Wistar rats (8 weeks old, with weight range of 250 to 300 g at the beginning of the experiment) were purchased from the Masaryk University breeding facility (Brno, Czech Republic). The rats were housed individually in standard rodent plastic cages. Environmental conditions during the whole study were constant: relative humidity 50-60 %, room temperature 23 ºC ± 1 ºC, inverted 12-hour light-dark cycle (6 a.m. to 6 p.m. darkness). Food and water were available *ad libitum*. There were two experimental groups: SHAM = sham operated rats (n=10 at the beginning of the study) and OBX = olfactory bulbectomized rats (n=15 at the beginning of the study). The final number of animals was n=9 in the sham operated group and n=6 in the OBX group. The reasons for exclusion of the animals were as follows: death after surgery (n=3), behavioural deficits (n=1), death during the study (n=2), incomplete OBX (n=4). All procedures were performed in accordance with EU Directive no. 2010/63/EU and approved by the Animal Care Committee of the Faculty of Medicine, Masaryk University, Czech Republic and Czech Governmental Animal Care Committee, in compliance with Czech Animal Protection Act No. 246/1992.

2.2. Drugs and treatments

Ketamine (KET) solution was prepared by diluting a ready-made preparation Calypsol® inj. sol. (50 mg in 1 ml) with saline to obtain desired concentration. For IV self-administration a solution of 0.5 mg/kg per infusion was used. The solutions were prepared for specific animals depending on their body weights rounded to the closest category of 250 g, 300 g, 350 g, etc. This paradigm is adapted from Emmett-Oglesby MW (Fort Worth, USA) [31] and routinely used in our laboratory [26, 30, 32, 33]. For the locomotor

activity testing concentrations of the solutions ranged from 5 to 15 mg/kg and were prepared in the same manner.

2.3. Olfactory bulbectomy surgery

At the beginning of the study the rats were randomly divided into two groups and the bilateral ablation of the olfactory bulbs was performed in accordance with the standard method as described earlier [27, 30]. In brief, animals were anaesthetized with ketamine 50 mg/kg and xylazine 8 mg/kg given intraperitoneally. The top of the skull was shaved, swabbed with an antiseptic solution, after which a midline frontal incision was made through the skin on the skull. After exposure of the skull, 2 burr holes were drilled at the points 7 mm anterior to the bregma and 2 mm lateral to bregma suture. Both olfactory bulbs were aspirated while paying particular attention not to damage the frontal cortex. Prevention of blood loss was achieved by filling the dead space with a haemostatic sponge. The skin above the lesion was closed with suture and the antibacterial neomycin and bacitracin powder was applied. Sham operated rats underwent the identical anaesthetic and drilling procedures as OBX animals, but their bulbs were left intact. A period of 14 days was allowed for the recovery from the surgical procedure and the development of the depressive-like phenotype. During this period, animals were handled daily for few minutes to eliminate aggression, which could otherwise arise [25, 34]. At the end of the experiment, rats were euthanized by an anaesthetic overdose and the brains were dissected for confirmation of the successful removal of the olfactory bulbs. Animals with incomplete removal of the olfactory bulbs were eliminated from the analysis (n=4).

2.4. Food self-administration protocol

Food self-administration was employed to develop self-administration operant behaviour in the animals. The training was conducted as already described [35] in 10 operant boxes

(30x25x30 cm, Coulbourn Instruments, USA) using nose-poke operandi under a fixed ratio 1 (FR-1) schedule of reinforcement, i.e. animal had to make 1 nose-poke to the active operandum to obtain a single palatable pellet (BioServ, sweet dustless rodent pellets, F0021-Purified Casein Based Formula - 45mg). Each cage was provided with two nose-poke holes allocated on one side and programmed by software Graphic State Notation 3.03 (Coulbourn Instruments, USA). The cage was illuminated by a house light during the whole session. Self-administration sessions lasted 30 minutes during the dark period of the inverted light-dark cycle. The length of the training was 5 days. All animals ate the vast majority of the gained pellets.

2.5. Intravenous drug self-administration surgery

The IV self-administration catheter was implanted after completion of the food self-administration training following standard procedure described earlier [35, 36]. In brief, animals were deeply anesthetized with IP injections of 50 mg/kg ketamine plus 8 mg/kg xylazine. Catheter was inserted 3.7 cm into the right external jugular vein to the right atrium and securely sutured. A subcutaneous tunnel was made and the catheter exited the skin in the midscapular area. Since the implantation, the catheters were flushed daily by enrofloxacine (17 mg/kg) solution followed by 0.1 ml of a heparinized (1%) sterile saline solution to prevent infection and occlusion. When a catheter was found to be blocked or damaged, the animal was excluded from the analysis.

2.6. Intravenous self-administration protocol

Ketamine self-administration was conducted as previously described [18, 37] in 10 standard experimental boxes (30x25x30 cm, Coulbourn Instruments, USA) using nose-poking as operandum. Each cage was provided with two nose-poke holes allocated on one side and programmed by software Graphic State Notation 3.03 (Coulbourn Instruments,

USA). Nose-pokes in the active hole led to the activation of the infusion pump and administration of a single infusion followed by a 10 sec timeout, while nose-poke stimulation was recorded but not rewarded, i.e. fixed ratio 1 (FR-1) schedule of reinforcement. Infusions were delivered by a syringe within an automatic infusion pump located outside the chamber. The infusion pumps were connected to liquid swivels which were fixed to the catheters via polyethylene tubing withinside a metal spring tether. The cage was illuminated by a house light during the session. The light was flashing when infusion was being administered (5 sec) and off during the time-out period. Self-administration sessions lasted 120 minutes and took place 7 days/week between 8 a.m. and 3 p.m. during the dark period of the cycle.

After 21 days of daily ketamine intake at FR-1 the maintenance phase was terminated and rats were kept in their home cages for the 14 days of the forced abstinence period. On the day of reinstatement, rats were placed into self-administration chambers for the last session taking 120 minutes and the numbers of responses on the active drug paired nose-poke and the inactive nose-poke were recorded but the drug was not delivered. Responses on the active nose-poke are considered to reflect reinstatement of drug seeking behaviour, while responses on inactive nose-poke are interpreted to reflect general locomotor and exploratory activity.

2.7. Forced swim test (FST)

A modified FST [38] was used to measure immobility of the rats, as described previously [5, 39]. Briefly, the rats were individually placed into a plexi-glass cylinder filled with 30 cm of water (24±1 °C). The sessions were video-taped for later scoring and the water was changed after every animal. A time-sampling scoring technique was used, whereby the predominant behaviour, i.e. immobility, swimming or climbing, in each 5-s period of the 5

minutes test was recorded. OBX rats acquire the depressive-like phenotype by surgery, therefore they should exhibit spontaneous immobility in the forced swim test. Furthermore, the aim of the test was to assess spontaneous behaviour, i.e. not a drug effect. Hence, there is no need to have a pre-test exposure to forced swimming the day before to induce helplessness [5, 40]. The test was performed twice: in order to assess the basal depressive-like phenotype the first test took place before the IVSA surgery, the second test aimed to assess the potential change in the depressive-like profile after ketamine self-administration – first day of forced abstinence period. There were in total four weeks between the tests.

2.8. Statistical Data analysis

Primary data were summarized using arithmetic mean and standard error of the mean estimate (SEM). Food self-administration data were analysed by repeated measures (RM) ANOVA model (factor: group, repeated variable: day) and Bonferroni post-test for the group*time-point interaction. IV self-administration behaviour showed different dynamics every week. Therefore, the data were summarized as weekly means for each animal and then analysed by repeated measures ANOVA model (factor: group, repeated variable: day) and Bonferroni post-test. Data from the reinstatement session and forced swim test were analysed by t-test or Mann-Whitney U test (MWU test) depending on the result of Kolmogorov-Smirnov test of normality. The analyses were calculated using Statistica 12 (StatSoft, USA). A value $p<0.05$ was recognized as boundary of statistical significance in all applied tests.

3. Results

3.1. Food self-administration in SHAM and OBX rats

The acquisition of food taking behaviour (sweet pellets) was used in order to train the operant behaviour. **Figure 1** shows daily mean numbers of active nose-poking, inactive nose-poking, number of pellets eaten and the mean day of reaching the acquisition criterion. There was no difference in the active nose-poking (RM ANOVA, $F=1.5888$, $p=0.18806$, n.s.) and pellet intake between the groups (RM ANOVA, $F=1.3493$, $p=0.2615$, n.s.). However, inactive nose-poking reveals highly significantly increased activity in the OBX group on the first two days (RM ANOVA, $F=16.218$, $p=0.0000$, Bonferroni post-test, $p<0.001$). All rats acquired operant behaviour during 5 days of training; however, the OBX group met the acquisition criteria (day when the animals started to prefer the active nose-poke more than 75 %) significantly later: SHAM animals 1.2 day and OBX rats 2.2 days, T-test, $p=0.006$.

3.2. Ketamine IV self-administration in SHAM and OBX rats

Ketamine IV self-administration data are presented as the daily mean values of number of active nose-pokes, inactive nose-pokes, infusions and dose of ketamine (mg/kg). Our system uses nose-poke operandi, therefore, the number of nose-pokes and infusions does not correspond (as in retractable lever systems). Furthermore, ketamine solution used for self-administration was prepared for body weight categories with 50 g resolution. To assess even this potential source of inaccuracy, we convert the number of infusions to drug dose using the exact body weight. As visible on the **Figure 2**, the number of active and inactive nose-pokes did not differ throughout the whole 3 weeks of the protocol (RM ANOVA, effect of group: $F=1.361$, $p=0.264$ and $F=0.015$, $p=0.904$ respectively). However, both number of infusions and ketamine dose showed development towards

higher drug intake in the OBX group as compared to SHAM controls. Infusions: RM ANOVA, effect of group: F=11.188, p=0.005, effect of group*time-point interaction: F=3.940, p=0.032. Bonferroni post-test for the group*time-point interaction: week 1, n.s.; week 2, n.s.; week 3, p=0.009. Ketamine dose: RM ANOVA, effect of group: F=10.692, p=0.006, effect of group*time-point interaction: F=4.068, p=0.029. Bonferroni post-test for the group*time-point interaction: week 1, n.s.; week 2, p=0.822; week 3, p=0.0104. Furthermore, the characteristics of the ketamine intake were different between the groups. **Figure 3** shows individual data on self-administered dose over 3 weeks of the study to provide better overview of the findings.

3.3. Reinstatement of ketamine seeking behaviour

In the reinstatement session only, active and inactive nose-poking can be assessed (no drug delivery). **Figure 4** compares the mean number of both types of nose-pokes in this session together with mean nose-poking in the last week of the maintenance phase in SHAM and OBX rats. There was no difference between the performance in the reinstatement session and last maintenance week in SHAM rats (MWU test, n.s.) but OBX rats exhibited lower drug seeking behaviour in the reinstatement session that in the maintenance phase (T-test, p=0.016). However, when the active responding in the reinstatement session was converted to a percent of mean nose-poking in the week 3 (reinstatement / mean week 3 x 100) a robust difference was shown: SHAM did mean 252 % of nose-poking in the week 3 while OBX animals only 60 %. This difference does not reach full statistical significance with 5% threshold (T-test, p=0.059) but the result may deserve some attention despite of comparing reinforced and non-reinforced responding. In order to understand the finding better we prepared the **Table 1** showing individual data. Lastly, there was no difference in

the numbers of inactive nose-pokes neither within groups or in SHAM vs. OBX comparison.

3.4. Forced swim test

The first test assessing the basal condition of the OBX and SHAM rats revealed significant decrease of swimming behaviour and increase of immobility in the OBX group (t test, p=0.0003, p=0.0386 respectively). Chronic ketamine self-administration lead to important changes in the behaviour of the OBX animals, in the second FST OBX rats showed increased climbing and decreased immobility scores as compared to SHAM controls (t test, p=0.0155, p=0.050 respectively) suggesting alleviation of the depressive-like condition (**Figure 5**).

4. Discussion

This study observed different characteristics of ketamine intake behaviour in the IV self-administration model between OBX rats and SHAM rats. These differences cannot be explained by changes in natural reward seeking behaviour as active nose-poking during training was similar between the groups. The higher inactive nose-poking in the OBX group on the first two days of the training which led to a delay in meeting acquisition criteria (i.e. 75% preference of active operandum) could be explained by hyperactivity of the OBX group. Typical novelty induced hyperactivity is commonly found in OBX rats during the initial phases of behavioural assessments, typically in open-field test (Song and Leonard, 2005).

In accordance with our hypothesis, OBX rats exhibited a higher drug intake during the maintenance phase of IV ketamine self-administration. Similar behaviour was reported previously in different drugs of abuse such as amphetamine (Holmes et al., 2002), methamphetamine (Kucerova et al., 2012) and CB1 agonist WIN55,212-2 (Amchova et al., 2014) with a similar time-course, i.e. higher drug intake in the OBX rats is developed approximately ten days after initiation of IVSA. This behaviour mimics the enhanced rate of drug addiction in depressive humans, which is believed to be an attempt of self-medication (Hall and Queener, 2007; Khantzian, 1985).

In this study the depressive-like behaviour was demonstrated in the first (basal) forced-swim test (FST) where OBX animals showed increased immobility scores. Consequently, this phenotype was reversed after chronic ketamine exposure, i.e. decreased immobility scores and increased climbing behaviour similarly as shown earlier (Fraga et al., 2014). This is in accordance with clinical case reports of patients abusing ketamine to relieve their depression (Bonnet, 2015; Liu et al., 2015). However, it seems that in preclinical

experiments even a single dose leads to protracted antidepressant-like effect and longer exposure to the drug has a similar outcome (Browne and Lucki, 2013). So far, there is just one report on acute ketamine in the OBX model showing positive results (Holubova et al., 2016). Therefore, we cannot conclude that the antidepressant-like effect detected in this study was exclusively owing to chronic ketamine exposure.

Interestingly, in this study OBX and SHAM rats exhibited quite different intake dynamics. While approximately one half of the SHAM animals kept low intake, the other half exhibited an escalation of ketamine intake in the second week of maintenance phase. Majority of the OBX rats escalated their drug intake during first week and kept it for the rest of the study. This may be a result of different neurochemical adaptations in the OBX model to chronic ketamine exposure, which yet to be fully explained. The most probable mechanism underlying ketamine effectiveness in reversing depressive (and depressive-like) symptoms would be its ability to modulate glutamatergic signalling which consequently influences synaptic plasticity (Vasquez et al., 2014).

Ketamine acts in the brain mainly by inhibition of NMDA (N-methyl-D-aspartate) and activation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors while ketamine also stimulates glutamate synthesis (Browne and Lucki, 2013; Tizabi et al., 2012). The overall result of these actions is an increase in glutamate signalling. Aside from its actions on the glutamatergic system, ketamine acts to some extent also as a muscarinic receptor antagonist, acetylcholinesterase inhibitor, dopamine D2 receptor partial agonist and serotonin 5-HT2A receptor agonist, monoaminergic transporters' inhibitor and opioid receptor agonist (Browne and Lucki, 2013; Kapur and Seeman, 2002).

Based on clinical findings, glutamatergic dysregulation is considered to be one of the core characteristics of major depression as suggested by the glutamate hypothesis of depression

(Sanacora et al., 2012). Numerous animal models of depression (surgical, behavioural and genetic) exhibit impaired glutamate signalling in which glutamate enhancing treatments rescued depressive phenotypes (Tokita et al., 2012). In the OBX model, the glutamatergic system is generally considered to be hypoactive. Studies have shown decreased extracellular glutamate levels in the olfactory cortex along with increased glutamate decarboxylase activity. Lower NMDA receptor density was also found in several brain regions, e.g. prefrontal and piriform cortexes, lateral amygdaloid nucleus and thalamic nucleus (Harkin et al., 2003; Song and Leonard, 2005). However, the exact nature of glutamatergic dysregulation seems to be much more complex as some studies showed increased NMDA density in the prefrontal cortex (Webster et al., 2000) and amygdala (Nakanishi et al., 1990). Moreover, our previous results showed increased extracellular glutamate levels in the nucleus accumbens shell in OBX rats (Ruda-Kucerova et al., 2015b). Nevertheless, novelty induced cortical glutamate release in the OBX model is believed that to be responsible for some of the typical depressive phenotypes, especially irritability, hyperactivity in unknown environment and maladaptation to stress (Ho et al., 2000). This is in accordance with our previous results showing increased locomotor activity corresponding to enhanced glutamate levels measured using in vivo microdialysis in OBX rats (Ruda-Kucerova et al., 2015b).

At the same time, increased ketamine self-administration seen here in OBX rats may also be explained by the effects of ketamine on the dopaminergic system. Chronic administration of ketamine increases dopamine levels in many brain regions together with decreased expression of D2 receptors (Li et al., 2015; Tan et al., 2012). Ketamine is also known to act as a D2 partial agonist (Kapur and Seeman, 2002). These combined effects lead to a moderate enhancement of dopaminergic tone resulting in a positive reinforcing

effect. Furthermore, the OBX model is potentially more susceptible to dopaminergic drugs. Our previous study reported that OBX rats have decreased basal dopamine levels in the nucleus accumbens shell (Ruda-Kucerova et al., 2015b) which may explain the higher operant self-administration of addictive substances such as synthetic cannabimimetic drug (Amchova et al., 2014) and methamphetamine (Kucerova et al., 2012).

The glutamate hypothesis of depression implies that impaired production of BDNF following glutamatergic dysregulation leads to an abnormal neuroplasticity (Sanacora et al., 2012). This notion has been supported by clinical studies reporting reduced serum BDNF levels in patients with major depression, which can be normalized upon antidepressant treatments (Teche et al., 2013). Preclinical research demonstrated that ketamine increases BDNF levels in several brain regions (Duman et al., 2012). Clinical trials have also shown that plasma BDNF levels increased upon both acute (Duncan et al., 2013) and sub-chronic ketamine treatments (Haile et al., 2014) in patients with depression. However, different ketamine abusers were shown to have increased (Ricci et al., 2011) as well as decreased (Ke et al., 2014) BDNF levels. Preclinical evidence in OBX animals shows robust decrease of BDNF levels in brain tissue (Rinwa et al., 2013), which is in accordance with clinical findings. Rapid increase of BDNF is hypothesized as one of the critical components of the antidepressant mechanism of ketamine (Haile et al., 2014). Therefore, this mechanism could explain our results showing antidepressant effect upon chronic ketamine self-administration. However, the exact mechanisms of ketamine induced NMDA receptor blockade-mediated BDNF changes are yet to be elucidated.

During the drug-free reinstatement phase, there was no difference in the ketamine-seeking behaviour between OBX and SHAM rats in terms of active and inactive nose-poking. This confirms no effect of memory in the test, the preference of the active operandum remained

equal in both groups. OBX rats were less active during the reinstatement session compared to their behaviour in the last week of maintenance phase while there was no such difference in the SHAM rats. As previously suggested [35] we performed conversion of the active nose-poking in the reinstatement session to a percent of mean nose-poking in the week 3 and this attenuation of active operandum stimulation in the OBX group was more evident. However, usually only operandum performance is evaluated, therefore we summarize that there is apparently no difference between the groups. This unexpected result is contradictory to previous findings acquired in a study of methamphetamine relapse. In the same model, OBX rats were shown to increase methamphetamine-seeking behaviour in the reinstatement session (Babinska et al., 2016).

In conclusion, this study supports the validity of the self-medication hypothesis explaining the dual disorder of depression and drug abuse as shown by a reversal of the depressive-like phenotype upon ketamine self-administration. In accordance with previous studies, OBX animals show increased operant intake of the drug. However, ketamine-seeking behaviour in the model of relapse was lower in the OBX animals compared to SHAM animals. This finding contradicts previous studies reporting increased methamphetamine (Babinska et al., 2016) and cocaine (Frankowska et al., 2014) seeking behaviour in the reinstatement paradigm. More importantly, this indicates substantially different underlying neuroadaptation changes between chronic ketamine vs. psychostimulant exposure.

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6. Statement of interest

None to declare.

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Figures:

Figure 1: operant self-administration of sweet pellets

The line graphs present the mean ±SEM of daily numbers of active nose-pokes, self-administered pellets and inactive nose-pokes in SHAM and OBX animals. RM ANOVA detected a significant effect of group only in number of inactive nose-pokes and Bonferroni post-test identified significant differences between the groups on the first two days ($p<0.001$***). This indicates the hyperactive response to novel environment typical for the OBX model. Furthermore, T-test revealed significant difference in the mean day when animals reached 75 % preference of the active operandum ($p=0.006$**).

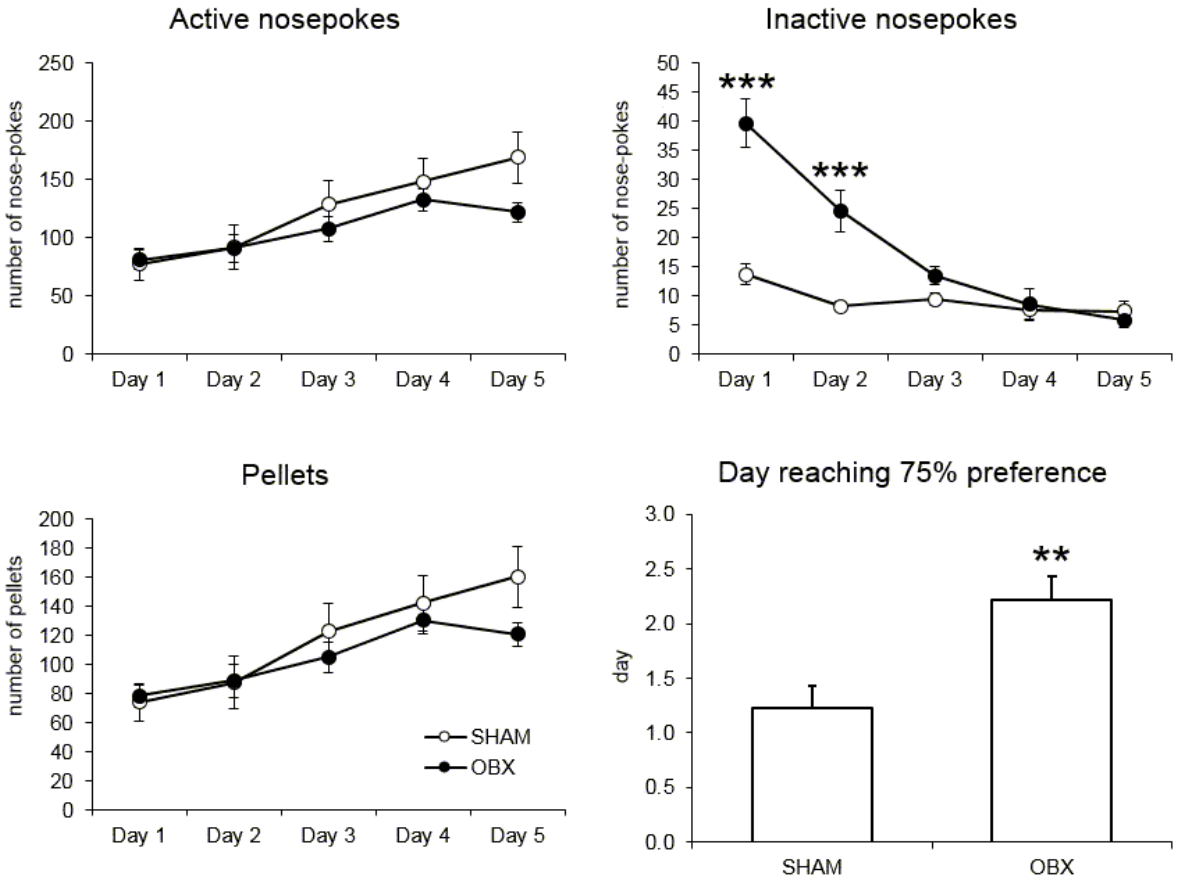

Figure 2: maintenance of ketamine self-administration

All data are presented as the daily mean ±SEM values of number of active nose-pokes, inactive nose-pokes, infusions and dose of ketamine (mg/kg). The number of active and inactive nose-pokes did not differ throughout the whole 3 weeks of the protocol (RM ANOVA, effect of group: n.s.). Number of infusion was found to be significantly increased in the OBX group: RM ANOVA, Bonferroni post-test for the group*time-point interaction: week 1, n.s.; week 2, n.s.; week 3, **p=0.009. Similarly, self-administered ketamine dose was found to be higher: RM ANOVA, Bonferroni post-test for the group*time-point interaction: week 1, n.s.; week 2, p=0.822; week 3, **p=0.0104.

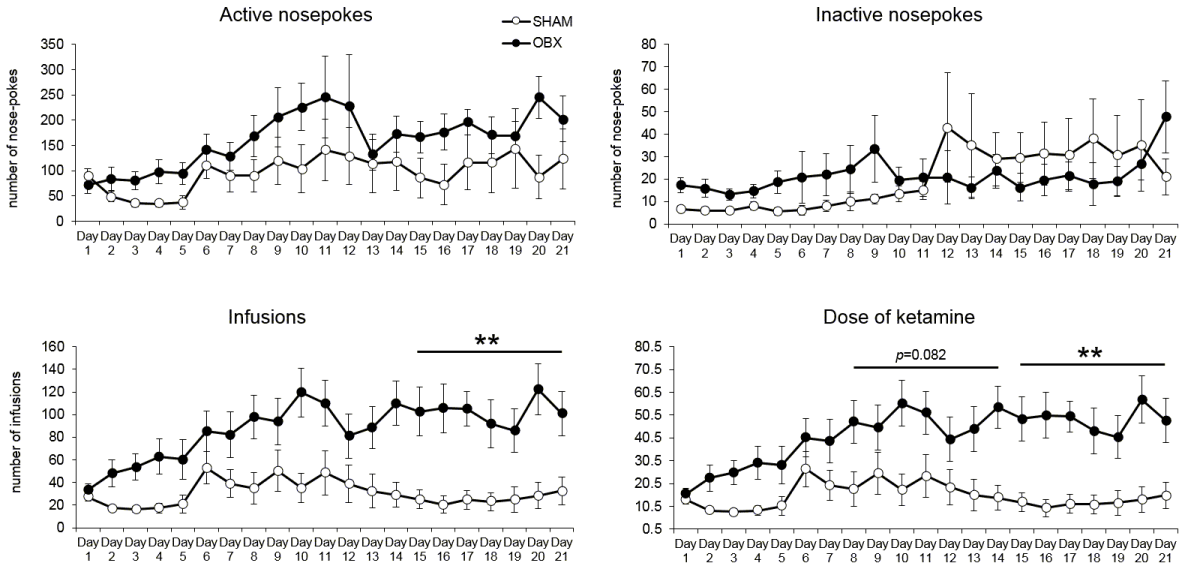

Figure 3: individual data on self-administered ketamine dose

SHAM animals show either low intake in all sessions (n=5) or escalation of intake in the second week (n=4). OBX rats show escalation of intake in the first week and then keep the same trend till the end (only 2 animals show low intake, yet higher than SHAM). The interrupted lines indicate excluded data (technical reasons).

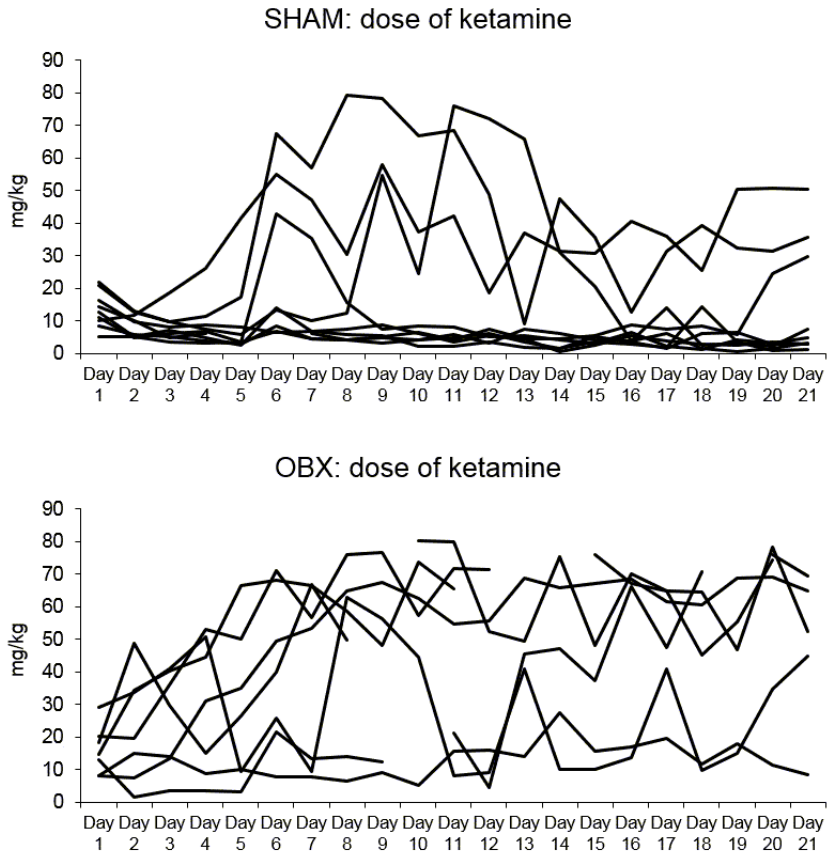

Figure 4: reinstatement of ketamine seeking behaviour

The bar graphs indicate the mean ±SEM number of both types of nose-pokes in the reinstatement session together with mean ±SEM nose-poking in the last week of the maintenance phase in SHAM and OBX rats. There was no difference between the performance in the reinstatement session and last maintenance week in SHAM rats (MWU test, n.s.) but OBX rats exhibited lower drug seeking behaviour in the reinstatement session that in the maintenance phase (T-test, *p=0.016). However, when the active responding in the reinstatement session was converted to a percent of mean nose-poking in the week 3 (reinstatement / mean week 3 x 100) a robust difference was shown: SHAM did mean 252 % of nose-poking in the week 3 while OBX animals only 60 % (T-test, p=0.059). There was no difference in the numbers of inactive nose-pokes neither within groups nor in SHAM vs. OBX comparison (MWU test n.s., T-test n.s., respectively).

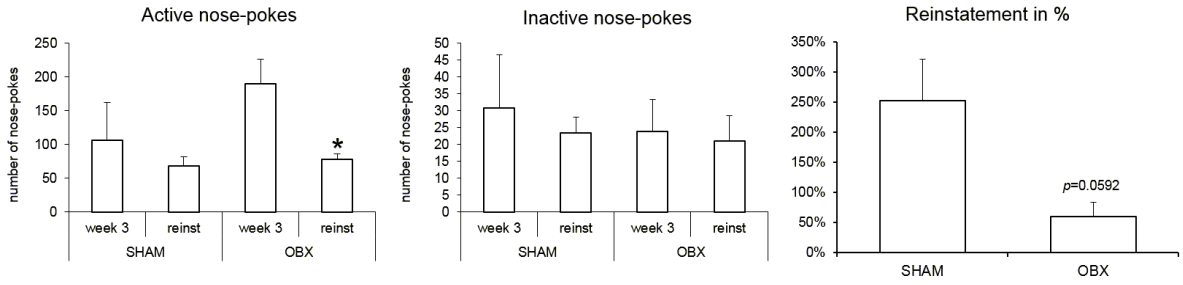

Figure 5: Forced swim test

All data are presented as the mean ±SEM values. The first – basal – test revealed significant decrease of swimming behaviour and increase of immobility in the OBX group (t test, ***p=0.0003, *p=0.0386 respectively). Chronic ketamine intake lead to important changes in the behaviour of the OBX animals, in the second FST OBX rats showed increased climbing and decreased immobility scores (t test, *p=0.0155, *p=0.050 respectively).

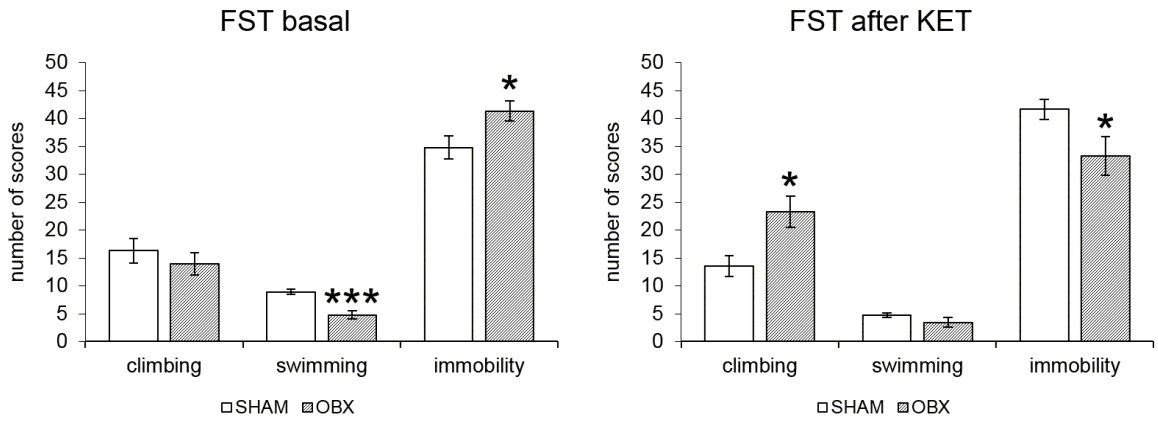

Table 1: reinstatement individual data

The table provides a detailed overview of individual drug seeking behaviours (active nose-pokes). The column "week 3 min" and "week 3 max" show the minimum and maximum number of active nose-pokes in the last week of maintenance phase. Column "week 3 mean" shows the weekly mean and "reinstatement" the number of active nose-pokes in the reinstatement session. Last "%" column indicates the calculation: reinstatement / mean week 3 x 100. As clearly visible, the high variability in the SHAM group attributes to only two animals with very high responding in the last maintenance week (rendering low percentage of the reinstatement activity). On the other hand the OBX group shows relatively consistent % of responding with only one exception of 188 %.

animal	week 3 min	week 3 max	week 3 mean	reinstatement	%
SHAM 1	279	711	**414.0**	60.0	**14%**
SHAM 2	101	539	**363.9**	37.0	**10%**
SHAM 3	4	59	**21.6**	95.0	**440%**
SHAM 4	3	77	**24.1**	31.0	**128%**
SHAM 5	3	48	**14.7**	26.0	**177%**
SHAM 6	11	155	**36.6**	49.0	**134%**
SHAM 7	23	108	**49.7**	118.0	**237%**
SHAM 8	8	13	**10.6**	55.0	**520%**
SHAM 9	6	47	**23.9**	145.0	**608%**
OBX 1	167	242	**207.0**	89.0	**43%**
OBX 2	54	276	**175.3**	82.0	**47%**
OBX 3	126	328	**254.5**	42.0	**17%**
OBX 4	175	306	**217.1**	61.0	**28%**
OBX 5	23	80	**51.7**	97.0	**188%**
OBX 6	200	323	**251.0**	96.0	**38%**

3. Schizophrenia and addiction comorbidity

3.1. Background

The "self-medication hypothesis" was developed to explain not only the comorbidity of depression and drug abuse but also for other psychiatric disorders (Hall and Queener, 2007, Khantzian, 1985, Khantzian, 2013, Khantzian and Albanese, 2009). The clinical evidence indicates, that the incidence of drug abuse is higher in the population with psychiatric morbidity (Wedekind et al., 2010), including schizophrenia (Koskinen et al., 2009, Mesholam-Gately et al., 2014). Almost 50 % of patients with schizophrenia suffer comorbid addiction (Lybrand and Caroff, 2009) and this comorbidity is associated with substantially higher burden of the disease (Schmidt et al., 2011, Hartz et al., 2014), higher suicide attempt rate (McLean et al., 2011, Melle et al., 2010) and also non-adherence (Wilk et al., 2006)to antipsychotic therapy. All drug classes were shown to be abused by the patients with schizophrenia. The most common was nicotine (Wing et al., 2012, Chambers et al., 2001, Mackowick et al., 2014) and alcohol (Krystal et al., 2006, Kalyoncu et al., 2005, Kerner, 2015, Regier et al., 1990) but abuse of opiates (Kern et al., 2014), amphetamines (Grant et al., 2012) and Cannabis drugs (McLoughlin et al., 2014) is reported as well.

There is certain association between development of schizophrenia and drug addiction (Volkow, 2009). A typical example is a risk of schizophrenia onset in young people smoking Cannabis (Kucerova et al., 2014, Caspi et al., 2005, Hall and Degenhardt, 2015, Semple et al., 2005). Methamphetamine induced psychosis is a well-known condition in drug addicts (Yui et al., 2000, Gururajan et al., 2012).

Therefore, similarly as in the depression – addiction dual disorder, a common distortion of neurobiological mechanisms underlying schizophrenia and substance abuse is expected. The most obvious is the dopaminergic system, which is dysregulated in both psychiatric disorders. It was already proposed as the main factor increasing the vulnerability of patients with schizophrenia to drug abuse (Chambers et al., 2001). Conformable with the self-medication hypothesis, patients with schizophrenia might relieve their negative and cognitive symptoms (Mackowick et al., 2014, Ng et al., 2013).

There are only several preclinical studies examining drug abuse behaviours in schizophrenia-like phenotype, mostly using different neurodevelopmental models (Micale et al., 2013) This approach to model schizophrenia seems to be highly valid (Kucerova et al., 2014, Micale et al., 2013). Most pre-clinical studies on this dual diagnosis used neonatal ventral hippocampal lesion (NVHL) model of schizophrenia (Tseng et al., 2009). Rats in this model were subjected to cocaine operant self-administration where they responded more for the drug and needed more days to extinguish the drug-seeking behaviour. This indicates higher motivation to obtain the drug and also showed higher drug-induced (Chambers and Self, 2002) and cue-induced (Karlsson et al., 2013) reinstatement. Similarly in methamphetamine self-administration study NVHL rats achieved higher break-points in progressive ratio paradigm confirming higher motivation while there was no difference in responding at fixed ratio for either the drug or food (Brady et al., 2008). Alcohol drinking was repeatedly tested in the NVHL model as well showing rather inconclusively increased vulnerability of the schizophrenia-like phenotype (Berg et al., 2011, Jeanblanc et al., 2014).

Prenatal immune activation models have demonstrated certain validity as well. Prenatal lipopolysaccharide exposure lead to increased alcohol intake in adulthood (Liu et al., 2004). Rats in the poly I:C model were reported to show enhanced amphetamine induced reinstatement of conditioned place preference (Richtand et al., 2012).

A recently developed neurodevelopmental model of schizophrenia induced by prenatal treatment with DNA-alkylating mitotoxin methylazoxymethanol acetate (MAM) has been (Lodge and Grace, 2009) seems to be suitable for studying schizophrenia-addiction comorbidity. MAM-treated animals show higher behavioural response to amphetamine challenge dose resembling sensitization to the drug (Lodge and Grace, 2012). However, despite the proven aberrant dopaminergic functioning, the influence of the MAM phenotype on addictive behaviour was not confirmed in a cocaine self-administration study (Featherstone et al., 2009).

Our team has only recently developed and validated the MAM model. We have recorded a complex behavioural profile indicating the schizophrenia-like phenotype is present in these animals (Stark et al., 2015). We have performed two studies on drug taking behaviour in this animal model: one with methamphetamine and another with alcohol drinking

paradigm. The findings from the experiment with methamphetamine are mostly in accordance with the negative data previously shown with cocaine (Featherstone et al., 2009). However, the alcohol drinking study showed some promising data (Ruda-Kucerova et al., 2016).

3.2. Aims

The research on the animal model of the schizophrenia and addiction comorbidity aimed to:

1. Establish the rat model of the dual disorder using prenatal methylazoxymethanol treatment as a model of schizophrenia while methamphetamine abuse was modelled by intravenous self-administration
2. Establish the rat model of the dual disorder using prenatal methylazoxymethanol treatment as a model of schizophrenia while alcohol abuse was modelled by drinking in the dark paradigm with sucrose fading procedure

Both studies were published together, Ruda-Kucerova *et al.*, 2016.

3.3. Methods

3.3.1. Animals

Time mated female albino Sprague-Dawley rats were purchased from Charles River (Germany) at gestational day 13 and housed individually. The rats were housed individually in standard rodent plastic cages. Environmental conditions during the whole study were constant: relative humidity 50-60 %, room temperature 23◦C ± 1◦C, inverted 12-hour light-dark cycle. Food and water were available *ad libitum*. All experiments were conducted in accordance with all relevant laws and regulations of animal care and welfare. The experimental protocol was approved by the Animal Care Committee of the Masaryk University, Faculty of Medicine, Czech Republic, and carried out under the European Community guidelines for the use of experimental animals.

3.3.2. Methylazoxymethanol (MAM) model of schizophrenia

Methylazoxymethanol acetate (MAM) was administered intraperitoneally to the dams on gestational day (GD) 17, saline was used as vehicle (Lodge, 2013, Lodge and Grace, 2009). The average surviving litter size was n=9.6 in control and n=11.5 in MAM treated mothers. The average proportion of male and female offspring was 52 % of males and 48 % of females. No cross-fostering was used, the mothers were regularly weighted and no differences were observed between control and MAM treated mothers. The offspring were weaned on the postnatal day (PND) 22 and housed in sections of 5 and later individually during the drug addiction studies initiated at age of 9 weeks.

3.3.1. Alcohol drinking paradigm

Alcohol intake was assessed by using the drinking in dark paradigm with the sucrose fading procedure (Samson, 1986, Czachowski, 2005). The drinking sessions lasted 90 minutes daily and started at 10 a.m. (3 hours after start of the dark period of the day). For the session the water bottle was switched for another one containing the alcohol solution. At the end of the daily session alcohol bottles were removed and standard water bottles were returned to the home cage. All solutions were presented at room temperature. The sucrose fading training phase was performed as follows: 10% alcohol and 5% sucrose (3

days), 15% alcohol and 5% sucrose (3 days), 20% alcohol and 5% sucrose (4 days), 20% alcohol and 2% sucrose (3 days), 20% alcohol and 1% sucrose (4 days). The training lasted 17 days in total. From day 18 onward the animals were given under the same conditions 20% alcohol only. This phase of stable alcohol intake lasted 18 days (maintenance of the alcohol drinking). In continuation the rats were subjected to 14 days of forced abstinence when the alcohol solution was not available. After this period 20% alcohol was given again at the same time for 5 more days to model relapse of the alcohol drinking behaviour after abstinence. Rats were not food or water deprived throughout the study. Ethanol intake was calculated as grams of ethanol per kg of body weight (animals were weighed daily).

3.3.2. IV self-administration (IVSA) surgery and procedures

The IV self-administration study was performed in the same manner as described in the section 2.3.3.

3.3.3. Locomotor activity

Locomotor activity was assessed as described in the section 2.3.5.

3.3.4. Sucrose preference

Sucrose preference test was performed as described in the section 2.3.7.

3.4. Results

3.4.1. Reactivity to addictive drugs in the methylazoxymethanol (MAM) model of schizophrenia in male and female rats

This study validated the rat model of the dual disorder of schizophrenia and addiction using prenatal methylazoxymethanol treatment as a neurodevelopmental model of schizophrenia (Lodge, 2013, Lodge and Grace, 2009) while methamphetamine abuse was modelled by intravenous self-administration and alcohol abuse was modelled by drinking in the dark paradigm with sucrose fading procedure (Czachowski, 2005, Samson et al., 1988, Thiele and Navarro, 2014). Furthermore, both studies (alcohol and methamphetamine) were designed to cover maintenance, forced abstinence and reinstatement to the respective drug in order to provide data on all stages of drug addiction modelling. Both male and female rats were used to address the potential sex dependent differences.

The study suggests that the female sex and schizophrenia-like phenotype induced by the prenatal MAM exposure may work synergistically to enhance alcohol consumption. Different models of schizophrenia were used in alcohol studies which all have demonstrated to have some merit in modelling escalation of alcohol consumption (Berg et al., 2011, Jeanblanc et al., 2014). However, there was only a minor alteration of addictive behaviours towards methamphetamine in the MAM animals. This is in accordance with a methodologically similar study with cocaine (Featherstone et al., 2009).

At this stage, the NVHL model seems to be of more interest but the application of MAM model to study this type of substance abuse remains understudied. Therefore, it is not possible to conclude that the reward related processes in MAM model are intact.

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ORIGINAL INVESTIGATION

Reactivity to addictive drugs in the methylazoxymethanol (MAM) model of schizophrenia in male and female rats

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ABSTRACT

Objectives: Patients with schizophrenia often suffer comorbid substance abuse regardless of gender. However, the vast majority of studies are only conducted in male subjects. Therefore, the aim of these experiments is to assess addictive behaviors of both sexes in a neurodevelopmental model of schizophrenia induced by prenatal methylazoxymethanol (MAM) acetate exposure.

Methods: MAM (22 mg/kg) was administered intraperitoneally on gestational day 17. Two studies were performed in the offspring: (1) an alcohol-drinking procedure to assess daily intake of 20% alcohol and relapse-like behavior after a period of forced abstinence; (2) Methamphetamine (METH) intravenous self administration (IVSA) followed by forced abstinence and reinstatement phases.

Results: MAM exposure during the prenatal period did not change alcohol drinking regardless of sex. However, MAM females showed higher alcohol consumption in comparison to MAM males. The METH IVSA study revealed only a modest increase of drug consumption in MAM males, while there was no difference between the female groups. Reinstatement data showed no effect of the MAM model in either sex, but suggested increased responding in female rats.

Conclusions: This study suggests that female sex and schizophrenia-like phenotype may work synergistically to enhance alcohol consumption. However, future research is needed to establish paradigms in which these findings would be readily assessed to test anti-addiction treatments.

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Introduction

Drug addiction is known to be more prevalent in patients with psychiatric morbidity than in the general population (Wedekind et al. 2010). This is particularly well documented in affective disorders such as anxiety, depression (Volkow 2004) and schizophrenia (SCZ; Koskinen et al. 2009; Mesholam-Gately et al. 2014). Almost 50% of SCZ patients suffer comorbid addiction (Lybrand and Caroff 2009), which is linked with a substantially higher burden of the disease as these patients have a higher rate of hospitalizations, a shorter life expectancy (Schmidt et al. 2011; Hartz et al. 2014) and a higher suicide attempt rate (Melle et al. 2010; McLean et al. 2011). The most common drug addiction is nicotine, with a prevalence of 70–90% in SCZ patients compared to 26% in the general population (Chambers et al. 2001; Wing et al. 2012; Mackowick et al. 2014). The high prevalence of other substances use, such as alcohol (Regier et al. 1990; Kalyoncu et al. 2005; Krystal et al. 2006; Kerner 2015), opiates (Kern et al. 2014), amphetamine psychostimulants (Grant et al. 2012) and cannabis (McLoughlin et al. 2014) is also alarming. Recently, emerging clinical studies have suggested differential abuse patterns in men and women to different substances in a variety of research paradigms (Johnson et al. 2010; Bahorik et al. 2013), indicating a growing research interest (Mendrek 2015).

Several lines of evidence support the association between SCZ and addiction (Volkow 2009). There is a known risk of triggering SCZ by cannabis use especially during adolescence (Caspi et al. 2005; Semple et al. 2005; Kucerova et al. 2014; Hall and Degenhardt 2015), and by psychostimulants, e.g., methamphetamine (METH; Yui et al. 2000; Gururajan et al. 2012) and cocaine (Malave and Broderick 2014). This suggests a common distortion of neurobiological mechanisms underlying both SCZ and substance abuse, namely the dopaminergic (DAergic) system.

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DAergic abnormalities have been well described in both SCZ and substance abuse. It is possible that the DAergic dysfunction in SCZ patients disrupts normal reward pathways predisposing individuals to higher risks for drug abuses (Chambers et al. 2001).

Dopamine (DA) dysregulation in SCZ is complex. Positive symptoms of SCZ are associated with increased DA signaling from enhanced subcortical DA release (mostly via D2 receptors). In contrast, negative symptoms are believed to be due to decreased DA signaling as a result of decreased D1 receptor activation in the prefrontal cortex and alterations of the D3 signaling (Brisch et al. 2014). Due to this decreased DA signaling, SCZ patients rarely ever experience reward feelings (Brisch et al. 2014; Mesholam-Gately et al. 2014). Substance abuse is thought of as a self attempt by SCZ patients to relieve this symptom, and this abuse is essentially a drug-induced release of DA in the prefrontal cortex. In SCZ, it is probably to relieve negative and cognitive symptoms. Similar notions about substance abuse in psychiatric morbidity have led to the proposal of the self-medication hypothesis (Khantzian 1985). Currently, there is a discussion about potential positive effects of nicotine in SCZ patients (Mackowick et al. 2014), which is well supported by pre-clinical data (Ng et al. 2013). This, however, remains controversial due to the efficacy of nicotine and in contrast the harmful effects of smoking (Hahn et al. 2013). Also, this self-medication strategy could potentially be secondary to DA-suppressing antipsychotic treatment (Samaha 2014).

Despite the high prevalence of drug addiction and SCZ comorbidity, there are few pre-clinical studies examining drug-abuse behaviors in the SCZ-like phenotype. A composite translational animal model could provide a basis for investigation of the mechanisms underlying the interaction between the two disorders. Valid assessment of substance-abuse characteristics in the SCZ-like phenotype requires chronic animal models of SCZ, i.e., neurodevelopmental models seem to be the most useful (Micale et al. 2013). Furthermore, operant approaches to model drug abuse are considered the most relevant for the human disorder (O'Connor et al. 2011).

Until recently, most pre-clinical studies on the dual diagnosis used a neonatal ventral hippocampal lesion (NVHL) model of SCZ (Tseng et al. 2009). Rat males in this model were subjected to cocaine-operant self-administration testing and the animals were found to respond more for the drug. The animals also took longer to extinguish drug-seeking behaviors, indicating a higher motivation to obtain the drug which can be interpreted as higher drug-induced (Chambers and Self 2002) and cue-induced (Karlsson et al. 2013) reinstatement. Similarly, in a METH self-administration study, NVHL rats achieved higher break-points in a progressive ratio paradigm confirming higher motivation, while there was no difference in responding at a fixed ratio for either the drug or food (Brady et al. 2008). Alcohol drinking was repeatedly tested in the NVHL model, showing rather inconclusively an increased vulnerability in the SCZ-like phenotype (Berg et al. 2011; Jeanblanc et al. 2014). Furthermore, prenatal immune activation models have also demonstrated certain validity. Prenatal lipopolysaccharide exposure led to increased alcohol intake in adulthood (Liu et al. 2004). Rats in the poly I:C model were reported to show enhanced amphetamine-induced reinstatement of conditioned place preference (Richtand et al. 2012). DNA-alkylating mitotoxin methylazoxymethanol (MAM) acetate prenatal treatment has been established as a well validated neurodevelopmental model of SCZ (Lodge and Grace 2009). This model seems suitable for studying SCZ-addiction comorbidity given its chronic nature (as opposed to acute pharmacological models) as well as its high face and construct validity described by the behavioral (i.e., augmented locomotor response to amphetamine, social deficits and cognitive impairments) and neurochemical (i.e., enhanced activity in the mesolimbic DAergic system and decreased parvalbumin interneuron density in medial prefrontal cortex and hippocampus) changes (Micale et al. 2013). Moreover, prenatally MAM-treated animals show a higher behavioral response to an amphetamine challenge dose resembling sensitization to the drug (Lodge and Grace 2012). Interestingly, despite the proven aberrant dopaminergic functioning, the influence of this phenotype on addictive behavior has not been identified in a cocaine self-administration study assessing both fixed and progressive schedules of reinforcement, extinction and drug-induced reinstatement (Featherstone et al. 2009). However, this finding still does not rule out differential reactivity to drugs of abuse because only one psychostimulant drug was tested in a single paradigm.

More importantly, in all these studies only male offspring were used and the vast majority of both preclinical and clinical studies are conducted on male subjects only. Despite the fact that the absolute number of women suffering SCZ is less than men, this issue should be properly addressed because women also suffer from comorbid substance abuse (Abel et al. 2010) and, unlike men, do not improve after hospitalization intervention (Bahorik et al. 2013). The validity of the MAM model in female offspring was already shown in a previous study reporting similar behavioral

alterations to MAM exposure regardless of gender (Hazane et al. 2009).

Therefore, the aim of this study is to assess possible differences in: (1) alcohol drinking, one of the most commonly abused substances (Kalyoncu et al. 2005; Krystal et al. 2006), and (2) operant METH intravenous self administration (IVSA) between MAM-exposed and control rats of both sexes. Furthermore, both studies were designed to cover maintenance, forced abstinence and reinstatement to the respective drug to provide data on all stages of drug addiction modelling. A forced abstinence model where the animal does not have access to the operant box was used because it mimics human behaviour. In rehabilitation centres, patients usually discontinue drug use and protected from exposure to drug-related environments. A preclinical paradigm based on this approach is readily used (Fuchs et al. 2006; Reichel and Bevins 2009; Yahyavi-Firouz-Abadi and See 2009; Ruda-Kucerova et al. 2015a). It provides perhaps a more translational alternative to extinction procedures because it measures drug-seeking behavior following a period of involuntary drug withdrawal, when the motivation of drug-response behavior is not influenced by any training procedures. Furthermore, this paradigm can be used in both alcohol drinking and METH IVSA studies allowing a better comparison.

Material and methods

Animals

Time mated female albino Sprague-Dawley rats were purchased from Charles River (Germany) at gestational day (GD) 13 and housed individually. MAM acetate was administered intraperitoneally on GD 17 to 37 rats, while the vehicle was administered to 13 control rats. The average surviving litter size was $n = 9.6$ in control and $n = 11.5$ in MAM-treated mothers. Two litters were lost (killed by the mother), one control and one MAM treated. The average proportion of male to female offspring was 52% male and 48% female. No cross-fostering was used, the mothers were regularly weighed and no differences were observed between control and MAM-treated mothers. The offspring were weaned on postnatal day 22 and housed in sections of five and later individually, during the drug addiction studies, initiated at the age of 9 weeks. All females were left with intact gonads to assess addictive behavior in a population with natural estrous cycle (Ruda-Kucerova et al. 2015a). For the alcohol drinking study, 20 male (10 vehicle and 10 MAM treated) and 20 female (10 vehicle and 10 MAM treated) offspring were used. For the METH IVSA study different groups of 20 male (10 vehicle and 10 MAM treated) and 30 female (11 vehicle and 19 MAM treated) offspring were used. The alcohol study was finished by all animals, but the final numbers in the IVSA study were lower due to surgery or catheter patency. At the end of the study, there were $n = 9$ male vehicle (M VEH), $n = 8$ male MAM (M MAM), $n = 9$ female vehicle (F VEH) and $n = 16$ female MAM (F MAM) included in the analysis. All experimental groups were composed of offspring from four to five different mothers. Environmental conditions during the whole study were constant: relative humidity 50–60%, temperature $23 \pm 1\,^{\circ}C$, inverted 12-h light–dark cycle (from 07:00 to 19:00 h). Food and water were available ad libitum throughout the study. All experiments were conducted in accordance with all relevant laws and regulations of animal care and welfare. The experimental protocol was approved by the Animal Care Committee of the Masaryk University Faculty of Medicine, Czech Republic, and carried out under the European Community guidelines for the use of experimental animals.

Drugs and treatments

MAM acetate (Midwest Research Institute, Kansas City, USA) was dissolved in saline and administered intraperitoneally at dose 22 mg/kg in a volume of 1 ml/kg on GD 17, as previously described (Moore et al. 2006). Saline was administered to the control group as vehicle.

Ethanol 96% was purchased from a local pharmacy and dissolved using distilled water to the desired concentration (from 10 to 20%, see alcohol-drinking protocol).

METH (Sigma, St Louis, MO, USA), available in the operant cage for IVSA was 0.08 mg/kg per infusion with the maximum number of infusions obtainable in one session set to 50 as previously described and validated (Kucerova et al. 2009, 2012; Amchova et al. 2014; Ruda-Kucerova et al. 2015a).

Alcohol-drinking study

Sucrose preference test

A two-bottle choice procedure was used to determine the sucrose intake at two timepoints during the alcohol study. Test 1 was conducted to assess possible anhedonia before alcohol training. Test 2 was performed on the first day of alcohol abstinence to reveal the possible development of alcohol-induced anhedonia (Kalejaiye et al. 2013). During a 24-h training phase,

each rat was provided with two water bottles in their home cage in order to adapt the rats to drinking from two bottles. After training, one bottle (counterbalanced across rats) was randomly switched to contain 1% sucrose solution. After 24-h, both bottles were removed and the amount of liquid remaining in each bottle was measured. The sucrose preference score was calculated as the percentage of sucrose solution ingested relative to the total amount of liquid consumed.

Alcohol-drinking procedure

The drinking-in-the-dark paradigm was used along with the sucrose-fading procedure for training and adapted from published protocols (Samson et al. 1988; Czachowski 2005). The drinking sessions lasted 90 min daily and started at 10:00 h (3 h after the lights went off). During this time the water bottle was switched for another one containing the alcohol solution. At the end of the daily session alcohol/sucrose bottles were removed and standard water bottles were returned to the home cage. The alcohol/sucrose solutions were presented at room temperature. The sucrose-fading training phase was organized as follows: 10% alcohol and 5% sucrose (3 days), 15% alcohol and 5% sucrose (3 days), 20% alcohol and 5% sucrose (4 days), 20% alcohol and 2% sucrose (3 days), 20% alcohol and 1% sucrose (4 days). The training lasted 17 days in total. From day 18 onward the animals were given 20% alcohol only under the same conditions. This phase of stable alcohol intake lasted 18 days (maintenance of the alcohol drinking). In continuation, the rats were subjected to 14 days of forced abstinence when the alcohol solution was not available. After this period 20% alcohol was given again at the same time for a further 5 days to model the relapse of the alcohol-drinking behavior after abstinence. Rats were not food or water deprived throughout the study. Ethanol intake was calculated as grams of ethanol per kg of body weight (animals were weighed daily). Blood alcohol levels were not assayed in the study as it was shown previously that the alcohol levels do not differ in male and female rats (Murawski and Stanton 2011).

METH IVSA study

Locomotor activity test

Before starting the METH IVSA study, the baseline behavioural profile was assessed in all animals. In brightly lit room, rats were individually tested for locomotor activity using the Actitrack system (Panlab, Spain) as previously described (Pistovcakova et al. 2008; Ruda-Kucerova et al. 2015a). Each Plexiglas arena ($45 \times 45 \times 30$ cm) was surrounded by two frames equipped with photocells located one above another at 2 and 12 cm over the cage floor. Animals were placed in the centre of arena and the spontaneous behavior was tracked for 10 min. In the test, horizontal locomotor activity (the trajectory calculated by the system as beam interruptions that occurred in the horizontal sensors) and vertical activity (number of rearing episodes breaking the photocell beams of the upper frame) were recorded. At the end of the session, animals were returned to their home cage, and the arenas were wiped with 1% acetic acid to avoid olfactory cues. The test was carried out during 1 day (morning hours) in all animals starting with males and continuing with females. The animals were brought to the test room individually.

Food self-administration protocol

Food self administration was employed to develop self-administration operant behaviour in the animals. The training was conducted as already described (Ruda-Kucerova et al. 2015a) in 10 operant boxes ($30 \times 25 \times 30$ cm, Coulbourn Instruments, USA) using nose-poke operandi under a fixed ratio 1 (FR-1) schedule of reinforcement, i.e., animal had to make one nose-poke to the active operandum to obtain a single palatable pellet (BioServ, sweet dustless rodent pellets, F0021-Purified Casein Based Formula – 45 mg, sweet taste attributed by 276 g/kg of monosaccharides and 310 g/kg of sucrose). Each cage was provided with two nose-poke holes allocated on one side and programmed by software Graphic State Notation 3.03 (Coulbourn Instruments). The cage was illuminated by a house light during the whole session. Self-administration sessions lasted 30 min during the dark period of the inverted light–dark cycle 7 days/week, and at the end the rats were returned to their home cages.

IVSA protocol

Animals were deeply anesthetized with an intraperitoneal injection of 50 mg/kg ketamine plus 8 mg/kg xylazine. Under aseptic conditions, a permanent intracardiac silastic catheter was implanted through the external jugular vein to the right atrium. The outer part of the catheter exited the skin in the midscapular area. After surgery, a 1-week recovery was allowed. The catheters were flushed daily using enrofloxacine (17 mg/kg) solution followed by 0.1 ml of a heparinized (1%) sterile saline solution to prevent infection and occlusion of the catheter. METH IVSA was conducted as previously described (Kucerova et al. 2009; Kucerova

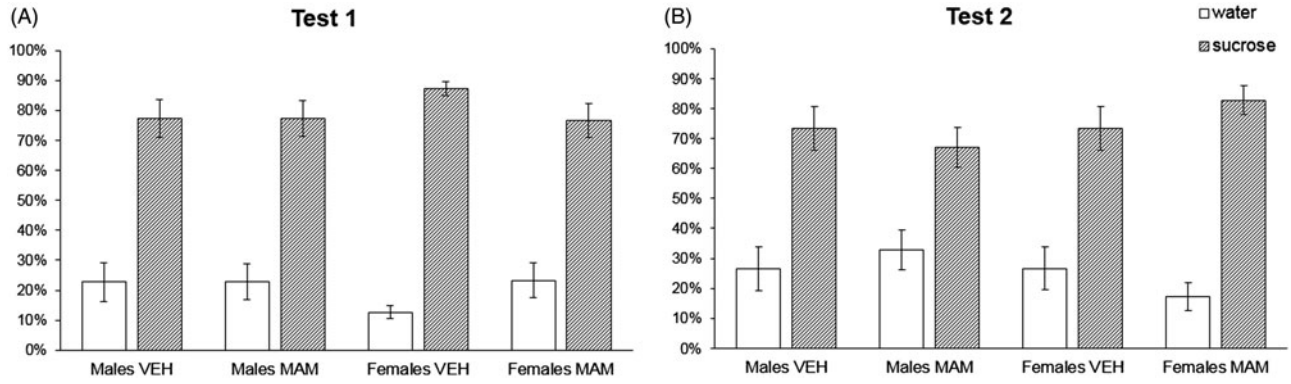

Figure 1. The sucrose preference test. The test was conducted in male and female control and MAM animals. Data are percentages (±SEM) of sucrose solution consumed in 24 h at two timepoints, i.e., Test 1 (A) was conducted before alcohol drinking training and Test 2 (B) on the first day of forced abstinence from maintenance of 20% alcohol drinking. Two-way ANOVA (two factors: sex and MAM model), not signficant.

et al. 2012; Amchova et al. 2014; Ruda-Kucerova et al. 2015a) in the same operant boxes (Coulbourn Instruments) using nose-poke operandi under a FR-1. Nose-poking in the active hole led to the activation of the infusion pump and administration of an infusion followed by a 10-s timeout, when nose-poking was recorded but not rewarded. The cage was illuminated by a house light during the session. The light was flashing when the system was administering infusion (5 s), and was off during the timeout period to provide an environmental cue associated with METH infusion. IVSA sessions lasted 90 min and took place 7 days/week between 08:00 and 15:00 h during the dark period of the inverted light–dark cycle, and at the end rats were returned to their home cages. After 14 days of METH intake the maintenance phase was terminated and rats were kept in their home cages for the 14 days of the forced abstinence period. On day 15 of abstinence, rats were placed into IVSA chambers for the last 90-min reinstatement session which was, apart from drug delivery, identical to the maintenance sessions. The numbers of responses on the active drug-paired nose-poke and the inactive nose-poke were recorded but the drug was not delivered.

Statistical data analysis

Primary data were summarized using arithmetic mean and standard error of the mean (SEM) estimates. Sucrose preference and open-field data were analyzed using two-way analysis of variance (ANOVA) (factors: sex, MAM model, repeated factor: day) and Bonferroni post-hoc test for multiple comparisons. For evaluation of alcohol intake, food intake and maintenance variables in the METH study, repeated measures ANOVA with the same factors and Bonferroni post-hoc test was employed. Two-way ANOVA (factors: sex, MAM model) and Bonferroni post-hoc test were also used for analysis of METH reinstatement and alcohol-relapse data. The data on the percentage of alcohol intake during the relapse phase were non-parametric (Kolmogorov-Smirnov test of normality), therefore the Kruskal-Wallis test was used. The analyses were calculated using Statistica 12 (StatSoft, USA). A value $P < 0.05$ was recognized as boundary of statistical significance in all applied tests.

Results

Alcohol-drinking study

Sucrose preference test

As shown in Figure 1, all rats consumed the same proportion of sucrose solution (approximately 80%) both before the beginning of the alcohol study and during abstinence. Two-way ANOVA (two factors: sex and MAM model) did not reveal any significant differences induced by sex, MAM model or their interaction ($F = 1.144$, $F = 0.363$ and $F = 0.811$, respectively).

Alcohol drinking

Figure 2 shows mean daily intake of pure (theoretical 100%) ethanol per kg of body weight in all groups during both the maintenance phase and the relapse after abstinence. Repeated measures ANOVA (two factors: sex and MAM model) detected a significant effect of sex–model interaction: $F = 2.931$, $P = 0.0241$, but not sex or MAM model alone. Bonferroni post-hoc test indicated that the F MAM group consumed significantly more alcohol than the M MAM group in both the maintenance and relapse phases of the study (P values indicated in the graph by * symbols). However, control animals (M VEH vs. F VEH) showed

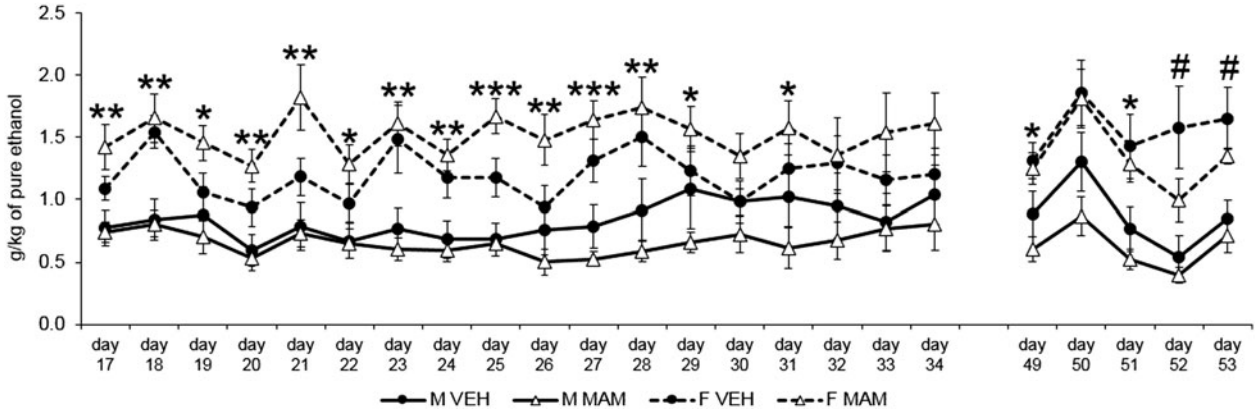

Figure 2. Alcohol drinking. Data are shown as mean (±SEM) daily intake of pure (theoretical 100%) ethanol per kg of body weight during both maintenance and the relapse phase. The F MAM group consumed more alcohol than the M MAM in both maintenance and relapse phases of the study: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, repeated measures ANOVA followed by Bonferroni post-hoc test. On days 52 and 53 the F VEH group consumed more alcohol than the M VEH: $\#P < 0.05$, repeated measures ANOVA followed by Bonferroni post-hoc test.

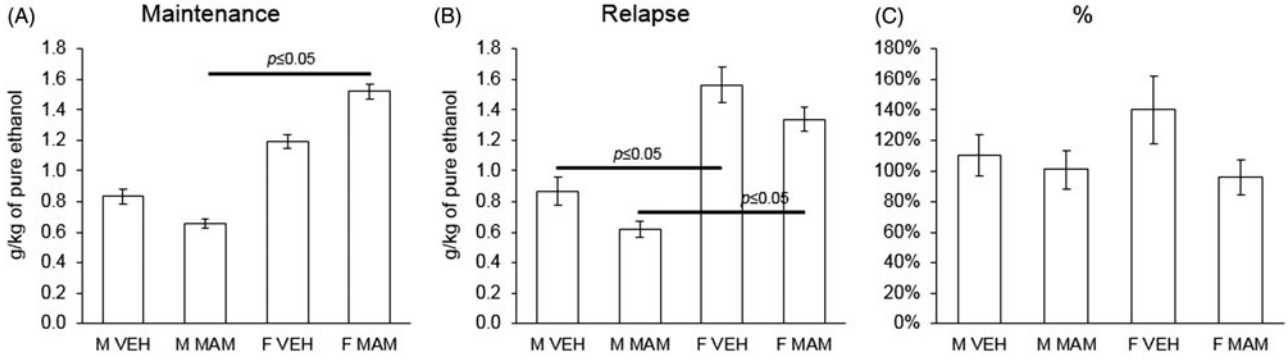

Figure 3. Alcohol drinking summary. (A) The overall (17 days) mean (±SEM) values of (theoretical 100%) alcohol intake during the maintenance phase of the study. Repeated measures ANOVA followed by Bonferroni post-hoc test has revealed significant difference between the M MAM and F MAM groups ($P < 0.05$). (B) The overall (5 days) mean (±SEM) values of (theoretical 100%) alcohol intake during the relapse phase of the study. Repeated measures ANOVA followed by Bonferroni post-hoc test revealed significant difference in the M VEH vs. F VEH ($P < 0.05$) and M MAM vs. F MAM ($P < 0.05$) groups. (C) The mean (±SEM) percent of baseline alcohol intake (mean intake in relapse sessions divided by mean intake in maintenance in each animal) in all groups. Kruskal-Wallis non-parametric test, not significant.

only few significant data points during the relapse phase (*P* values indicated in the graph by # symbols). Therefore, these data indicate a trend to increased alcohol intake in the females, which is strongly enhanced by the MAM model. For better visualization this is also shown in the Figure 3, which depicts the summarized maintenance (A) and relapse (B). Furthermore, these numbers were converted to a percent of mean baseline alcohol intake (mean intake in relapse sessions divided by mean intake in maintenance) to assess the effect of the period of abstinence on the drug-intake behaviour (C). However, due to a very high variability and the non-Gaussian distribution of the data no difference was detected (Kruskal-Wallis non-parametric test), despite a trend to higher intake in relapse in the control female rats (140%).

METH IVSA study

Baseline locomotor characteristics

Before starting the IVSA protocol, baseline locomotor and exploratory activity was assessed in all groups to exclude the possibility that these characteristics would lead to different drug-taking behaviour. Figure 4 illustrates the results on total horizontal (A) and vertical (B) activity (number of rearing episodes) and the number of faecal droppings during the session (C). Two-way ANOVA (two factors: sex and MAM model) revealed no significant differences in the distance travelled but detected the main effect of sex in the numbers of rearing episodes ($F = 11.099$, $P = 0.002$) and droppings ($F = 9.894$, $P = 0.003$). The Bonferroni post-hoc test added one significant difference in the number of droppings between the M VEH and F VEH groups

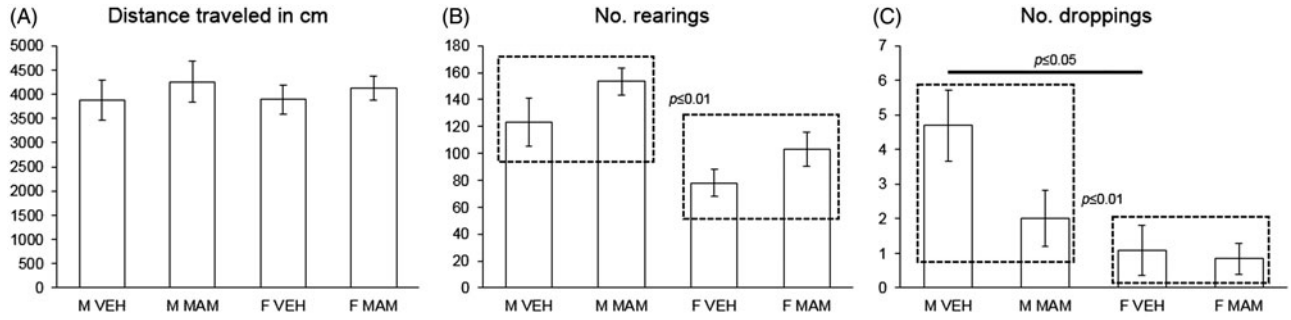

Figure 4. Baseline behavioural profile assessed by open-field test. (A) Total distance travelled (in cm), (B) number of rearing episodes (vertical activity) and (C) mean number of faecal droppings. All data are shown as means (±SEM). Two-way ANOVA revealed the main effect of sex (marked by dotted line frames) in the number of rearings and droppings. Bonferroni post-hoc test identified only a significant difference in number of droppings between M VEH and F VEH ($P < 0.05$).

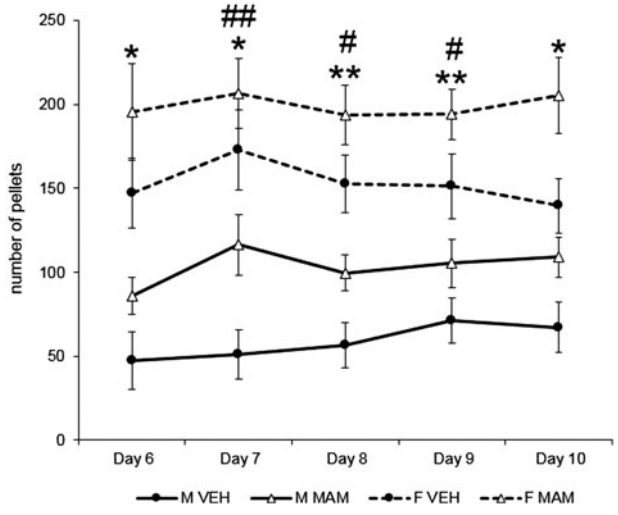

Figure 5. Maintenance of food self administration. The graph shows the mean (±SEM) number of pellets self administered in all groups (*t*-test, $*P \leq 0.05$, $***P \leq 0.001$). The F VEH group consumed more pellets than the M VEH group: $\#P < 0.05$, $\#\#P < 0.01$. Similarly F MAM animals consumed more pellets than M MAM animals: $*P < 0.05$, $**P < 0.01$, repeated measures ANOVA followed by Bonferroni post-hoc test.

($P = 0.015$), showing a lower number in females. This baseline behavioural profile indicates only sex-dependent differences which are unlikely to contribute to dissimilar behaviour in the operant cage. Males exhibited rather higher anxiety-related behaviours visible mainly in the number of droppings as a measure of general emotionality.

Food self administration

Food-taking behaviour was assessed as a mean number of self-administered pellets during the last 5 days of training when the intake was stable. Figure 5 depicts the significantly higher pellet intake in both groups of female rats compared to the respective male groups as indicated by repeated measures ANOVA (two factors: sex and MAM model), main effect of sex, $F = 5.250$, $P = 0.002$. The Bonferroni post-hoc test further detected a significant increase of pellet intake in both F VEH and F MAM groups as compared with M VEH and M MAM rats, respectively (detailed *P* values are indicated in the graph). Generally, all the pellets delivered were also eaten by the animals during the session, rarely were a few of them (several, never more than 10) left intact on the cage floor or in the feeder. Furthermore, this happened only on the first days of the training and was not repeated on subsequent days in the same animals.

Maintenance of METH IVSA

The acquisition and maintenance of METH-taking behavior was assessed firstly in terms of mean number of nose-pokes, infusions self administered per session, and secondly by the mean METH dose per session in mg/kg. As shown in Figure 6, there was no difference between the groups in the active (A) or inactive (B) nose-poking as well as number of infusions (C), repeated measures ANOVA with two factors: sex and MAM model. However, when the number of infusions was converted to a METH dose per kg of body weight, ANOVA detected a main effect of the model: $F = 3.059$, $P = 0.022$ and Bonferroni post-hoc test indicated a significant difference between the M VEH and M MAM groups, with the MAM model leading to a decrease of METH intake (day 10, $P = 0.023$). This effect was no longer significant in the following days.

Reinstatement of METH IVSA

After the 2-week period of forced abstinence, one last reinstatement session was performed with no drug availability. The only measure of the drug-seeking behaviour was the responding operandi. Figure 7 shows the mean number of active (A) and inactive (B)

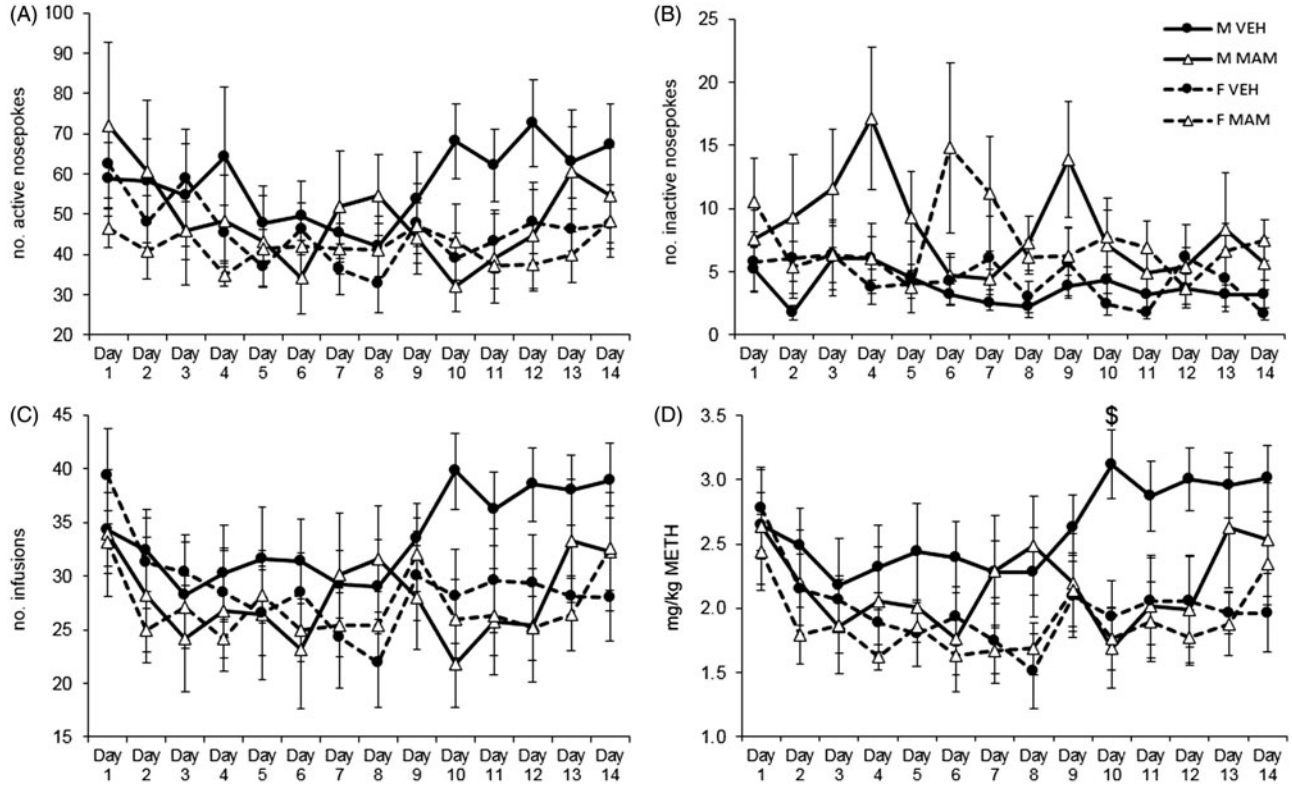

Figure 6. Maintenance of METH intake. (A) The mean numbers of active (drug-paired) nose-pokes over the 14 days of the METH IVSA in all groups; analogously (B) depicts mean numbers of inactive (non-drug-paired) nose-pokes, (C) The mean numbers of METH infusions and (D) the mean METH dose in mg/kg. All data are shown as means (±SEM). The only significant difference between the M VEH and M MAM groups was identified in the METH dose (day 10, $\$P = 0.023$), repeated measures ANOVA followed by Bonferroni post-hoc test.

nose-pokes during this session, the number of active responses as a percent of mean baseline nose-poking in maintenance (C) and the percentage of active nose-poke preferences calculated as the number of active nose-pokes in the reinstatement session/mean nose-poking during the 14 days of maintenances phase (D) as previously described (Ruda-Kucerova et al. 2015a).

Two-way ANOVA (two factors: sex and MAM model) has indicated only one difference in the percent of mean baseline nose-poking (C) with the main effect of sex, $F = 6.890$, $P = 0.012$.

Discussion

Alcohol-drinking study

This study has provided evidence that the differential addictive behaviour in the MAM model can be partially sex-dependent. There were no differences in the sucrose preference test in either phase of the study (i.e., naive and abstinent animals). All animals naturally preferred the sucrose solution. This indicates that the animals did not have impaired behaviour related to a natural reward such as anhedonia or other depressive-like phenotype. Furthermore, prenatal MAM exposure did not lead to changes of 20% alcohol-drinking behaviour in either gender. However, alcohol consumption was significantly higher in females compared to males in the MAM-treated groups (F MAM vs. M MAM). This suggests that the prenatal MAM exposure may increase vulnerability for alcohol drinking in female rats. During the relapse phase, this phenomenon was still present when sex difference in control animals reached significance, i.e., control females consumed more alcohol than control males. To our knowledge, this is the first report on alcohol-drinking behaviour in the MAM model.

Regarding **sex differences**, Piano et al. (2005) showed Sprague-Dawley female rats to have a higher consumption of a liquid ethanol at ethanol concentrations from 5 to 8% in the diet available 24 h/day. This effect can be, to a large extent, explained by presence of female gonadal hormones because the ovariectomized group showed similar consumption as males. Interestingly, blood ethanol levels were similar in all groups (Piano et al. 2005). In a later analogous study, the same team found no significant differences in alcohol drinking (Piano et al. 2007). Long-Evans female rats were shown to reduce 10% alcohol drinking after

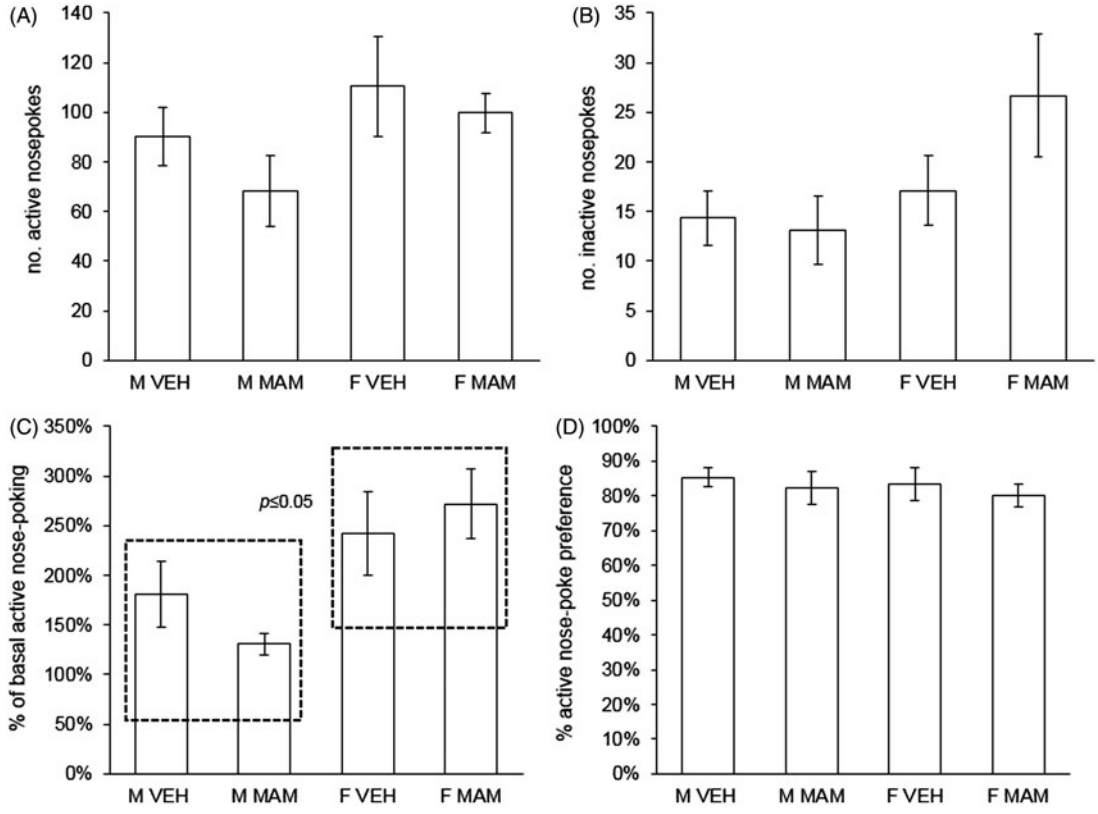

Figure 7. Reinstatement of METH-seeking behaviour. (A) The mean numbers of active (drug-paired) nose-pokes in the reinstatement session in all groups; analogously (B) the mean numbers of inactive nose-pokes, (C) mean percent of baseline active nose-poking (mean number of nose-pokes during maintenance phase/number of nose-pokes ×100 in each animal) and (D) the mean active nose-poke preference in the reinstatement session. All data are shown as means (±SEM). Two-way ANOVA revealed the main effect of sex (marked by dotted line frames) in the percent of mean baseline nose-poking (C), while Bonferroni post-hoc test did not show any other significances.

ovariectomy, which was counteracted by oestrogen supplementation (Ford et al. 2002). Furthermore, intact Wistar female rats were shown to have a higher intake of 6% alcohol than a male group (Juarez and Barrios de Tomasi 1999). However, these sex specificities are not easy to replicate in all designs. Moreover, there are also studies showing no differences between genders in different strains (Piano et al. 2007; Anderson et al. 2012). In addition, behavioural traits other than absolute alcohol consumption or blood level may differ between the sexes, such as memory test performances or depressive- and anxiety-like phenotype. This suggests a higher vulnerability of females to the same level of alcohol exposure (Gomez and Luine 2014).

Regarding the **SCZ-like phenotype,** most studies were performed using the NVHL neurodevelopmental model in male rats. These studies have that shown animals can easily develop behavioural sensitization to ethanol compared with sham rats (Conroy et al. 2007). NVHL rats were found to have higher operant self administration of sweet alcohol solution, but not sucrose or alcohol alone (Berg et al. 2011). A new study evaluating intermittent drinking in home cage and operant self administration of alcohol identified a loss of control of over 20% alcohol drinking in adulthood after animals had been exposed to light drinking in adolescence (10% solution). These animals also reached higher break-points in a progressive ratio schedule of alcohol self administration, and a higher intake in a drug-primed reinstatement session after extinction training. Interestingly, there were no differences between control and NVHL rats in either adolescence or adulthood drinking alone or sucrose consumption (Jeanblanc et al. 2014). Another neurodevelopmental model of SCZ-like phenotype induced by prenatal lipopolysaccharide administration in male offspring showed enhanced alcohol drinking (Liu et al. 2004). Therefore, it seems that the SCZ-like phenotype induced by different neurodevelopmental models enhances vulnerability to alcohol addiction and allows further study of the dual disorder in pre-clinical setting.

METH self-administration study

This set of experiments has shown rather sex-dependent differences that significantly altered addictive

behaviour in the MAM model. Consistent with our previous data, female sex leads to significant increases in operant self administration of sweet pellets (Ruda-Kucerova et al. 2015a), which was also apparent in the MAM animals. Food self administration was very different from the METH-taking behaviour, suggesting a higher motivation for natural reward in females. This was shown as approximately three times more self-administered pellets in females than in males, corresponding with earlier results showing fewer unreinforced responses of female rats when testing for food-reward-lever holding (van Hest et al. 1987). However, the natural reward-oriented outcome is very different from METH-related operant behaviour which rules out the possibility of a general gender-specific difference in the reward processes.

Furthermore, MAM exposure in male rats induced only sporadic differences in self-administered doses of METH, suggesting a trend to lower consumption of the drug in the model. This effect was not apparent in female rats which showed a lower intake than males in the maintenance of METH self administration (Ruda-Kucerova et al. 2015a). In the reinstatement session, the only sex-dependent difference was identified in accordance with increased relapse-like behaviour in females (Ruda-Kucerova et al. 2015a). To our knowledge, this is the first study evaluating potential interactions of sex and the SCZ-like phenotype. However, there are published papers on sex differences in psychostimulant addiction and relapse, and also studies on addiction in neurodevelopmental models of SCZ.

There is a large body of pre-clinical evidence on **sex differences** to psychostimulants showing female rats to be more vulnerable to their behavioural effects (Robinson et al. 1982; Stohr et al. 1998; Becker et al. 2012), readily developing behavioural sensitization after repeated treatment (Robinson 1984; van Haaren and Meyer 1991; Harrod et al. 2005). However, the results on METH addiction in male and intact (not gonadectomized) female rats are quite contradictory. It was shown that there is lower self administration of METH in females (Ruda-Kucerova et al. 2015a), no differences in METH Conditioned Place Preference (Schindler et al. 2002), higher METH intake in both short and 6-h long IVSA sessions or progressive IVSA paradigm (Roth and Carroll 2004; Reichel et al. 2012). These contradictions might originate in different experimental paradigms and dose ranges. Furthermore, a higher reinstatement (relapse-like behaviour) in intact female rats was also reported with both METH (Holtz et al. 2012; Cox et al. 2013; Ruda-Kucerova et al. 2015a) and cocaine (Lynch and Carroll 2000; Lynch and Taylor 2004). This study is consistent with the current results confirming a higher trend of reinstatement to METH seeking in females as shown by the percentage of basal responding.

Other addiction studies of the **SCZ-like phenotype** have shown quite contradictory data on the differential psychostimulant intake in male rats. The NVHL rats were demonstrated to reach higher break-points and gained more METH infusions in a progressive ratio schedule of reinforcement (Brady et al. 2008). In a similar study with cocaine, NVHL males were also reported to meet later extinction criteria and dose-dependently increased drug-primed reinstatement (Chambers and Self 2002). However, a cocaine self-administration study in the MAM model did not show any differences, either in drug taking under fixed or progressive schedules at several doses or in extinction and drug-induced reinstatement (Featherstone et al. 2009). The authors of the study stated that while MAM treatment is known to increase reactivity of the mesocorticolimbic DAergic system, this effect might not be sufficient to alter the reinforcing properties of cocaine. It is important to keep in mind that increased behavioural response to amphetamine is apparently not fully reproducible. The same laboratory has even reported once an increased response of MAM rats to an amphetamine dose of 0.5 mg/kg and not 2 mg/kg (Perez et al. 2013) in a subsequent study with the same design otherwise (Perez et al. 2014). Therefore, the result of no effect reported in the cocaine IVSA study (Featherstone et al. 2009) or a trend of lower METH intake in MAM males in the current study could be a characteristic hyperactive response of the MAM-exposed animals to amphetamine due to basal hyperdopaminergia (Lodge and Grace 2012). If true, this could indicate that MAM-exposed rats may need lower doses for the same DA release in the reward pathway and consequently to experience a similar level of pleasure. This hypothesis could be proven by an in vivo microdialysis study. However, a similar behavioural effect to psychostimulant challenge was demonstrated in the NVHL model (Chambers and Taylor 2004), while reports on IVSA studies were positive (Chambers and Self 2002; Brady et al. 2008).

Another explanation could be based on the pharmacology of amphetamine-like drugs. Given the fact that psychostimulant administration leads to very strong dose-dependent dopamine release in the reward circuit reaching up to several hundreds of percent of the basal level (Di Chiara et al. 2004), a ceiling

effect of the DA release could play a role (Ruda-Kucerova et al. 2015b).

Conclusions

Our study suggests that the female sex and the SCZ-like phenotype induced by prenatal MAM exposure may work synergistically to enhance alcohol consumption. Different SCZ models were used in alcohol studies which have all been demonstrated to have some merit in modelling escalation of alcohol consumption. Future research will be needed to establish paradigms that would be readily assessed to test anti-addiction treatments. Furthermore, adolescence in the prenatally MAM-exposed animals seems to be a vulnerable period during which the MAM-induced phenotype can be pharmacologically changed or modified including drugs of abuse as proven in the case of alcohol (Jeanblanc et al. 2014) and diazepam treatment (Du and Grace 2013).

The usefulness of prenatal MAM exposure for modelling psychostimulant addiction can be questioned. At this stage, the NVHL model seems to be of more interest, but the application of the MAM model to study this type of substance abuse remains under studied. Therefore, it is not possible to conclude that the reward-related processes in the MAM model are intact. Furthermore, sex-dependent variables are not readily assessed in dual-disorder pre-clinical studies; therefore, any conclusion on the matter would not go beyond speculation.

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Statement of interest

None to declare.

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4. Sex-dependent specificities in the drug abuse

There is a large body of clinical evidence suggesting differential characteristics of the disorder in men and women. Despite the absolute number of female methamphetamine abusers being lower than the male ones, women usually appear more dependent, show higher escalation rates (Dluzen and Liu, 2008, Becker and Hu, 2008) and most importantly tend to experience more frequent relapses (Bobzean et al., 2014, Fattore et al., 2014). The abuse of psychostimulant drugs (cocaine, methamphetamine, etc.) is currently on the rise among women, and it has been shown that women experience higher cravings and suffer more relapses than men (Becker and Hu, 2008). These gender specific differences require specific treatment strategies for men and women (Brecht et al., 2004, Munro et al., 2006, Terner and de Wit, 2006). This particularly applies to relapse-prevention which represents a key treatment challenge especially for women (Brecht and Herbeck, 2014). Preclinical studies of drug addiction were carried out with male subject only for a long time because significant influence of the oestrous cycle is well known in terms of behavioural and neurochemical effects but recently this approach has been abandoned on order to identify the gender differences and develop new more specific treatments.

There are four main biological factors accounting for gender differences in the drug addiction:

1) Different levels of sex hormones
2) Gender dependent dimorphism in the brain reward system (unrelated to actual hormonal levels)
3) Different pharmacokinetics and pharmacodynamics of the drugs in men and women
4) Genetic differences linked to sex chromosomes

Levels of male and female gonadal hormones strongly affect the behaviour of people which concerns the addictive one as well (Becker et al., 2012). However, there have been reported also gender dependent differences in the brain structure in both humans and animals (Carroll and Anker, 2010). Another source of the gender differences comprise metabolic adjustments leading to pharmacokinetic changes of the drugs including different

fat deposition, amount of water in the body or proportion of skeletal muscles (Graziani and Nistico, 2015, Fattore and Fratta, 2010) as confirmed in animal models (Milesi-Halle et al., 2015, Milesi-Halle et al., 2007) and clinical experience (Frackiewicz et al., 2000). Pharmacodynamical changes could be underlined by changes in connectivity of the neuronal tracts and neurotransmitter systems which are modified prenatally by gonadal hormones or chromosomes (Graziani and Nistico, 2015, Fattore et al., 2008). These pharmacodynamical specificities are the major source of subjective differences in the effects of the abused drugs and to the variable tendency to develop the addiction, tolerance or sensitization to the drug. Namely dopaminergic system was suggested to be sexually dimorphic, which contributes to the differential reactivity of men and women towards drugs of abuse (Melis et al., 2005). However, there is apparently also a strong hormonal effect on pharmacokinetics of abused substances as shown in cocaine (Niyomchai et al., 2006).

We have focused primarily on the effect of oestrogens and we found an increased methamphetamine intake in females with high levels of oestrogens (Kucerova et al., 2009). This is in accordance with other preclinical and also clinical experience. Clinical studies have shown increased subjective rewarding properties of amphetamine during follicular phase of the menstrual cycle (Justice and De Wit, 2000, Justice and de Wit, 1999) and also higher craving (Carpenter et al., 2006). Preclinical evidence suggests a strong relationship between oestrogens and enhanced addictive behaviour (Becker et al., 2001) while progesterone has opposite effect (Justin et al., 2010, Quinones-Jenab and Jenab, 2010). This approach was already evaluated as a treatment option for cocaine addiction in clinical trials (Evans and Foltin, 2006, De Wit, 2011).

One of the main factors of drug addictions faced by clinicians is the relapse. The hormonal effects on relapse in humans or relapse-like behaviour in animal models were mostly conclusively described showing once again enhancement by oestrogens (Larson and Carroll, 2007, Anker and Carroll, 2011) and suppression by progestins (Lynch and Sofuoglu, 2010, Anker et al., 2007). However, there is a lack of more naturalistic studies which should evaluate the behaviour of female abusers without simplifying the matter to just hormonal levels. Therefore, we designed a simple study to assess relapse-like behaviour in male rats and their female counterparts with a free oestrous cycle and we confirmed increased vulnerability of the female group towards methamphetamine abuse (Ruda-Kucerova et al., 2015a).

4.1. Aims

The research on the sex differences in addictive behaviours aimed to:

1. Investigate the effect of sex and oestrogen levels on intravenous self-administration of methamphetamine
    - Section 4.3.1., Kucerova *et al.*, 2009
2. Extend the approach to a potentially differential influence of behavioural sensitization to methamphetamine in both sexes
    - Section 4.3.1., Kucerova *et al.*, 2009
3. Further validate the model by assessing the relapse-like behaviour towards methamphetamine in both sexes
    - Section 4.3.2., Ruda-Kucerova *et al.*, 2015

4.2. Methods

4.2.1. Animals

Adult rats of Wistar or Sprague-Dawley strain of both sexes were used in the studies. The rats were housed individually in standard rodent plastic cages. Environmental conditions during the whole study were constant: relative humidity 50-60 %, room temperature 23◦C ± 1◦C, inverted 12-hour light-dark cycle. Food and water were available *ad libitum*. All experiments were conducted in accordance with all relevant laws and regulations of animal care and welfare. The experimental protocol was approved by the Animal Care Committee of the Masaryk University, Faculty of Medicine, Czech Republic, and carried out under the European Community guidelines for the use of experimental animals.

4.2.1. Ovariectomy and oestrogen supplementation

In one study female animals were gonadectomized during the IVSA surgery. The ovaries were removed and the uterus below was ligated. Access to the ventral cavity was allowed by one central incision (Caine et al., 2004).

Oestrogen (Estradiol benzoate salt suspension in AGOFOLLIN DEPOT®, Biotika a.s., Slovak Republic dissolved in saline) was administered once a week intramuscularly as a depot formulation. The dose of 0.28 mg/kg used is expected to maintain the hormone plasma levels in the physiological range of rat oestrous cycle (Mendoza-Rodriguez et al., 2003). The control group of ovariectomized rats received the same volume of saline solution instead.

4.2.2. IV self-administration (IVSA) surgery and procedures

The IV self-administration study was performed in the same manner as described in the section 2.3.3.

4.2.3. Locomotor activity

Locomotor activity was assessed as described in the section 2.3.5.

4.3. Results

4.3.1. Impact of repeated methamphetamine pretreatment on intravenous self-administration of the drug in males and estrogenized or non–estrogenized ovariectomized female rats

The present study was designed to evaluate the effects of gender, oestrogen, and potential behavioural sensitization (Robinson and Berridge, 1993, Vanderschuren and Pierce, 2010) expected to be associated with repeated methamphetamine pretreatment on methamphetamine intake in the intravenous self-administration paradigm in male rats and ovariectomized female rats with and without oestrogen substitution.

The highest spontaneous methamphetamine intake in the IV self- administration was demonstrated in estrogenized ovariectomized females, with lower intake in males, and the lowest intake in non-estrogenized ovariectomized females. Repeated pre-exposure to methamphetamine produced a significant decrease in the mean number of methamphetamine infusions self-administered per sessions in males, as well as, in estrogenized ovariectomized females, but not in non-estrogenized ones. This may indicate that methamphetamine infusions self-administered during the sessions produced stronger reinforcing effects in rats previously exposed to methamphetamine than in drug naïve animals and that the lack of oestrogen in ovariectomized females may provide protection from the development of such changes in drug effects. In humans, it has been demonstrated that higher levels of oestrogen are associated with greater subjective stimulation after amphetamine in women (White et al., 2002), but amphetamine-stimulated dopamine release can be greater in men (Dluzen and Liu, 2008), which could perhaps increase vulnerability of men to neurotoxic effects of amphetamines (Munro et al., 2006). The findings from the pre-clinical and clinical studies should be taken into an account when creating specific prevention and treatment programs for men and women.

Kucerova J, Vrskova D, Sulcova A. Impact of repeated methamphetamine pretreatment on intravenous self-administration of the drug in males and estrogenized or non-estrogenized ovariectomized female rats. Neuro Endocrinol Lett. 2009, 30(5): 663-70.

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Impact of repeated methamphetamine pretreatment on intravenous self-administration of the drug in males and estrogenized or non–estrogenized ovariectomized female rats

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Key words: **methamphetamine; IV self-administration; methamphetamine intermittent pretreatment; gender differences; ovariectomy; estrogen; wistar rats**

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Abstract

OBJECTIVE: The female animals were already recorded to respond differently to methamphetamine (MET) abuse than males. This gender dissimilarity may be caused by the influence of estral cycles and different susceptibility to behavioural sensitization.

METHODS: Influences of gender and pre-exposure to MET were studied in the rat model of MET intravenous self-administration (IVSA). The fixed ratio (FR) paradigm was employed in male rats (M) and estrogenized (F-ESTR) and non-estrogenized ovariectomized female rats (F-OVX) either pre-exposed or not-exposed to MET pretreatment.

RESULTS: In rats that were not pre-exposed to MET, F-ESTR self-administered more MET infusions than each of the other groups, but F-OVX self-administered less than each of the other groups; the same trend was apparent in the MET pre-treated groups. MET pre-exposure decreased subsequent MET IVSA in all groups except F-OVX.

CONCLUSION: Thus, pre-exposure to MET and the loss of inherent estrogen in females notably decreased the intake of MET by rats, suggesting that abuse liability was reduced. Estrogen's effects on MET self-administration here correspond with accumulating evidence of stronger behavioural responses of females to drugs of abuse.

Abbreviations :

MET	- methamphetamine
SAL	- saline
M	- males
OVX	- ovariectomy
F-OVX	- ovariectomized females
F-ESTR	- estrogenized ovariectomized females
IVSA	- IV self-administration

INTRODUCTION

Although the role of gender in the mechanisms of drug action remains unclear, both preclinical and clinical studies indicate that ovarian hormones, particularly estrogen, play a role in producing sex differences in drug abuse (Lynch *et al.* 2002). These differences in the behavioural reactivity to drugs in men and women (Brecht *et al.* 2004, Munro *et al.* 2006) will probably require different strategies in the prevention and treatment of addiction depending on gender. So far, most experimental studies have been conducted with male subjects; many researchers have chosen to ignore the influences of the estrous cycle, which is more difficult to study, but has an important impact on animal behaviour. The reasons for differences in female reactivity to drugs can be due to pharmacokinetic specifics (Milesi-Halle *et al.* 2007) such as distinct metabolising enzyme activities, distribution volume and other parameters. Pharmacodynamic effects of drugs can also be dependent on structural changes induced in early life by the physiological hormonal levels in the brain or on particular receptor gene expression modulated by sex hormones (Hu *et al.* 2004). Experiments in laboratory rodents showed that estrogen levels can regulate behavioural responses to drugs of abuse, especially psychomotor stimulants, including methamphetamine (MET). During estrus, the effects of abused drugs are more pronounced, and this is reflected in gender differences in general patterns of drug abuse (Sell *et al.* 2002, Becker & Hu, 2008)

Behavioural sensitization to drugs and the adaptations in striatal neurotransmission that are associated with this sensitization are thought to play an important role in certain aspects of addiction (Ohmori *et al.* 2000). Dopaminergic activity in the brain is enhanced by estrogen in positive correlation with behavioural effects (Thompson & Moss, 1994, Thompson *et al.* 2000, White *et al.* 2002). However, positron emission tomography (PET) scans performed after amphetamine administration in humans showed increased reactivity of the striatal dopamine system in men compared with women (Munro *et al.* 2006). The interactions between ovarian hormones, dopamine, and drugs of abuse are not clear yet, and more studies are necessary for further elucidation. Nevertheless, in a number of studies females were more prone to develop sensitization than males (Phillips *et al.* 1997, Becker *et al.* 2001, Sell *et al.* 2002, Hu & Becker, 2003, Kawakami *et al.* 2007, Kucerova *et al.* 2008).

Intravenous drug self-administration by laboratory animals is a model for testing dependence potential and abuse liability of drugs (Collins *et al.* 1984). Compared to males, females are reported to become addicted more rapidly (and subsequently to relapse more readily following abstinence) when drugs of abuse are offered in the drug self-administration model at lower doses (Becker & Hu, 2008). The acquisition rate was found to be faster in female compared to male rats self-administering nicotine, alcohol, heroin, cocaine or MET (Lynch *et al.* 2002), presumably indicating that the reinforcing effects of these drugs are stronger in females. Rates of acquisition are also dependent on variables such as drug dose, circadian variability in access to drug and previous drug exposure (potentially leading to behavioural sensitization) (Roth & Carroll, 2004).

Understanding the influences of gender and sex hormones on drug self-administration in animals may lead to improved strategies for treatment and prevention of drug abuse in humans. While progress is beginning to be made in this area, much remains to be done. In particular, methamphetamine is a widely-abused and highly-addictive drug that has serious health consequences, yet has received less research attention than other major drugs of abuse. Therefore, the present study was designed to evaluate the effects of gender, estrogen, and potential behavioural sensitization expected to be associated with repeated MET pretreatment on MET intake in the intravenous self-administration (IVSA) paradigm in male rats (M) and ovariectomized female rats with (F-ESTR) and without estrogen substitution (F-OVX).

MATERIAL AND METHODS

Animals

The present study with Wistar rats (purchased in the Laboratory Animal Breeding and Experimental Facility, Masaryk University Brno, Czech Republic) consisted of 6 experiments (2 with males – M: n=36, 2 with ovariectomized females – F-OVX: n=36, 2 with ovariectomized females substituted regularly with estrogen – F-ESTR: n=36). Adult male rats weighing 350–400 g and female rats weighing 250–300 g at the beginning of experiment (in order to assure the catheter position stability in animals not growing much to the length anymore) were housed in sections of five in standardized rat plastic cages during the first two weeks of the experiment. After the catheter implantation surgery was performed, rats were housed individually in plastic cages standardized for separate stabling. During the whole experiment the environmental conditions were constant: relative humidity 50%, temperature 23 °C ± 1 °C, inverted 12-hour light-dark cycle (5 a.m. to 5 p.m. darkness). Food and water were available *ad libitum*. All experiments were conducted in accordance with all relevant laws and regulations of animal care and welfare. The animal study protocol was approved by the Animal Care Committee of the Masaryk University Faculty of Medicine, Brno, Czech Republic, and carried out under the European Community guidelines for the use of experimental animals.

Surgery

Under general anaesthesia (xylazine 8 mg/kg + ketamine 50 mg/kg intraperitoneally in combination with

isoflurane inhalation for induction to anaesthesia) a permanent intracardiac silastic catheter (our own production) was implanted through the external jugular vein into the right atrium. The outer part of the catheter exited the skin in the midscapular area. A small nylon bolt was fixed on the skull with dental acrylic to stainless-steel screws embedded in the skull; this served as a tether to prevent the catheter from being pulled out while the rat was in the self-administration chamber. During the surgery all the female animals were gonadectomized. The ovaries were removed and the uterus below was ligated. Access to the ventral cavity was permitted by one central incision. The catheters were flushed daily before all the sessions with 0.2 ml of heparinized cephalosporine (VULMIZOLIN 1.0 inj sic, Biotika a.s., Slovak Republic) solution (0.05 mg/kg in saline with 2.5 I.U./kg) and 0.05 ml of heparin (HEPARIN LECIVA inj. sol. 1x10ml/50 I.U.) solution (5 I.U.) to prevent infection and occlusion of the catheter. During this procedure the blood was aspired daily to assess the patency of the catheter, and changes in general behaviour, weight and other circumstances were recorded. When a catheter was found blocked the animal was excluded immediately from the analysis.

Drugs and treatments

Methamphetamine (MET) from Sigma Chemical, Co., St Louis, MO, USA was used for both intraperitoneal drug pretreatment and IVSA. The administration of MET prior to IVSA was according to the following dosing regimen, which was successfully used in our previous studies (Landa *et al.* 2006, Landa *et al.* 2008) to induce behavioural sensitization: 0.5 mg/kg/day, intraperitoneally, for 14 days, administered in home cages. The identical volume (1 ml/kg/day) and route of administration of saline solution (SAL) were used for all control treatments. The MET dose available for IVSA was 0.08 mg/kg per single infusion with the maximum number of infusions during one session set to 50 (Vinklerova *et al.* 2002).

Estrogen (Estradiol benzoate salt suspension in AGOFOLLIN DEPOT®, Biotika a.s., Slovak Republic dissolved in saline) was administered to ovariectomized females (F-ESTR) once a week intramuscularly as a depot (Shansky *et al.* 2004). The dose of 0.28 mg/kg used is expected to maintain the hormone plasma levels in the physiological range of rat estrous cycle (Mendoza-Rodriguez *et al.* 2003). The other group of ovariectomized rats (F-OVX) received the same volume of saline solution instead.

Each animal group (M, F-ESTR, and F-OVX) was randomly divided into four subgroups ($n_{1,2,3,4}$) for the following treatments: a) n_1=6: 14 days of daily pretreatment with saline (SAL), 1.0 ml/kg, intraperitoneally + the 15th day surgery procedure + 14 days of recovery and drug washout + 21 days of IVSA of SAL; b) n_2=12: 14 days of daily pretreatment with SAL, 10.0 ml/kg, intraperitoneally + the 15th day surgery procedure + 14 days of recovery and drug washout + 21 days of IVSA of methamphetamine (MET); c) n_3=6: 14 days of daily pretreatment with MET, 0.5 mg/kg, intraperitoneally + the 15th day surgery procedure + 14 days of recovery and drug washout + 21 days of IVSA of SAL, 1.0 ml/kg, intraperitoneally; d) n_4=12: 14 days of daily pretreatment with MET, 0.5 mg/kg, intraperitoneally + the 15th day surgery procedure + 14 days of recovery and drug washout + 21 days of IVSA of MET, 0.5 mg/kg, intraperitoneally. The MET or SAL pretreatment was given in the home-cage daily at the same time within the dark period of the light cycle.

Self-administration apparatus and procedure

Standard experimental chambers (with all accessories provided by Coulbourn Instruments, USA) with two nose-poke holes allocated on one side of the cage were programmed by software L2T2 (Coulbourn Instruments, USA). IVSA sessions were initially conducted under the fixed ratio (FR) schedule of reinforcement starting at FR1 (each correct response reinforced). Fixed-ratio requirements were raised (e.g. FR2 – two correct responses required, FR3 – three correct responses required, etc.) when the animal fulfilled the following conditions for three consecutive sessions: a) at least 70% preference for the active nose-poke; b) minimum intake of 10 infusions per session; c) stable intake of the drug (maximum 10% deviation). Active nose-pokes led to the activation of the infusion pump and administration of a single infusion paired with a 2-s light cue, followed by a 30-sec time-out. Nose-pokes in the other (non-active) hole were recorded but had no programmed consequences. The cage was illuminated by a house light, which was off during the time-out. There were 21 daily sessions in 21 consecutive days, each lasting 120 minutes and taking place regularly between 7 a.m. and 4 p.m. during the dark period of the reversed light cycle. After the session the animals were returned to the home-cage.

Statistical Data analysis

For statistical analysis of differences in either saline or MET IVSA the Mann-Whitney U test was applied (comparing nose-poke responses on the active lever to those on the inactive lever), and for evaluation of the IVSA acquisition rates a Survival Data Analysis (Peto-Peto-Wilcoxon test) was used. Level of statistical significance was determined to $p<0.05$.

RESULTS

Table 1 demonstrates the reinforcing properties of the dosing IVSA MET schedule as all groups of rats (M, F-ESTR, F-OVX) regardless of repeated pretreatment (SAL or MET) exhibited preference for active (reinforced) nose-poke over the inactive (non-reinforced) nose-poke when nose-poking was reinforced by MET (0.08 mg) infusions. In each of these groups the number

Tab. 1. The table shows the mean number of nose-pokes in the 21 IVSA sessions (non-active: not associated with IVSA; active: associated with IVSA) exhibited during the whole experiment by rat males (M) and ovariectomized females with presence (F-ESTR) or absence (F-OVX) of estrogen substitution (depot suspension of estradiol benzoate, 0.28 mg/kg/week) after 14 days of withdrawal from 14 day intraperitoneal pretreatment with either saline (SAL) or methamphetamine (MET – 0.5 mg/kg/day). Statistical evaluation was processed by the Mann-Whitney U test.

Group	Pretreatment	IVSA	Mean No. Of nose-pokes per session		Mann Whitney U-test result
			active	non-active	
Males (M)	saline	saline	**9.44**±3.01	**6.95**±1.94	**NS**
	saline	MET	**48.34**±10.76	**8.39**±2.47	**p=0.0001**
	MET	saline	**8.38**±1.37	**3.92**±0.76	**NS**
	MET	MET	**40.67**±9.80	**7.66**±1.81	**p=0.0001**
Female estrogenized castrates (F ESTR)	saline	saline	**9.73**±3.91	**7.22**±1.69	**NS**
	saline	MET	**51.32**±8.88	**13.29**±1.96	**p=0.0001**
	MET	saline	**9.16**±2.70	**5.49**±1.61	**NS**
	MET	MET	**44.86**±8.51	**9.68**±3.18	**p=0.0001**
Female castrates (F OVX)	saline	saline	**12.92**±4.04	**3.40**±0.83	**NS**
	saline	MET	**20.71**±2.47	**8.34**±1.47	**p=0.0001**
	MET	saline	**19.41**±5.86	**4.60**±1.83	**NS**
	MET	MET	**25.16**±9.84	**11.56**±3.11	**p=0.0147**

of active nose-pokes reinforced by MET was significantly higher than number of inactive nose-pokes. On the other hand, the number of nose-pokes into active vs. inactive hole was not significantly different in groups that were allowed to self-administer saline, regardless of repeated pretreatment (SAL or MET).

Figure 1 shows the percentage of rats from each group (M, F-ESTR, F-OVX) acquiring MET self-administration after 14 days of withdrawal after 14 day pre-treatment with saline (A) or methamphetamine (B) that met the criteria for increasing FR from initial FR1 to FR2. Differences between acquisition rates of MET IVSA of individual rat groups are shown in both parts of the figure (A and B) were not significant according to a Survival Data Analysis (Peto-Peto-Wilcoxon test). However, there is an apparent trend in both conditions (A-absence or B-presence of MET pre-exposure) showing that non-estrogenized female castrates (F-OVX) exhibited the slowest rate in reaching higher FR conditions of all three groups, and the lowest incidence of meeting this criterion.

This trend is also apparent in Figures 2A and B: at the end of experiment (21st day of consecutive sessions) the highest cumulative percentage of animals staying on FR1 conditions were non-estrogenized female castrates (F-OVX). In contrast, the highest FR7 requirement for MET IVSA was performed only by the group of male rats (M) after MET pre-exposure (Figure 2B). However, there was no significant difference in acquisition rate of MET self-administration between SAL and MET pretreated animals, although some of the latter animals were able to reach one-step higher FR as a more demanding IVSA condition (more nose-pokes needed to obtain one MET infusion) compared to saline pretreated rats (Figure 2).

Figure 3 shows acquisition of MET self-administration over 21 consecutive sessions in all three groups of rats (M, F-ESTR, F-OVX) with (Figure 3B) or without (Figure 3A) repeated pretreatment with MET. The F-OVX group self-administered the lowest while F-ESTR group the highest number of MET infusions over the course of the experiment under both pretreatment conditions (MET or SAL). In the groups that received repeated SAL pretreatment (Figure 3A), the number of MET infusions received was significantly higher in F-ESTR group compared to M (p=0.005) and F-OVX (p=0.0001) groups, and F-OVX animals were consuming significantly (p=0.0001) lower number of MET infusions than both M and F-ESTR animals. The same trend was apparent in the MET pretreated groups (Figure 3B). The number of infusions self-administered by F-ESTR group was significantly higher than in the M (p=0.0001) and F-OVX (p=0.0001) groups while F-OVX animals self-administered significantly (p=0.0001) lower number of MET infusions (Figure 3B).

Figure 4B shows that M and F-ESTR groups repeatedly exposed to MET self-administered a significantly lower number of MET infusions than those to saline (M: p<0.0005 and F-ESTR: p<0.001). The MET pretreatment had no effect on number of infusions self-administered in the F-OVX group (p=0.0849). Figure 4A shows that there were no significant differences in the SAL IVSA in any of experimental groups after both, pretreatment with SAL and MET. The only exception was the group F-OVX which self-administered higher number of SAL infusions after repeated

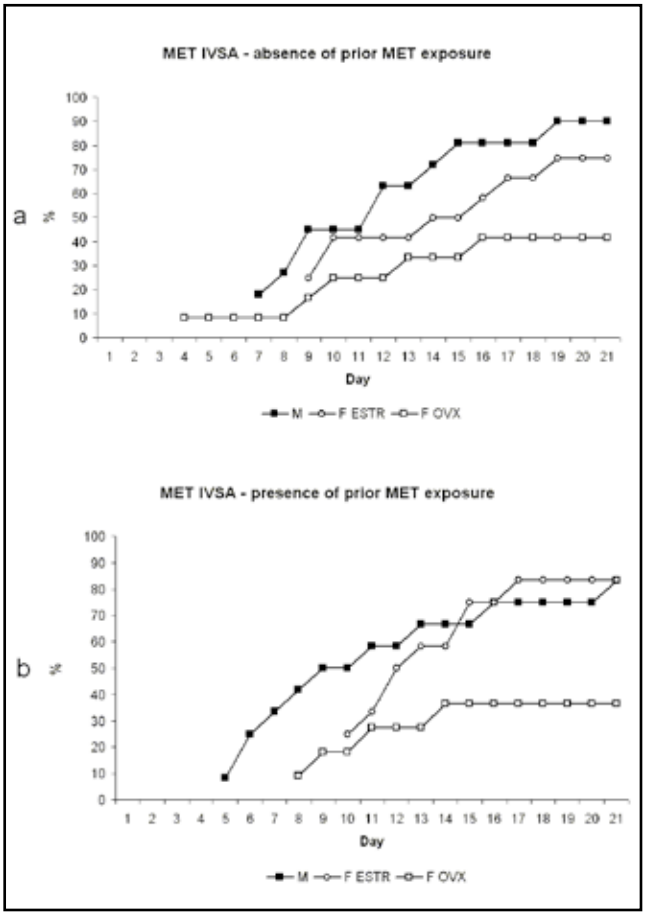

Fig. 1. The cumulative percentage of males (M) and ovariectomized females either with (F-ESTR) or without (F-OVX) estrogen substitution (depot suspension of estradiol benzoate, 0.28 mg/kg/week) after 14 days of withdrawal from 14-day intraperitoneal pretreatment with either (a) saline (SAL) or (b) methamphetamine (MET – 0.5 mg/kg/day) that met the criteria for MET IVSA for switching from FR1 to FR2 conditions in 21 consecutive sessions of the experiment. Evaluation: Survival Data Analysis (Peto-Peto-Wilcoxon test, non-significant).

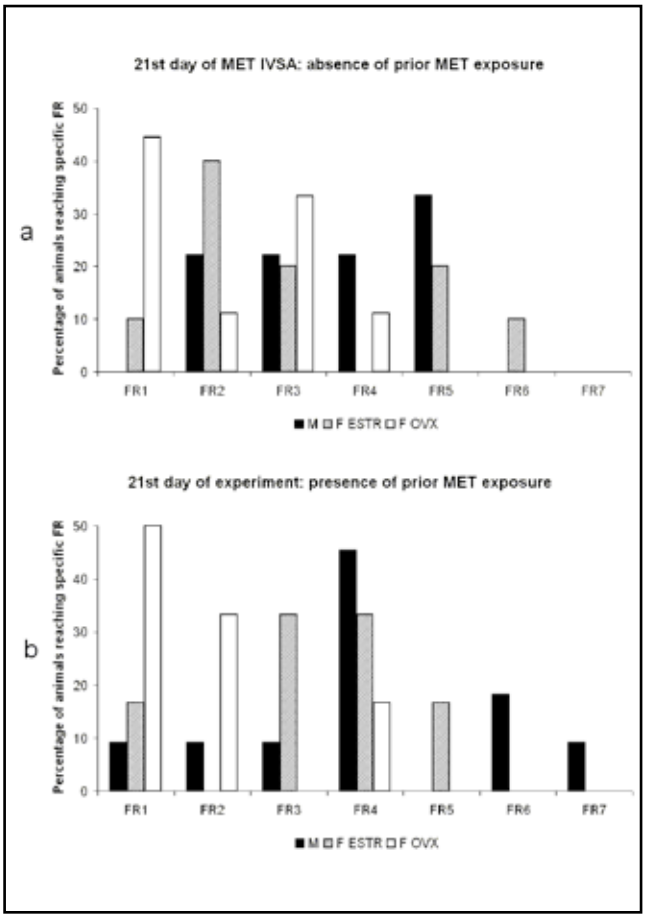

Fig. 2. The percentage of rat males (M) and ovariectomized females either with (F-ESTR) or without (F-OVX) estrogen substitution (depot suspension of estradiol benzoate, 0.28 mg/kg/week) meeting the criteria for MET IVSA under gradually increasing FR requirements after 14 days of withdrawal from 14-day intraperitoneal pretreatment with either (a) saline (SAL) or (b) methamphetamine (MET – 0.5 mg/kg/day).

pretreatment with MET. This difference was significant when compared with matching M and F-ESTR groups (M: $p<0.001$ and F-ESTR: $p<0.001$). In all groups the mean intake was notably lower than 10 infusion/session criterion used as an indicator of reinforcing effects.

DISCUSSION

This study demonstrates that Wistar rats repeatedly exposed to MET (14 daily doses of 0.5 mg/kg) self-administered a lower number of MET infusions under a fixed ratio schedule (FR) of MET infusions (0.8 mg/infusion) compared to animals pretreated with saline. The same trend was observed to some extent in all groups of rats (M, F-ESTR, and F-OVX) but differentially depending on the gender. Both estrogenized ovariectomized female groups (F-ESTR) regardless of prior repeated MET or SAL pretreatment self-administered higher numbers of MET infusions than corresponding male groups. The while non-estrogenized ovariectomized (F-OVX) female groups were self-administering the lowest number of MET infusions regardless of prior MET exposure, and also their acquisition rates were the lowest. Though there was no statistically significant difference, an apparent trend of facilitation of MET IVSA acquisition was present in rats repeatedly pre-exposed to MET. This increased drug-seeking behaviour in this model could be considered a sign of behavioural sensitization (Lorrain *et al.* 2000) , which however can be influenced by the IVSA experimental paradigm itself. A similar trend was described by Lorrain *et al.* (2000) in the rats self-administering amphetamine under a progressive ratio schedule but not under a fixed ratio schedule. The acquisition of IVSA behavior might also be influenced by a previous association of the drug effect and the experimental

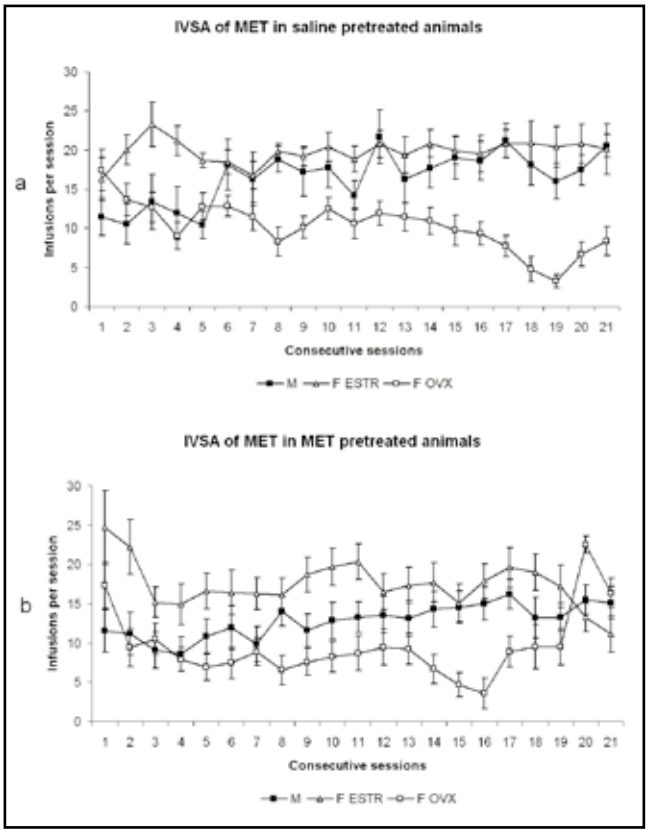

Fig. 3. The acquisition of methamphetamine (MET) infusions (0.08 mg/infusion) in all 21 IVSA daily sessions in the male rats (M - filled squares, A: n1=11, B: n2=12), ovariectomized female rats with estrogen substitution (F-ESTR - open circles, a: n1=11, b: n2=12), and ovariectomized female rats with no hormonal substitution (F-OVX - open squares, a: n1=11, b: n2=11) after 14-day withdrawal from 14 days of intraperitoneal pretreatment with either (**a**) saline (SAL) or (**b**) methamphetamine (MET – 0.5 mg/kg/day). Data are shown as daily means ± SEM.

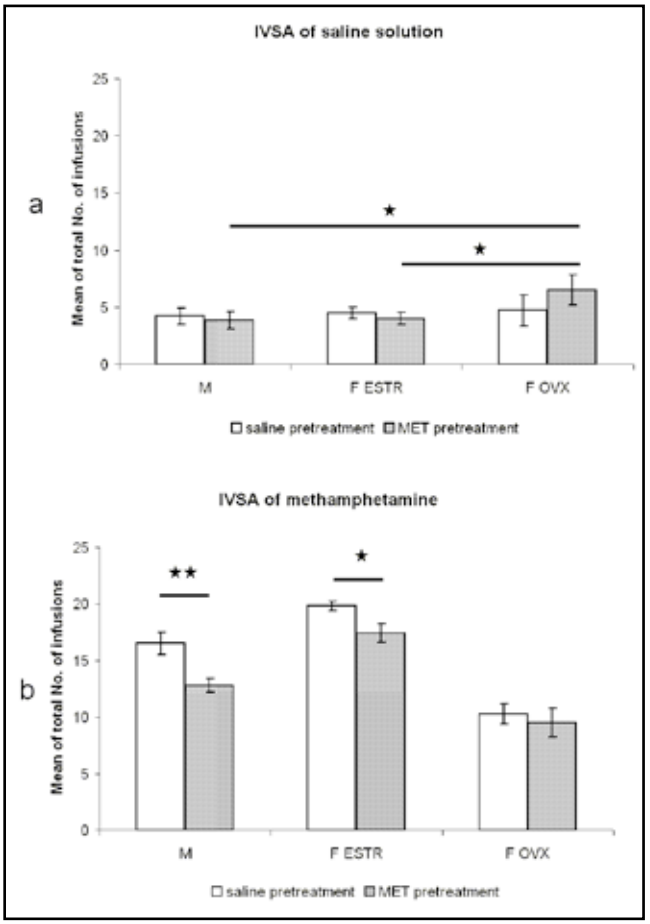

Fig. 4. The effect of methamphetamine (MET) pretreatment (0.5 mg/kg/day, for 14 days, intraperitoneally) on either saline (SAL) (**a**) or MET (**b**) self-administration after 14-day withdrawal from the pretreatment. The graphs show the mean number of IVSA infusions received during the whole experiment in male rats (M) and ovariectomized females rats either with (F-ESTR) or without (F-OVX) estrogen substitution (depot suspension of estradiol benzoate, 0.28 mg/kg/week). Groups of rats after control SAL pretreatment = open bars, groups of rats after MET pretreatment = filled bars. Data shown as means ± SEM. Statistical evaluation was done by using the Mann-Whitney U test (* $p<0.001$, ** $p<0.0005$).

cage environment (Reid *et al.* 1998). In this study, the animals were pre-exposed to MET in their home-cages. In a parallel experiment a different group of male rats were placed for 30 minutes after the intraperitoneal administration of MET pre-treatment into the operant cage used later for IVSA sessions. No significant differences in acquisition rates of MET IVSA were found when compared with the experimental design used in the present study (unpublished data).

The susceptibility of the female organism to effects of drugs of abuse, including the induction of behavioural sensitization, has mostly been reported by both pharmacological experimental studies and clinical trials as being higher and enhanced further by increased estrogen levels: (Lynch *et al.* 2002, Becker & Hu, 2008). The pharmacokinetic and metabolic profiles of a drug have also been suggested as playing significant roles in the differential pharmacological response to MET in male and female rats. Slower MET clearance and lower metabolite (amphetamine) formation were reported in the Sprague-Dawley female rats (Milesi-Halle *et al.* 2005, Milesi-Halle *et al.* 2007). The development of behavioural sensitization has also been reported to vary between rat strains, possibly due to different brain penetration of MET. In Wistar rats, the brain penetration was found to be increased in repeatedly and behavioural sensitization to the effects of MET were observed in MET-treated animals, but these effects were not found to occur in Long-Evans strain (Fujimoto *et al.* 2007). In the present experiment using Wistar rats with the IVSA method, which is the most widely used model for assessing the relative abuse liability of drugs of abuse, we confirmed that estrogen levels can influence intake of MET. The repeated pre-exposure to MET, which was proven to induce behavioural sensitization to stimulatory effects on locomotion in the same rat

strain (Landa *et al.* 2008), lowered the number of MET infusions self-administered during consecutive 21 daily sessions in all groups (M, F-OVX and F-ESTR). This effect is not likely to have been due to habituation as the control SAL-pretreated rats were allowed to self-administer MET at the same IVSA paradigm. It is also unlikely that the lower rates of IVSA seen after MET pre-exposure were actually due to sensitization (comparable to increasing the dose per infusion), since there was evidence that MET exposed rats were less likely to acquire the self-administration response. Under the fixed-ratio procedure used here, the decreased IVSA after MET pre-exposure as well as after ovariectomy were likely due to a reduced motivation to obtain MET or a reduced reinforcing effect of MET.

Reports in the existing literature on the relationship between stimulatory effects of drugs on rodent locomotion and their IVSA are not consistent. Nevertheless, the neurobiological basis of the brain systems underlying both locomotor activation (Schindler *et al.* 2002) and reward are believed to be sexually dimorphic (for review see: (Dluzen & Liu, 2008, Becker *et al.* 2001). The pharmacological mechanism of action of amphetamine and its derivatives (such as MET) involves indirect adrenergic action inducing a massive release of biogenic amines, particularly dopamine and noradrenaline, from the storage sites in nerve terminals to the synapses, and blockade of their reuptake (Kish, 2008). There are confirmed sex differences in changes of dopamine extracellular levels and turnover induced by methamphetamine in rodents (for review see: (Becker & Hu, 2008), as well as in dopamine release in humans (Munro *et al.* 2006). However, in rats, structural differences caused by the sex hormones were also found in the early brain ontogenesis, which was not dependent on the actual hormonal level (Hu *et al.* 2004). According to binding studies, there are sex differences reported in densities of dopamine receptor subtypes in the rat striatum and the nucleus accumbens fluctuating dependently on estrous cycle (Becker & Hu, 2008). The number of dopamine D1 (and to some extent also of D2) binding sites is higher in male rats compared to females and estradiol administration is shown to downregulate D2 receptors in dorsolateral striatum while enhancing basal dopamine extracellular concentrations ("dopaminergic tone") (Xiao & Becker, 1994). Thus, estradiol can elicit changes in dopamine release and dopamine receptor activity leading to greater behavioural response to psychostimulant drugs in intact females in estrus or estrogenized ovariectomized females. This correlates well with a report that under IVSA with FR conditions female rats obtained significantly more MET infusions (0.02 mg/infusion) compared to males (Roth & Carroll, 2004), as well as with the results of the present study in which the estrogenized ovariectomized females (F-ESTR with and without pre-exposure to MET) self-administered the highest number of MET infusions (0.08 mg/infusion). The higher number of MET infusions in M groups compared to F-OVX groups in our experiment corresponds with suggestion that due to higher basal dopamine tone in the male striatum and the nucleus accumbens (Xiao & Becker, 1994), a greater dopaminergic stimulation is required to achieve a rewarding effect (Becker & Hu, 2008).

Our results also showed a significantly higher saline intake in the F-OVX group repeatedly pre-exposed to MET compared to the rest of the SAL self-administering groups. Intravenous SAL is not usually found to have reinforcing effects, but it is known that estrogen can influence electrolyte homeostasis. In the rat model of angiotensin II-induced thirst, the chronic administration of estradiol attenuated water-seeking behaviour (Fregly & Thrasher, 1978). This was confirmed further by other rat experiments and evaluated as central interaction mechanism between this peptide hormone and estrogen on a genomic level (Kisley *et al.* 1999). Estrogens also may influence body fluid regulation by interacting with several neurotransmitters, including serotonin, dopamine and noradrenaline (Kucharczyk, 1984). In rats it was proven that water drinking can be initiated by administration of dopaminergic drugs (Zabik *et al.* 1993). This could be reason for higher IVSA saline intake in the F-OVX rat group lacking estrogen influence and moreover being pretreated with dopaminergically acting MET in the present study.

In summary, the highest spontaneous methamphetamine intake in our model of MET IV self- administration in rats was demonstrated in estrogenized ovariectomized females, with lower intake in males, and the lowest intake in non-estrogenized ovariectomized females. Repeated pre-exposure to MET (potentially inducing behavioural sensitization) produced a significant decrease in the mean number of MET infusions self-administered per sessions in males, as well as, in estrogenized ovariectomized females, but not in non-estrogenized ones. This may indicate that MET infusions self-administered during the sessions produced stronger reinforcing effects in rats previously exposed to MET than in drug naïve animals (perhaps due to behavioural sensitization) and that the lack of estrogen in ovariectomized females may provide protection from the development of such changes in MET effects. Thus, preclinical studies indicate that behavioural and neurobiological responses to psychostimulant drugs are sexually dimorphic and point to a particular role of estrogen, but all mechanisms underlying this dimorphism are not completely clear yet. In humans, it has been demonstrated that higher levels of estrogen are associated with greater subjective stimulation after amphetamine in women (White *et al.* 2002), but amphetamine-stimulated dopamine release can be greater in men (Dluzen & Liu, 2008), which could perhaps increase vulnerability of men to neurotoxic

effects of amphetamines (Munro *et al.* 2006). The findings from the pre-clinical and clinical studies should be taken into an account when creating specific prevention and treatment programs for men and women.

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4.3.2. Sex differences in the reinstatement of methamphetamine seeking after forced abstinence in Sprague-Dawley rats

The aim of this study was to assess gender differences in all stages of operant IV self-administration of methamphetamine in male and female rats (Fattore et al., 2014) while the gonads of all animals were kept intact assuring physiological oestrous cycle in females. Furthermore, possible gender differences in acquisition and maintenance of food self-administration were assessed in order to compare the operant behaviour towards natural reward (food) and the drug of abuse.

The data showed a lower consummator methamphetamine intake during maintenance phase of the self-administration together with higher vulnerability to the reinstatement of methamphetamine seeking behaviour in female rats after forced abstinence. These effects seem to be robust enough, thus relatively independent on the current hormonal level. Therefore, we propose this paradigm for preclinical screening for potential new medications specific for women.

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Sex differences in the reinstatement of methamphetamine seeking after forced abstinence in Sprague-Dawley rats

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Preventing relapse to drug abuse is one of the struggles faced by clinicians in order to treat patients with substance use disorders (DSM-5). There is a large body of clinical evidence suggesting differential characteristics of the disorder in men and women, which is in line with preclinical findings as well. The aim of this study was to assess differences in relapse-like behavior in methamphetamine (METH) seeking after a period of forced abstinence, which simulates the real clinical situation very well. Findings from such study might add new insights in gender differences in relapse mechanisms to previous studies, which employ a classical drug or cue-induced reinstatement procedure following the extinction training. Adult male and female Sprague-Dawley rats were used in IV self-administration procedure conducted in operant boxes using nose-poke operandi (Coulborn Instruments, USA). Active nose-poke resulted in activation of the infusion pump to deliver one intravenous infusion of METH (0.08 mg/kg). After baseline drug intake was established (maintenance phase), a period of forced abstinence was initiated and rats were kept singly in their home cages for 14 days. Finally, one reinstatement session in operant boxes was conducted. Females were found to self-administer significantly lower dose of METH. The relapse rate was assessed as a number of active nose-pokes during the reinstatement session, expressed as a percentage of active nose-poking during the maintenance phase. Females displayed approximately 300% of active nose-pokes compared to 50% in males. This indicates higher vulnerability to relapse of METH seeking behavior in female rats. This effect was detected in all females, independently of current phase of their estrous cycle. Therefore, this paradigm using operant drug self-administration and reinstatement of drug-seeking after forced abstinence model can be used for preclinical screening for potential new anti-relapse medications specific for women.

Keywords: methamphetamine, reinstatement of drug-seeking behavior, forced abstinence, sex/gender differences, Sprague-Dawley rats

Introduction

Methamphetamine (METH) addiction is a serious psychosocial problem, which leads to organic harm of the body as well as distortion of the normal functioning of affected people within the society and family. There is a large body of clinical evidence suggesting differential characteristics of the disorder in men and women. Despite the absolute number of female METH abusers being lower than the male ones, women usually appear more dependent, show higher escalation rates (1, 2) and most importantly tend to experience more frequent relapses (3, 4). These gender specific differences require specific treatment strategies for men and women (5–7). This particularly applies to relapse-prevention, which represents a key treatment challenge especially for women (8).

The preclinical approach to model drug addiction with the highest validity is usually considered as the operant drug self administration. To mimic relapse in this paradigm, a period of extinction procedure can be employed when the animal still has a regular access to the operant box but the drug delivered by infusion pump is replaced by vehicle. After certain number of sessions, the subject stops to respond to the active operandum (e.g., lever or nose-poke). After reaching a specific extinction criteria (number of active/inactive responses lower than a set number), one last session is conducted and the reinstatement of the drug-seeking behavior is primed by an environmental factor (stress, cues) or a drug dose. Such studies have repeatedly shown female rats to be more vulnerable to drug-primed relapse of METH seeking behavior at conditions of time limited sessions (2 h), which mimic rather consummatory behavior, as well as prolonged self-administration sessions. This is considered to provide a better model for loss of control over drug taking, leading to escalation of drug consumption (9) known from a clinical situation (3). Similarly, a higher relapse-like behavior was found in female rats after priming by conditioned cue and to even higher extent by METH dose (10). Earlier, analogous results were reported in studies with cocaine (11, 12) and fentanyl (13).

However, this paradigm does not mimic the human treatment very well, because the patient usually discontinues the drug abuse in the drug rehabilitation center and for some time does not have access to the drug-related environments. Therefore, a forced abstinence model was developed where the animal does not have access to the operant box and is kept in the home cage for some time (14–16); thus, the motivation of drug response behavior is not influenced by any training procedures.

Furthermore, many preclinical studies, which assess sex-dependent differences, isolate the hormonal effect either by ovariectomy and subsequent hormonal supplementation (17, 18) or by constant tracking of the estrous cycle phase (10, 19). These approaches already explained extensively the role of gonadal hormones in the reward processes showing enhancement of drug intake by estradiol (17, 18, 20–22) and attenuation of drug seeking by progesterone (4, 23). However, the possibilities of clinical applications of these findings are limited, so far only progesterone was tested as a treatment for nicotine relapse in women (24) and such treatment would have many undesirable side effects. Consequently, an ideal animal model with high face, construct, and predictive validity for testing new relapse-prevention treatments should not be based on hormonal levels only.

The intact animals (males and freely cycling females) showed no sex differences to effects of amphetamines in the animal model of conditioned place preference (CPP) (25, 26). Interestingly, CPP for METH did not occur in ovariectomized rats but developed in females treated with estradiol (27). Therefore, gender differences in the CPP paradigm might be biased by fluctuating hormonal levels in intact females. However, results supporting higher vulnerability to METH in intact female rats were reported too. Female rats displayed higher increase of locomotor activity, which lasted for longer time and had higher scores of stereotypies than male rats (28). These results indicate the sex differences may depend, besides hormonal influences, also on different pharmacokinetic processes in females (29).

Therefore, the aim of this study was to assess gender differences in all stages of operant IV self-administration of METH in male and female rats while the gonads of all animals were kept intact assuring physiological estrous cycle in females. We expected a higher variability in the female group, especially in the reinstatement of METH seeking behavior due to different hormonal stages. However, we hypothesized that this variability may be overpowered by all other significant gender differences. Furthermore, we assessed possible gender differences in acquisition and maintenance of food self-administration in order to compare the operant behavior toward natural reward (food) and the drug of abuse.

Materials and Methods

Animals

Eight-week-old male and female albino Sprague-Dawley rats weighing 175–200 g (females) and 200–225 g (males) at the beginning of the experiment were purchased from Charles River (Germany). The rats were housed individually in standard rat plastic cages, the experiments on males and females were performed separately, to assure the self-administration room is dedicated to one gender at a time only. Environmental conditions during the whole study were constant: relative humidity 50–60%, temperature 23 ± 1°C, inverted 12-h light–dark cycle (6 a.m. to 6 p.m. darkness). Food and water were available *ad libitum*. All experiments were conducted in accordance with all relevant laws and regulations of animal care and welfare. The experimental protocol was approved by the Animal Care Committee of the Masaryk University, Faculty of Medicine, Czech Republic, and carried out under the European Community guidelines for the use of experimental animals.

Drugs and Treatments

Methamphetamine from Sigma Chemical, Co., St Louis, MO, USA available in the operant cage for IV self-administration was 0.08 mg/kg per infusion with the maximum number of infusions obtainable in one session set to 50. The solutions were prepared for specific animals depending on their body weights rounded to the closest category of 250, 300, 350 g, etc. This paradigm is adapted from Emmett-Oglesby MW (Fort Worth, TX, USA) (30) and routinely used in our laboratory (17, 31–33).

Locomotor Activity Test

After adaptation period at the beginning of the study basal behavioral profile was assessed in both males and females. In brightly lit room, rats were individually tested for locomotor activity using the Actitrack system (Panlab, Spain) as previously described (34, 35). Each Plexiglas arena (45 cm × 45 cm × 30 cm) was equipped with 2 frames equipped with photocells located one above another 2 and 12 cm above the cage floor. Each animal was placed in the center of arena and the spontaneous behavior was tracked for 10 min. During the test, the horizontal locomotor activity (the trajectory as calculated by the system from beam interruptions that occurred in the horizontal sensors) and vertical activity (number of rearing episodes breaking the photocell beams of the upper frame) were recorded. At the end of the session, animals were returned to their home cage and arenas were wiped with 1% acetic acid to avoid olfactory cues.

Intravenous Drug Self-Administration Surgery

Animals were deeply anesthetized with i.p. injections of 50 mg/kg ketamine plus 8 mg/kg xylazine. Under aseptic conditions, a permanent intracardiac silastic catheter was implanted through the external jugular vein to the right atrium. The outer part of the catheter exited the skin in the midscapular area. After surgery, each animal was allowed for recovery, individually in its home cage with food and water freely available. Since the implantation, the catheters were flushed daily by heparinized cephazoline (Vulmizolin 1.0 g) solution followed by 0.1 ml of a heparinized (1%) sterile saline solution to prevent infection and occlusion of the catheter. During recovery, changes in general behavior and body weight were monitored. When a catheter was found to be blocked or damaged, the animal was excluded from the analysis. At the end of the study, there were $n = 6$ male and $n = 6$ female rats included to the analysis.

Intravenous Self-Administration Protocol

Methamphetamine self-administration was conducted as previously described (17, 32) in 10 standard experimental boxes (30 cm × 25 cm × 30 cm, Coulbourn Instruments, USA) using nose-poking as operandum under a FR-1 schedule of reinforcement, i.e., animal had to make 1 nose-poke on the active hole to obtain a single drug infusion. Each cage was provided with two nose-poke holes allocated on one side and programed by software Graphic State Notation 3.03 (Coulbourn Instruments, USA). Nose-pokes in the active hole led to the activation of the infusion pump and administration of a single infusion followed by a 10 s timeout, while nose-poke stimulation was recorded but not rewarded. The cage was illuminated by a house light during the session. The light was flashing when administering infusion and off during the time-out period. Self-administration sessions lasted 90 min and took place 7 days/week for 2 weeks in total between 8 a.m. and 3 p.m. during the dark period of the inverted light–dark cycle.

After 14 days of stable METH intake, the maintenance phase was terminated and rats were returned to their home cages for the 14 days of the forced abstinence period. On day 15, rats were placed into self-administration chambers for the last 90 min reinstatement session. The numbers of responses on the active drug-paired nose-poke and the inactive nose-poke were recorded but the drug was not delivered. Responses on the active nose-poke are considered to reflect the reinstatement of drug-seeking behavior, while responses on inactive nose-poke reflect non-specific locomotor and exploratory activity.

Food Self-Administration Protocol

Food self-administration was conducted in the same experimental boxes as METH study (Coulbourn Instruments, USA) in a separate batch of animals. Under the FR-1 schedule of reinforcement 1 nose-poke lead to activation of a feeder and delivery of a single palatable pellet (BioServ, sweet dustless rodent pellets, F0021-Purified Casein Based Formula – 45 mg). The cage was illuminated by a house light during the whole session. Self-administration sessions lasted 30 min during the dark period of the inverted light–dark cycle.

Statistical Data Analysis

Primary data were summarized using arithmetic mean and SE of the mean estimate. Behavioral data were analyzed by *t*-test. IV METH self-administration data during the 14 days of maintenance were analyzed at individual days by *t*-test and at 5-day intervals by mixed ANOVA model with Greenhouse–Geisser correction. Acquisition of food self-administration was evaluated by comparison of mean day of reaching 70% preference of active nose poke by Mann–Whitney *U* test. Maintenance of food self-administration was analyzed at individual days by *t*-test. Statistical analyses were computed using SPSS 19.0.1 (IBM Corporation, 2010). A p-value <0.05 was recognized as boundary of statistical significance in all applied tests.

Results

Basal Locomotor Characteristics

Before starting the IV self-administration protocol, basal locomotor and exploratory activity was assessed in both males and females to exclude the possibility that these characteristics would lead to different drug taking behavior. Horizontal and vertical locomotor activity was measured and a proportion of each in the inner zone of the arena was calculated in order to evaluate differences in the status of anxiety in male and female rats. **Figure 1** illustrates the results on total distance traveled, vertical activity (number of rearing episodes), and inner part of arena preference. There were no basal behavioral differences between the sexes, which could contribute to dissimilar behavior in the operant cage. As expected, both sexes avoided the central part of the arena, which represents normal rodent behavior and neither one shows highly anxiogenic behavior or locomotor hyper- or hypo-activity.

Acquisition and Maintenance of Methamphetamine Self-Administration in Male and Female Rats

The acquisition and maintenance of METH taking behavior were assessed, first, in terms of mean number of infusions self-administered per session and, second, by the mean METH dose per session in milligram per kilogram. **Figure 2A** shows number of infusions obtained per daily session and mean number of infusions during the entire acquisition phase in male and female

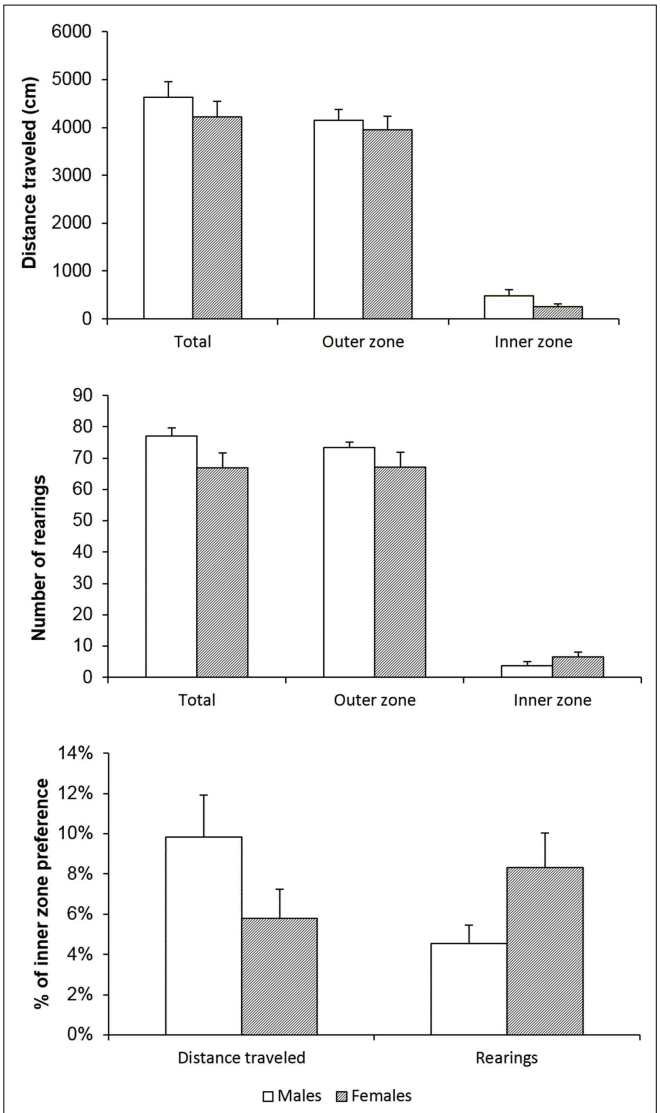

FIGURE 1 | Male and female rats have the same basal behavioral profile. Total distance traveled (in centimeters), number of rearing episodes (vertical activity), and % of preference of the central part of the arena did not reveal any gender difference. Data are shown as means (±SEM), *t*-test, n.s.

rats during the acquisition phase of METH self-administration training. ANOVA revealed no significant effects over the whole period. However, when the number of infusions was converted to a METH dose per kilogram of body weight, males were found to self-administer higher dose at the end of the acquisition phase as compared to females. More specifically, as depicted in the **Figure 2B**, mean METH intake during the last 5 days of training was significantly higher in males than in females, i.e., 2.5 and 1.5 mg/kg, respectively (mixed ANOVA model: $p = 0.038$).

Reinstatement of Methamphetamine Self-Administration in Male and Female Rats

After the 2-week-long period of forced abstinence one last reinstatement session was performed with no drug availability. The only measure of the drug-seeking behavior is the number of active operandum responses. This number was converted to a percent of mean basal nose-poking (14 days of acquisition and maintenance). There was a massive difference between the sexes recorded: male rats showed mean percent of responding 48.3% whereas females showed 295.7% (mixed ANOVA model, $p = 0.001$). Results are reported on the **Figure 3**.

Acquisition of Food Self-Administration in Male and Female Rats

The acquisition of food taking behavior (sweet pellets) was assessed in terms of day when the animals started to prefer the active nose-poke more than 70%. **Figure 4A** shows the development of active nose poke preference (%) over all sessions in male and female rats. **Figure 4B** reports the mean day for reaching 70% preference of the active operandum, which was 4.7 in males and 2.2 in females (Mann–Whitney *U* test, $p = 0.014$). The maintenance phase of the food self-administration was evaluated as a mean number of self-administered pellets during the last 5 days when the intake was stable. **Figure 5** depicts the significantly higher pellet intake in female rats as compared to males (138–175 and 51–73, respectively, $p \leq 0.05$).

Discussion

Findings of the present study demonstrated that male and female rats had equal basal locomotor and exploratory activity. Thus, differences in operant IV self-administration cannot be accounted for differences in locomotor activity. Furthermore, the food self-administration has shown a very different dynamics than the METH study, suggesting higher motivation to obtain natural reward (sweet pellet) in females, which learned the operant procedure faster (acquisition) and self-administered approximately three times more pellets than males. This behavior toward natural reward is very different from METH-related operant behavior, which rules out the possibility of general gender specific difference in the reward processes.

During the maintenance phase of the METH self-administration, female rats were found to self-administer the same number of infusion, but their METH intake in terms of dose per kilogram of body weight was found lower. This measure is not widely used among the self-administration studies, usually only the numbers of nose pokes (or lever presses) and infusions are reported. However, we propose this measure to be considered as highly valid for several reasons. Despite the solution of the drug being available in the operant box matches the body weight of the particular animal, the solutions are prepared for certain body weight category, e.g., solution for animal weighting 300 g can be used for rats reaching approximately 280–320 g (this fact is usually not described exactly in the Section "Materials and Methods" of the papers). This discrepancy, aggravated by the fact that the body weight of the animal changes over the course of the experiment, could be a source of significant differences in the dose taken even at conditions of the same number of infusions. This is a confounding factor, which complicates the comparison of findings from different laboratories. Furthermore, this approach should be used when the number of behavioral

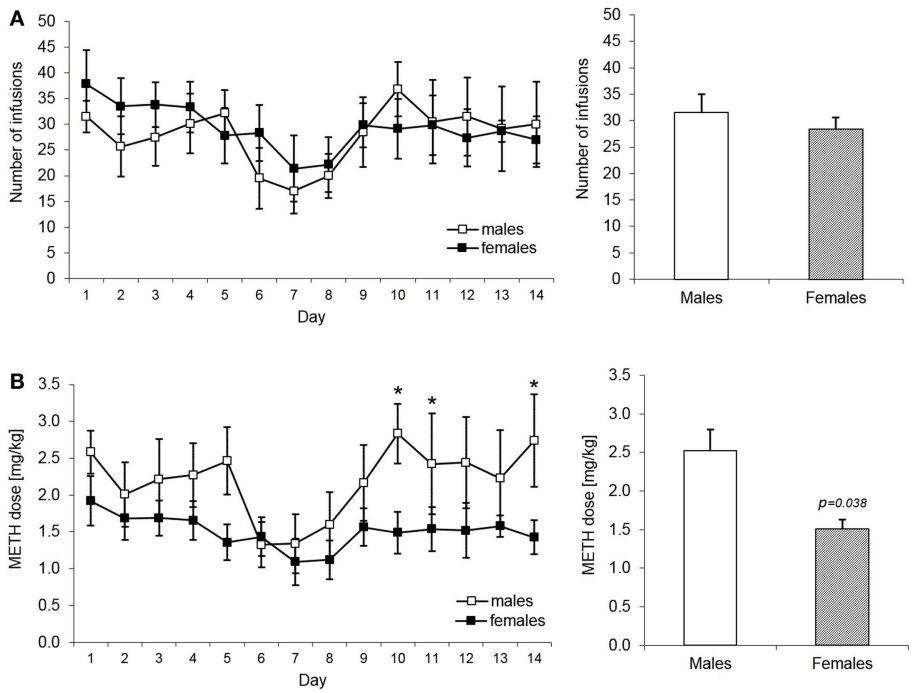

FIGURE 2 | Acquisition and maintenance of methamphetamine intake in male and female rats. The **(A)** part shows number of infusions expressed as daily means over the 14 days of acquisition and maintenance of the METH IV self-administration. The bar graph depicts the mean number of infusions over the whole 14 days period. There were no statistically significant differences in this measure (mixed ANOVA model). The **(B)** part shows in an analogical way the mean dose in milligram per kilogram of METH self-administered by male ($n = 6$) and female ($n = 6$) rats. The groups start to differ significantly from the day 10 with *t*-test results: day 10 ($p = 0.021$), day 11 ($p = 0.049$), and day 14 ($p = 0.048$). The bar graph shows the mean number of infusions over the last 5 days of the maintenance period (day 10–14) when the drug intake started to be significantly higher in male rats ($p = 0.038$, mixed ANOVA model).

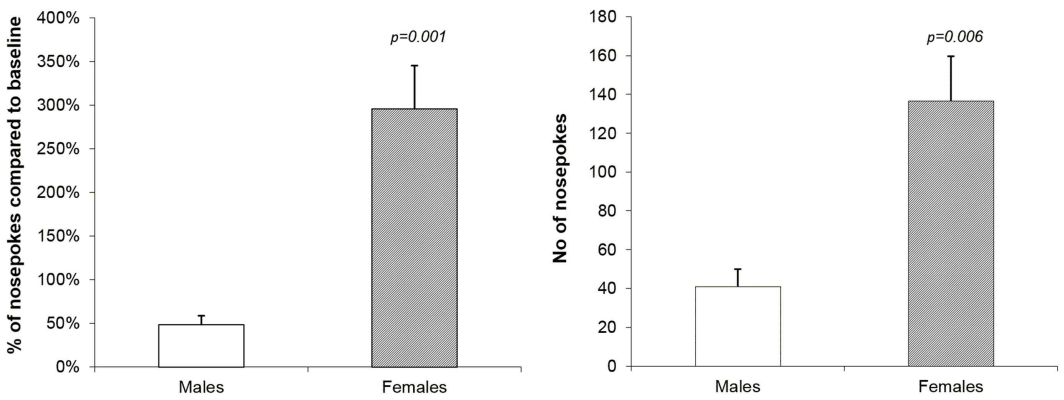

FIGURE 3 | Reinstatement of methamphetamine seeking behavior in male and female rats. The graphs show a percent of mean basal nose-poking (14 days of acquisition and maintenance) and number of nose-pokes in the reinstatement session in male and female rats. There was a statistically significant difference between the sexes in both measures: male rats showed mean % of responding 48.3% and females 295.7% (mixed ANOVA model, $p = 0.001$). The apparent difference between the sexes is further confirmed by behavioral activity reflected in a mean number of nose-pokes: 41.0 in males and 136.5 in females (mixed ANOVA model, $p = 0.006$).

responses (nose pokes or lever presses) does not match the number of infusions delivered. This is always the case when the system uses nose poke operandi (and in some cases levers which do not retract after infusion delivery).

Previous studies have shown that female rats to be more vulnerable to behavioral effects of psychomotor stimulants including cocaine (36–38) and, in particular, amphetamines (including METH), which elicited a higher increase of locomotion in females than males or reach the same behavior profile at lower dose (25, 28, 39, 40). Other studies have repeatedly shown that females with intact gonads tend to develop readily behavioral sensitization to psychostimulant drugs after repeated treatment (41–43).

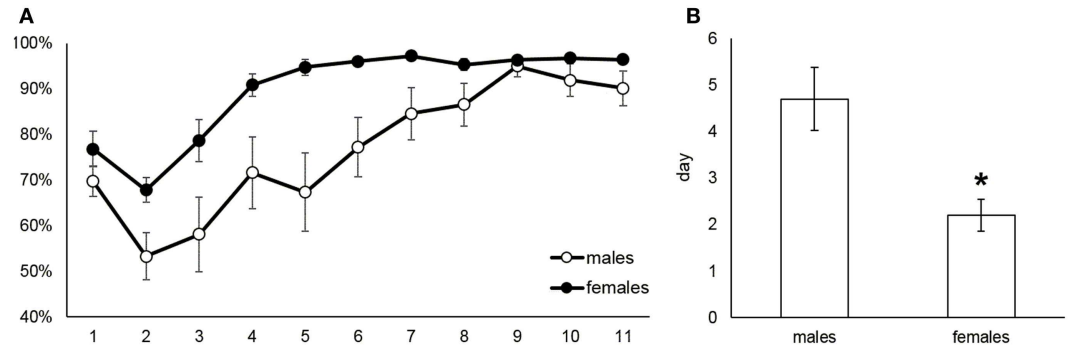

FIGURE 4 | Acquisition of food self-administration behavior in male and female rats. The **(A)** part shows the course of the active nose poke preference development during acquisition and maintenance of the food self-administration. The bar graph **(B)** depicts the mean day the animals reached and kept 70% preference of the active operandum: 4.7 in males and 2.2 in females (Mann–Whitney *U* test, $p = 0.014$).

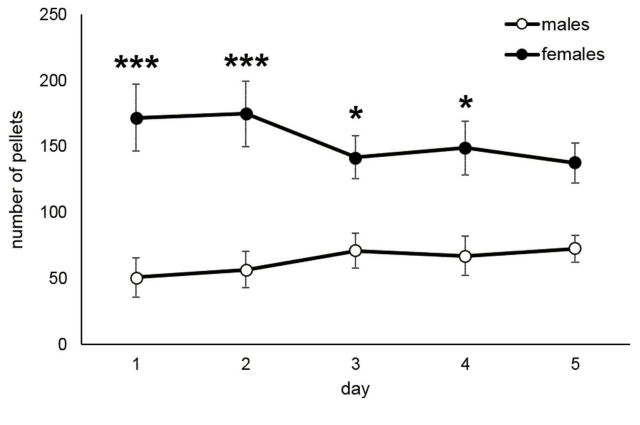

FIGURE 5 | Maintenance of food self-administration in male and female rats. The graph shows pellet intake in female rats as compared to males (*t*-test, $^{*}p \leq 0.05$, $^{***}p \leq 0.001$).

Furthermore, there is new evidence of specific pharmacokinetic differences in METH self-administration studies, where males were shown to have lower area under curve (AUC) of METH probably due to rapid drug elimination (29). The apparent higher efficacy of the amphetamines found in this and previously mentioned studies in female rats could be explained by the pharmacokinetic differences.

Similarly, in clinical studies, there has been shown that men are more sensitive to the reinforcing effects of a high dose of D-amphetamine than women, who respond rather to low doses at a random phase of the menstrual cycle (44). This is consistent with our data, which showed that males developed higher stable intake of METH than females (2.5 and 1.5 mg/kg daily, respectively). Furthermore, women were shown to experience greater increases in diastolic pressure and nausea than men at the same doses while the ability to discriminate D-amphetamine was equal in both sexes (45). These lines of evidence further support translational validity of our finding of lower METH intake in female rats.

However, in fixed ratio self-administration paradigms, the reports on gender differences in the maintenance phase are numerous and quite contradictory in both clinical (45, 46) and preclinical studies, showing both higher and lower drug intake in female subjects (21, 47).

Progressive ratio IV self-administration paradigm or prolonged access to the drug might be better tools to unravel gender differences as these may be linked to appetitive behavior (21). Female rats have been repeatedly shown to achieve higher breaking points in METH self-administration study suggesting higher motivation to obtain the drug (10, 48). This is consistent with the robust gender difference in the reinstatement found in this study, where the motivation of animals for the drug-seeking was not abolished by extinction training. At this point, active responses to the operandum are the only measure to report because the session is performed without delivering the drug. We found a highly significant difference in the percent of mean basal nose-poking, as well as in the absolute number of active operant responses. The enhancing effect of estradiol and attenuating effects of progesterone on psychostimulant (D-amphetamine, METH, cocaine) intake in female gender is repeatedly and consistently reported in both clinical (49–52) and preclinical studies (17, 20–22). Therefore, the higher variability in the reinstatement operant responding in the female group detected in this study probably originated from different hormonal stage. This conclusion can be supported by an earlier study, which employed the extinction and both drug- and cue-primed reinstatement, where females were found more vulnerable in both reinstatement procedures and also exhibited higher variability than males. Interestingly, the numbers of lever presses in the conditioned cue-primed reinstatement session were approximately 40 in males and 120 in females (10). These absolute numbers are similar to those reported in the present study: 41 and 136, respectively. Therefore, this effect seems to be well reproducible and strain independent (Long-Evans vs. Sprague-Dawley rats).

The forced abstinence model was proposed as a potentially better tool to model a spontaneous relapse in rodents (15, 53). To our knowledge, this is the first report of gender differences in the paradigm of reinstatement after forced abstinence. Extinction-based approach to study relapse-like behavior phase in the preclinical setting show contradictory results – females appear to meet the extinction criteria later than males (11), but negative results have been reported as well (54). Both studies were conducted with cocaine.

Taken together, this study reports lower consummatory METH intake during maintenance phase of the self-administration together with higher vulnerability to the reinstatement of METH seeking behavior in female rats after forced abstinence. These effects seem to be robust enough, thus relatively independent on the current hormonal level. Therefore, we propose this paradigm for preclinical screening for potential new medications specific for women. However, the main limitation for the translation of these results to human medicine is the absence of psychosocial aspects, which are impossible to reflect in animal studies.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5. Discussion

5.1. Depression-addiction comorbidity

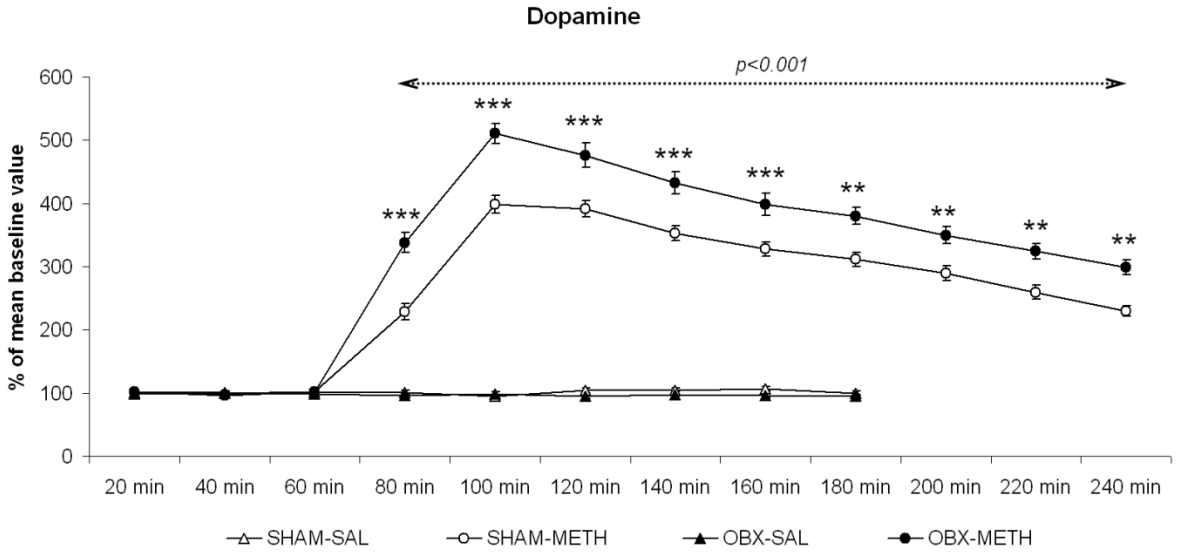
The self-medication hypothesis was recently challenged. The main reason is that different types of psychiatric patients should opt for different drugs of abuse. For example, a patient with attention deficit disorder would prefer amphetamines, while a person with elevated anxiety would prefer alcohol known for its anxiolytic properties. However, this paradigm does not reflect the real clinical situation where the population of psychiatric patients does indeed have increased rate of addiction over general population but the drug choice does not correlate with the disorder. Furthermore, the statement of resolving addiction issues with adequate treatment of the underlying psychiatric disease was not confirmed either (Lembke, 2012).

The scientific discussion was not concluded yet as there are new papers at least partially confirming this theory. Despite the questioned validity of the self-medication hypothesis, the existence of shared neurochemical and functional distortions in psychiatric disorders and addiction (Goodkind et al., 2015) remains valid. This fact is widely supported by the clinical experience showing a strong association of the psychiatric morbidity with drug abuse (Conner et al., 2008a, Conner et al., 2008b, Nunes and Levin, 2004) and interestingly also non-drug addictions such as internet addiction (Ho et al., 2014) or pathological gambling (Lorains et al., 2011).

Our model of the depression and addiction comorbidity combining the olfactory bulbectomy (OBX) and operant drug self-administration seems to mimic the clinical situation quite well showing increased rate of drug taking similarly as in clinical reports (Kushnir et al., 2013, Pettinati et al., 2013). Specifically, in this model the depressive-like rats showed higher intake of amphetamine (Holmes et al., 2002), methamphetamine (Kucerova et al., 2012), synthetic CB1 receptor agonist WIN55,212-2 (Amchova et al., 2014), ketamine (Babinska and Ruda-Kucerova, 2016) and 10% alcohol (Grecksch and Becker, 2015). This indicates an analogous reaction of animals in the OBX model towards drugs of abuse with substantially different mechanisms of action. Therefore, we expect a general distortion of the reward mechanisms in the OBX model. We tried to shed some light on the matter by evaluating extracellular levels of dopamine before and after a drug

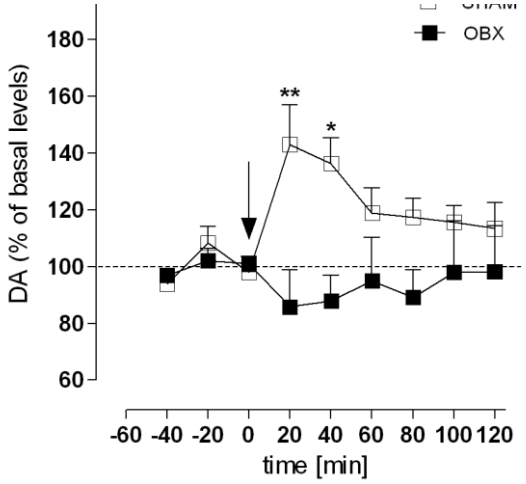
challenge and assessed a comprehensive profile of neurotransmitters in the *nucleus accumbens* shell in the OBX model. We observed lower basal levels of extracellular monoamines, i.e. dopamine, serotonin and their metabolites, and increased levels of glutamate and GABA. However, after a challenge dose of methamphetamine we detected a higher release of monoamines, lower levels of glutamate and no effect on GABA (Ruda-Kucerova et al., 2015b). Interestingly, in the study on WIN55,212-2 self-administration we observed an apparently opposite effect of the challenge dose of the drug on dopamine release in the *nucleus accumbens* shell (Amchova et al., 2014). Following Figure 6 and Figure 7 show data on the dopamine release reported in the corresponding studies.

Figure 6: relative dopamine release in the nucleus accumbens shell after methamphetamine challenge dose



(Source: Ruda-Kucerova et al., 2015b)

Figure 7: relative dopamine release in the nucleus accumbens shell after WIN55,212-2 challenge dose



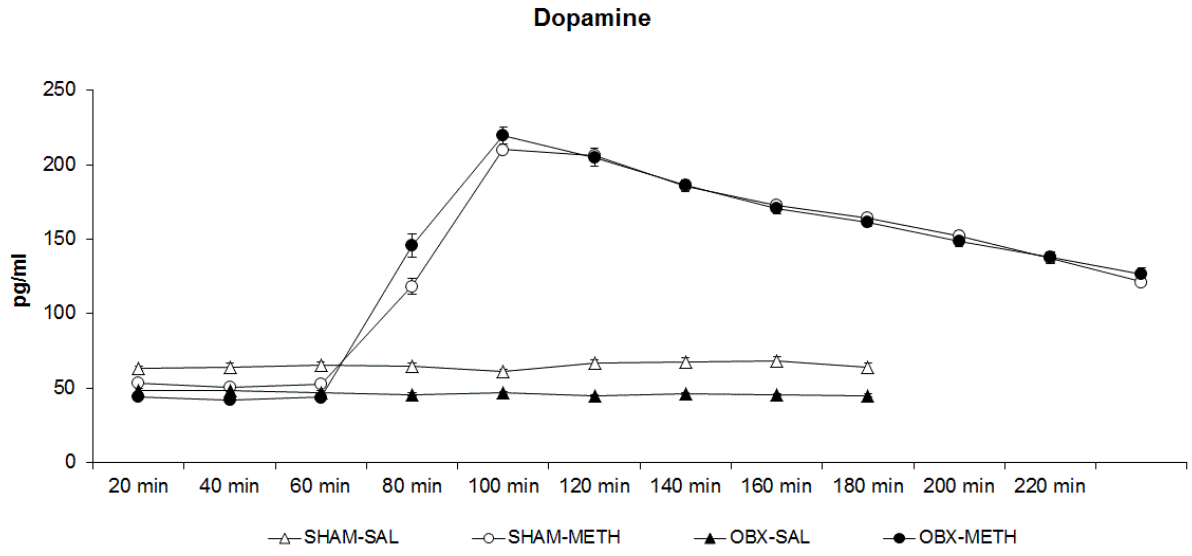
(Source: Amchova et al., 2014)

Both figures depict the same variable – a percent of basal dopamine level after the drug dose in intervals of 20 minutes. First, there is a high difference between the overall effects of the challenge dose. There is only moderate - 40% - release after WIN55,212-2 dose while methamphetamine dose has induced a 400% to 500% increase of the release. This can be explained by different mechanism of action of psychostimulant and cannabimimetic drugs. Psychostimulants act as indirect sympathomimetic agents producing a strong release of monoamines (Chiu and Schenk, 2012, Yu et al., 2015). Similar results of WIN55,212-2 (Lecca et al., 2006, Scherma et al., 2016) and methamphetamine (Izawa et al., 2006, Kai et al., 2015), effects on dopamine release in the *nucleus accumbens* shell were already reported.

There is a clearly different response of dopamine release in OBX animals to a challenge dose in both studies. We observed no effect in the OBX animals after the WIN55,212-2 treatment while there was significantly increased dopamine release after acute methamphetamine dose. The explanation is likely to be related to the drug doses used in the experiments. In case of WIN55,212-2 a dose of 0.3 mg/kg was chosen as this is the usual amount which is self-administered by control animals and was also found in the self-administration part of this study. However, the other set of experiments aimed to evaluate methamphetamine effects on many neurotransmitters and a dose known to exert a very strong effect was selected - 5 mg/kg. The usual operant intake of methamphetamine by

control animals is around 2 mg/kg and it rises up to 3 mg/kg in OBX rats. Similarly, we reported that OBX rats doubled their intake of WIN55,212-2 compared to control group. Taken together it seems that OBX rats need a higher dose to experience the same dopamine release in the *nucleus accumbens* shell and consequent rewarding effects which explains increased drug taking in the operant paradigms. Furthermore, the higher relative release of dopamine shown in the OBX rats after 5 mg/kg of methamphetamine may be baseline dependent. Figure 8 shows an unpublished comparison of the data presented in the paper as relative values (%). The dopamine release is depicted in absolute values (pg/ml) and the different basal level can be appreciated. In this comparison the peak levels are not statistically different.

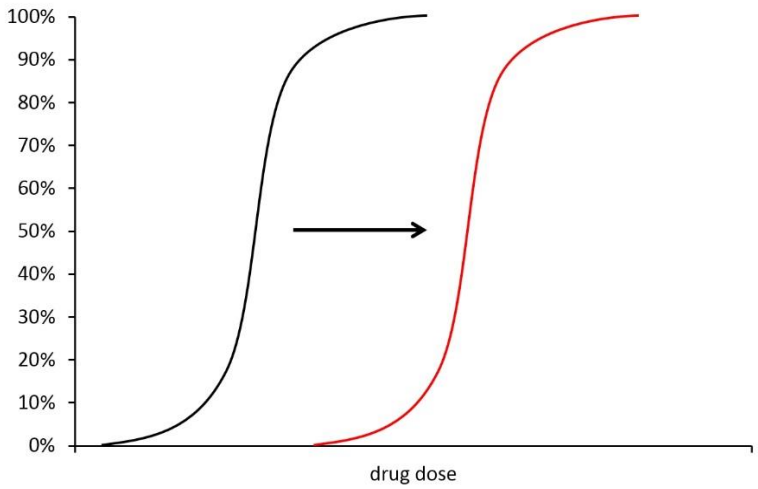
Figure 8: absolute values (pg/ml) of dopamine in the nucleus accumbens shell after methamphetamine challenge dose



(Source: unpublished comparison of data from Ruda-Kucerova et al., 2015b)

Findings from both studies point to a differential dose-response to drugs of abuse in the OBX model. We hypothesize a shift of the theoretical dose-response curve to right (Figure 9) in the OBX model. This assumption needs to be tested in order to conclude the matter.

Figure 9: theoretical dose-response curve



(Source: author's own figure)

This hypothesis can also be disputed by the fact that our *in vivo* microdialysis measurements evaluated only acute effects of the drugs. Besides the dose-response assessment of acute drug dose also a chronic study should be performed in order to estimate the effects of long-term exposure to the drug. Interestingly, the increased intake in the OBX animals in operant self-administration studies usually develops over time, usually after two or three weeks.

Relapse-like behaviour was not found to be consistently increased in the OBX group. While this effect was seen in studies using psychostimulants, i.e. methamphetamine (Babinska et al., 2016) and cocaine (Frankowska et al., 2014), we found an opposite effect in a ketamine self-administration study (Babinska and Ruda-Kucerova, 2016). However, the relapse after a period of abstinence induces quite different neurochemical adaptations than chronic exposure to the drug (See, 2005, Robinson, 1993, Self, 1998). Therefore, extracellular dopamine levels should be again assessed in this model of relapse to explain the reinstatement behaviours of the OBX animals.

5.2. Schizophrenia-addiction comorbidity

We have only recently extended our research of psychiatric dual disorders to an animal model of schizophrenia and drug abuse comorbidity. Also, we introduced a paradigm of alcohol drinking to our laboratory. This method is based on the drinking in the dark approach, where animals have a time limited access to alcohol during the dark phase of the light cycle (Samson et al., 1988, Czachowski, 2005). We have chosen this paradigm because it resembles the operant self-administration studies. To model schizophrenia-like phenotype in rats we employed a neurodevelopmental model, because human epidemiological data supports the fact that pre-perinatal environmental factors such as malnutrition, infection and obstetric complications increase the risk of developing schizophrenia (Brown et al., 2012). This knowledge has stimulated the development of models based on direct pre-perinatal damage of the central nervous system, which replicate several behavioural and neurochemical changes linked to the disease. In line with this approach, rats prenatally exposed to methylazoxymethanol (MAM), an antimitotic agent that methylates DNA, show behavioural (hyperactivity, cognitive and social deficits, disruption of pre-pulse inhibition) and histopathological (hyperdopaminergia) patterns similar to those observed in schizophrenia (Lodge et al., 2009, Lodge and Grace, 2009). The advantage of neurodevelopmental over pharmacological (acute) models of schizophrenia is the ability to perform behavioural and neurochemical investigations in the absence of confounding drugs and identification of new classes of antipsychotics by the use of agents operating on multiple pharmacological mechanisms (Micale et al., 2013).

So far we have published only one study with this dual disorder animal model which includes methamphetamine self-administration and alcohol drinking in a neurodevelopmental model of schizophrenia (Ruda-Kucerova et al., 2016). The most interesting finding of this study was that the female sex and schizophrenia-like phenotype induced by the prenatal MAM exposure may work synergistically to enhance alcohol consumption. There are other reports evaluating alcohol intake in other animal models of schizophrenia. Most of the papers presented findings from neonatal ventral hippocampal lesion (NVHL) model (Tseng et al., 2009, O'Donnell, 2012). Interestingly, in this model a key role of adolescence period was proven when a moderate exposure to alcohol was shown to sensitize the animals towards the same drug in adult age. The authors did not find differences in alcohol intake during adolescence or adult age without previous exposure

(Jeanblanc et al., 2014). A similar phenomenon was reported in the MAM model in the vulnerability to diazepam at adult age with adolescent exposure (Du and Grace, 2013). Therefore, adult animals in the MAM model might exhibit a different drug-taking behaviour after their early exposure to the drug. The part of our study focused on methamphetamine self-administration did not reveal any important significant differences between MAM and control animals. Negative results were also reported earlier in case of cocaine IV self-administration in the MAM model (Featherstone et al., 2009).

The usefulness of the MAM model in the study of dual disorders can be questioned. However, increased reactivity to amphetamine psychostimulants is routinely used as a test of positive-like symptoms of schizophrenia in the model (Gill et al., 2011, Lodge and Grace, 2009). Therefore, a hypothesis of distorted reward mechanism could be valid. However, so far the paradigm which would reveal these changes was not found.

5.3. Sex-differences in drug abuse

Sex differences in drug abuse were repeatedly assessed in both clinical studies (Becker and Hu, 2008) and animal models (Roth et al., 2004). Our team has contributed to the research by evaluation of oestrogen levels on methamphetamine self-administration. Furthermore, we included a variable of potential development of behavioural sensitization showing a decreased drug intake in previously sensitized rats of both genders (Kucerova et al., 2009). Later, we aimed at evaluation of relapse-like behaviour accepting physiological oestrous cycle of female rats as a confounding factor. Despite this limitation we observed higher drug-seeking behaviour after a period of abstinence, which reflects higher motivation of females to find methamphetamine (Ruda-Kucerova et al., 2015a). Together, our data indicate that despite a strong influence of the sex hormones, intact females do exhibit different relapse-like behaviour. This is in accordance with previous pre-clinical (Cox et al., 2013) and clinical studies (Grella and Lovinger, 2011).

There are already published many studies exploring all phases of drug abuse (i.e. acquisition, maintenance, withdrawal symptoms, abstinence, relapse) in both women and female animal subjects. However, gender specific issues in the dual disorders are still rarely assessed in the pre-clinical setting. So far, we attempted to assess differential reactivity of female MAM rats towards drugs of abuse and we actually found increased vulnerability in females in the MAM model towards alcohol drinking (Ruda-Kucerova et al., 2016).

6. Conclusion and future perspectives

Drug addiction is a serious condition harmful to all aspects of the afflicted person's life. Substance abuse is often comorbid with other psychiatric disorders where it may be either a primary cause or a secondary consequence of the disease. In order to develop innovative treatment strategies for these psychiatric dual disorders a downward translation to animal models is essential.

On the field of depression and addiction dual disorder modelling we propose a well validated combination of olfactory bulbectomy (OBX) and operant self-administration of drugs for testing of new anti-addiction treatments specific for depressive individuals. The ultimate aim of our research is to identify a pharmacological mechanism responsible for the OBX induced distortions in the reward pathways and test drugs which may reverse these neuroplastic changes. Such candidate drug might be low dose ketamine. There is currently an extensive research on its antidepressant effects in both preclinical studies (Scheuing et al., 2015) and clinical trials (Newport et al., 2015, Xu et al., 2015). We performed a pilot experiment evaluating the effect of acute pre-treatment (5 mg/kg of ketamine 30 minutes before the session) on IV self-administration of methamphetamine and observed a strong suppression of the drug-taking behaviour (unpublished data). This line of research deserves further investigation in order to explore this phenomenon in depth and publish a concise study.

There are only few preclinical studies aiming for evaluation of addictive behaviours in schizophrenia-like phenotype. In future we will design studies for identification of experimental approach sensitive enough to reflect the hypothesized distortions in the reward processes of the neurodevelopmental model of schizophrenia induced by prenatal administration of methylazoxymetanol acetate (MAM). Exposing adolescent rats to addictive substances and testing their abuse liability later in life will be the first because adolescence seems to be a vulnerable period as reported in an alcohol drinking study in another neurodevelopmental model of schizophrenia (Jeanblanc et al., 2014) and in vulnerability to benzodiazepines in the MAM model (Du and Grace, 2013).

The research on dual disorders should be extended to evaluation of sex differences in the animal models. So far, we have identified higher sensitivity of female MAM rats towards alcohol drinking. Female OBX rats are rarely used but the available evidence suggests they also exhibit a depressive-like phenotype (Stepanichev et al., 2016, Stock et al., 2000, Pudell et al., 2014) and may provide a useful tool to study sex differences in drug taking behaviours in context of a depression and addiction dual disorder.

7. List of papers related to the habilitation thesis

7.1. Publications *in extenso* in journals with IF

Ruda-Kucerova J, Babinska Z, Amchova P, Stark T, Drago F, Sulcova A, Micale V. Reactivity to addictive drugs in the methylazoxymethanol (MAM) model of schizophrenia in male and female rats. World J Biol Psychiatry. 2016, doi: 10.1080/15622975.2016.1190032, in press.
IF (2015) **4.159**
Citations (WOS): 0

Babinska Z, **Ruda-Kucerova J**, Amchova P, Merhautova J, Dusek L, Sulcova A. Olfactory bulbectomy increases reinstatement of methamphetamine seeking after a forced abstinence in rats. Behav Brain Res. 2016, 297: 20-7, doi: 10.1016/j.bbr.2015.09.035.
IF (2015) **3.002**
Citations (WOS): 0

Ruda-Kucerova J, Amchova P, Havlickova T, Jerabek P, Babinska Z, Kacer P, Syslova K, Sulcová A, Sustkova-Fiserova M. Reward related neurotransmitter changes in a model of depression: An *in vivo* microdialysis study. World J Biol Psychiatry. 2015, 16(7): 521-35. doi: 10.3109/15622975.2015.1077991.
IF 4.159
Citations (WOS): 0

Kucerova J, Babinska Z, Horska K, Kotolova H. The common pathophysiology underlying the metabolic syndrome, schizophrenia and depression. A review. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2015, 159(2): 208-14. doi: 10.5507/bp.2014.060.
IF 0.924
Citations (WOS): 2
This paper is included in this thesis as Appendix 1.

Amchova P, **Kucerova J**, Giugliano V, Babinska Z, Zanda MT, Scherma M, Dusek L, Fadda P, Micale V, Sulcova A, Fratta W, Fattore L. Enhanced self-administration of the CB1 receptor agonist WIN55,212-2 in olfactory bulbectomized rats: evaluation of possible serotonergic and dopaminergic underlying mechanisms. Front Pharmacol. 2014, 5: 44. doi: 10.3389/fphar.2014.00044.

IF 3.802

Citations (WOS): 5

Micale V, **Kucerova J**, Sulcova A. Leading compounds for the validation of animal models of psychopathology. Cell Tissue Res. 2013 Oct;354(1):309-30. doi: 10.1007/s00441-013-1692-9.

IF 3.333

Citations (WOS): 8

This paper is included in this thesis as Appendix 2.

Kucerova J, Pistovcakova J, Vrskova D, Dusek L, Sulcova A. The effects of methamphetamine self-administration on behavioural sensitization in the olfactory bulbectomy rat model of depression. Int J Neuropsychopharmacol. 2012, 15(10): 1503-11. doi: 10.1017/S1461145711001684.

IF 5.641

Citations (WOS): 8

Kucerova J, Vrskova D, Sulcova A. Impact of repeated methamphetamine pretreatment on intravenous self-administration of the drug in males and estrogenized or non-estrogenized ovariectomized female rats. Neuro Endocrinol Lett. 2009, 30(5): 663-70.

IF 1.047

Citations (WOS): 13

7.2. Publications *in extenso* in journals without IF

Ruda-Kucerova J, Amchova P, Babinska Z, Dusek L, Micale V, Sulcova A. Sex differences in the reinstatement of methamphetamine seeking after forced abstinence in Sprague-Dawley rats. Front Psychiatry. 2015, 6: 91. doi: 10.3389/fpsyt.2015.00091.
Citations (WOS): 1

Amchová P, **Kučerová J**. Pohlaví a drogová závislost: od animálních modelů ke klinické praxi. Česká a Slovenská Psychiatrie, Praha: Česká lékařská společnost J.E.Purkyně, 2015, 111(2): 72 -78.
This paper is included in this thesis as Appendix 3.

Babinská Z, **Kučerová J**. Spoločné neurobiologické mechanizmy depresie a metamfetamínovej závislosti. Alkoholizmus a drogové závislosti, Bratislava: Obzor, 2014, 49(3): 127-152.
This paper is included in this thesis as Appendix 4.

Kucerova J, Tabiova K, Drago F, Micale V. Therapeutic potential of cannabinoids in schizophrenia. Recent Pat CNS Drug Discov. 2014, 9(1): 13-25. Review.
This paper is included in this thesis as Appendix 5.

Kucerova J, Pistovcakova J, Vrskova D, Dusek L, Sulcova A. Aripiprazole does not influence methamphetamine I.V. self-administration in rats. Activitas nervosa superior rediviva, 2010. 52(4): 261-266.
This paper is included in this thesis as Appendix 6.

Kucerova J, Sulcova. Comparison of behavioural sensitization to ecstasy in mouse males and ovariectomized females with and without oestrogen substitution. Activitas Nervosa Superior, 2008. 50(1-2): 18-19.

Kučerová J. Vliv behaviorální senzitizace a pohlaví na metamfetaminovou závislost u člověka a ve zvířecím modelu. Alkoholismus a drogové závislosti, 2008. 43(5): 295-309.

Kucerova J, Novakova J, Landa L, Sulcova A. Gender differences in cannabinoid and ecstasy interacting effects in mice. Homeostasis in Health and Disease, 2006. 44(1-2): 95-96.

7.3. Book chapters

Micale, V., Tabiová, K., **Kučerová, J.** and Drago, F., Role of the endocannabinoid system in depression: From preclinical to clinical evidence. 2014, in Cannabinoids, Endocannabinoids, and Modulation of Emotion, Memory, and Motivation, Fattore & Campolongo, editors, Springer Science+Business Media New York 2015, DOI 10.1007/978-1-4939-2294-9_5, pp. 97-129 (Scopus)

Kučerová, J., Babinská, Z., Fattore, L.: Behavioral rodent models of eating disorders Appetite, Nova Science Publishers, Inc., ISBN: 978-1-63117-241-0, pp. 71-96. (Scopus)

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5. Babinska, Z. & Ruda-Kucerova, J. 2016. Differential characteristics of ketamine addiction in the olfactory bulbectomy model of depression in rats. *Behav Brain Res***,** submitted, under review.

6. Babinska, Z., Ruda-Kucerova, J., Amchova, P., Merhautova, J., Dusek, L. & Sulcova, A. 2016. Olfactory bulbectomy increases reinstatement of methamphetamine seeking after a forced abstinence in rats. *Behav Brain Res,* 297**,** 20-7.

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The common pathophysiology underlying the metabolic syndrome, schizophrenia and depression. A review

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Background. There is a growing interest in metabolic alterations in patients with psychiatric disorders due to their increased risk for metabolic syndrome (MetS) development. Inflammation is known to underlie the pathophysiology of schizophrenia and depression as well as MetS. Vulnerability factors for schizophrenia/depression and MetS hence appear to be shared.

Methods and Results. Based on a Web of Science search, this review examines current evidence for MetS pathophysiology involving dysregulation of adipose tissue signaling – adipokines and pro-inflammatory cytokine, both also known to be aberrant in schizophrenia/depression. Further, gender differences in the incidence and course of schizophrenia/depression were reported. The disturbances linked to the MetS are also described. Therefore, this review further maps the gender differences in the psychiatric-metabolic comorbidities.

Conclusion. There is evidence supporting a pathological predisposition to MetS in both schizophrenia and depression in both humans and animal models. This predisposition is dramatically enhanced by antipsychotic medication. Further, there are gender differences from clinical findings suggesting women with schizophrenia/depression are more vulnerable to MetS development. This has not yet been assessed in animal studies. We suggest further validation of existing schizophrenia and depression animal models for the assessment of metabolic disturbances to provide tools for developing new antipsychotics and antidepressants with "metabolically inert" profile or improving the metabolic status in schizophrenic/depressed patients.

Key words: metabolic syndrome, schizophrenia, depression, sex/gender differences, adipokines, leptin, adiponectin, resistin, AFABP

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INTRODUCTION

Psychiatric disorders are commonly associated with increased morbidity and mortality largely due to other medical conditions such as cardiovascular diseases, diabetes, respiratory and infectious diseases. There is a rapidly growing interest in the assessment of metabolic disturbances in patients diagnosed with psychiatric disorders (especially schizophrenia and depression) due to their higher risk of metabolic syndrome (MetS) than the general population. In addition, growing evidence indicates a role of inflammation in the pathophysiology of these diseases.

In the US and European populations, the prevalence of MetS in psychiatric patients ranges from 25-56% (depending on definition) and it constitutes major health and financial burdens[1-3]. It is estimated to be up to 50% higher than age-matched healthy control populations[4] with expectation to rise to 59% by 2020 (ref.[5]). Moreover, not only western populations are affected but also eastern regions report a high prevalence. More particularly, Asian populations report a mean prevalence of 20-40% (ref.[6]), South American regions with 14-30% and Australia with 20-30% (ref.[1]). Further, younger age groups also show a growing rate of MetS incidence[7]. A number of reports confirm the psychiatric populations have a substantially higher incidence of MetS than the general population, especially in the case of schizophrenia[8] and depression[9].

Obesity and MetS development are also known to differ according to gender as a result of differences in the amount and distribution of body fat and differences in adipose tissue metabolism and function between the sexes[10]. The incidence of MetS was reported to be higher in women than in men in Arabic populations[11], a finding consistent with European populations[12]. Therefore, a gender-specific approach may be more effective for the treatment and prevention of MetS development.

The need for reviewing current knowledge on the shared pathophysiology linking MetS and psychiatric disorders is supported by increasing numbers of publications on the topic of comorbid MetS and schizophrenia/depression. Web of Science search (performed in August 2014) for "metabolic syndrome" and "schizophrenia*" in publication titles currently provides 186 records, with the oldest from the year 2002 including 11 reviews. However, most papers (and reviews) focus on the side-effects of anti-

psychotics and not the pathophysiological causes according to the disorders *per se* resulting in MetS. Analogous search for depression ("depress*") returns 161 publications with the oldest from the 2003 including 5 review papers.

The aim of this review was to evaluate the available findings on the link between MetS and schizophrenia/ depression with a focus on gender differences.

METABOLIC SYNDROME AND INFLAMMATION

The epidemic spread of MetS has resulted in over 2 billion overweight and obese adults worldwide[13]. This syndrome is defined as a complex of risk factors closely related to the development of atherosclerosis and subsequent cardiovascular morbidity together with type-2 diabetes. In particular, these factors comprise mainly abdominal distribution of adipose tissue (abdominal obesity), dyslipidemia, hypertension and distortion of glycemic homeostasis[14]. The seriousness of this pathology lies in increased mortality due to cardiovascular conditions and diabetes type 2. Compared to normal populations, the incidence of myocardial infarction and stroke are 3-fold higher in MetS patients; the risk of type 2 diabetes development is 5-fold higher[15]. Individuals with MetS also often manifest pro-thrombotic and pro-inflammatory states reflected by higher blood levels of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (ref.[16]).

Dysregulation of adipokines as biomarkers of adipose tissue metabolism plays an essential part in all obesity-related diseases. Most relevant adipokines known to be dysregulated in MetS are leptin, adiponectin (both supporting insulin signaling functions), resistin and adipocyte fatty acid binding protein (AFABP). Also pro-inflammatory cytokines are considered adipokines such as interleukins, TNF-α and C-reactive protein (CRP), all generally suppressing insulin signaling functions.

Leptin is a peripheral signaling protein that regulates the hypothalamic satiety center and adipose reserves in the body[17,18]. It is key mediator of energy homeostasis involving regulation of appetite, lipid catabolism and inhibition neurotransmitter neuropeptide Y, a known appetite stimulator[19]. Plasma levels of leptin are generally higher in obese patients, which is considered to be a leptin-tolerant state[20,21].

Adiponectin is a protein that inhibits inflammatory reactions and protects against metabolic disease, by a wide range of mechanisms, including anti-diabetic, anti-inflammatory and anti-sclerotic[22]. It is involved in the regulation of carbohydrate and lipid metabolism, inhibiting gluconeogenesis in liver and increasing the transport and utilization of free fatty acids in the periphery. Furthermore, it significantly affects the function of insulin and plays an important role in energy homeostasis, causing a decrease in body weight without affecting food intake. However, it is believed that it also directly influences the regulation of appetite and weight control[21,23].

Resistin is a peptide hormone produced by mature adipocytes and regulates insulin sensitivity[24]. Its inhibitory effect on the differentiation of adipocytes probably underlies its role in the feedback between nutritional status and adipogenesis. Its plasma levels increase in correlation with inflammatory markers including CRP, soluble TNF-α receptor-2, IL-6 and lipoproteins in combination with phospholipase A2 under pathophysiological conditions related to inflammation[25]. Based on preclinical evidence, resistin may represent a key link between inflammation and its metabolic consequences[21,26-28].

AFABP is a newly discovered adipokine found at higher plasma levels in patients who have the MetS. Patients with higher levels of AFABP have worse prognosis and increased cardio-metabolic risk factors, reversible by atorvastatin treatment[29]. Further, TNF-α is constitutively expressed in adipose tissue and this condition leads to insulin resistance in animal models of obesity which supports the face validity of the models. Plasma levels of AFABP in humans closely correlates with degree of obesity and the development of insulin resistance and positively correlates with waist circumference, blood pressure values, and parameters of lipid metabolism, serum fasting insulin and insulin resistance index[21,30].

The growing evidence has resulted in the formulation of the inflammatory hypothesis of insulin resistance and MetS (ref.[31-33]) and MetS is also associated with other inflammatory diseases such as rheumatoid arthritis[34] showing secondary development of MetS on an inflammatory basis. The hypothesis assumes obesity is a consequence of excessive caloric intake representing a sub-clinical inflammatory process, which induces insulin resistance and following clinical and biochemical manifestations of MetS as demonstrated in numerous studies. For review see Alemany et al.[31] Moreover, the inflammation is mediated by pro-inflammatory cytokines produced by macrophages which tend to populate the growing adipose tissue in obesity at higher rates[35]. In mouse obesity models, an up-regulation of specific genes for macrophages - macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), macrophage-1 antigen (MAC-1), macrophage surface glycoproteins F4/80 and CD68; and genes promoting inflammatory processes in white adipose tissue are found. Molecular mechanisms leading to macrophage activation in obesity/ MetS are not fully understood. However, participation of adipokines (adiponectin, leptin, complement factor C3, MCP-1, cytokines, free fatty acids) is assumed[36]. Activated macrophages release several cytokines and chemokines such as TNF-α (ref.[37]), IL-1, IL-6 and MCP-1, distorting adipocyte sensitivity to insulin, which then in turn promote further activation and infiltration of macrophages. Therefore, impaired insulin signaling in adipocytes may lead to massive lipolysis, necrosis and development of insulin resistance[33,38].

PSYCHIATRIC DISORDERS AND INFLAMMATION

There is accumulating evidence suggesting that schizophrenia is associated with increased serum levels of pro-inflammatory cytokines, namely IL-1, IL-6, TNF-α and high-sensitivity CRP, even in patients with minimal or no exposure to antipsychotics[39].

Prenatal infections are also hypothesized to have serious impact on the brain, which is supported by validation of several neurodevelopmental animal models of schizophrenia based on immune or toxic prenatal insult namely: Immune: polyIC (polyriboinosinic-polyribocytidilic acid) model[40], toxic: MAM (methylazoxymethanol acetate) model[41] or Δ9-THC (Δ9-tetrahydrocannabinol) model[42]. Together with certain genetic factors, these findings provide convincing evidence that inflammation is a major factor in the pathology of this disorder[43].

There is a well-established concept of depression strongly associated with inflammation. An abundance of both clinical and preclinical data reported increases in pro-inflammatory cytokines such as IL-1, IL-6, TNF-α and CRP in depressed patients[44-47]. Similar findings were also discovered in preclinical studies[48] including the olfactory bulbectomized rodent model of depression[49].

PSYCHIATRIC DISORDERS AND METABOLIC SYNDROME

Schizophrenia and metabolic syndrome

Obesity or MetS are common in schizophrenic patients. MetS has an incidence of 3-4% in the general population, but up to 10% in schizophrenic patients even before initiation of the treatment with antipsychotics[8] which often results in typical changes in lipid metabolism[50]. It appears that not only antipsychotic treatment but the pathophysiology of schizophrenia itself is linked to MetS development suggesting a common underlying pathway- chronic inflammatory abnormality of cytokines[43]. Therefore, vulnerability factors for development of schizophrenia, diabetes, and MetS seem to be shared and interconnected. In patients with schizophrenia the risk is further greatly increased by the use of antipsychotic medication as reviewed repeatedly elsewhere[8,51-55].

More specifically, elevated blood levels of adiponectin have been reported in schizophrenia[56] and there is a correlation between serum leptin levels and body weight[51]. In addition, leptin concentration was shown to play an important part in the negative feedback against dopamine activity connected to positive symptoms of schizophrenia[57]. Furthermore, it is well known that antipsychotic treatment induces clinically relevant weight gain and rise in fasting plasma glucose levels[58].

Despite the high clinical relevance, relatively little research has been done in preclinical models of schizophrenia, which could then contribute to targeted drug development for MetS treatment in psychotic patients. Studies conducted in animals were mostly related to the evaluation of metabolic effects in all classes of antipsychotics rather than the assessment of the relation between MetS and schizophrenic phenotype *per se*. These drugs were shown to notably disturb lipid metabolism in drug naïve Sprague-Dawley rats as well as in kainic acid treated Fisher rats used as a model of schizophrenia[59,60]. This indicates that schizophrenic-like phenotype in rodent models and antipsychotic medication does lead to increase in vulnerability to metabolic disturbances further confirming their validity and translational potential evaluating gender differences, a key importance for developing new therapeutic strategies as described in the corresponding section of this text.

DEPRESSION AND METABOLIC SYNDROME

Multiple lines of evidence confirm higher incidence of MetS in depressed patients. A 50% higher prevalence of depression has been reported in individuals with MetS in an Australian population[61] and a 4-fold increased risk for MetS in patients with lifetime major depression episode in a Lithuanian population[62]. Similar outcomes in a German population were found[63]. Also, different ethnic groups were compared with consistent finding of higher MetS prevalence in depressed African-American, Caucasian[64], and Asian women[65]. Furthermore, not only major depression but also bipolar disorder has been shown to have association with MetS (ref.[66]). However, MetS seems to be specifically linked to depression as it has been repeatedly shown that anxiety is not associated with metabolic disturbances[62,67].

There are also clinical studies showing no[68] or only partial association of MetS symptoms (lipid profile) with depression[69]. However, recently, Pan et al. published an extensive meta-analysis (the first of its kind) reporting a strong link between depressive disorders and development of MetS in both genders. The results indicate a convincing bidirectional association between depression and MetS (ref.[9]). Further support (although sporadic) for the association between MetS and depression could provide a case study of a (Caucasian) woman treated with pioglitazone and showing strong antidepressant effect[70].

Suggested mechanisms underlying both disorders include HPA axis dysregulation following the inflammatory reaction. Moreover, two subtypes of depression (melancholic and atypical depression) were identified to be associated with the inflammatory and metabolic dysregulation. This highlights the possibility that not all forms of major depression possess this association with MetS (ref.[71]). Changes in important adipokine levels in depressed patients were reported compared to a healthy population suggesting predisposition to MetS development in depressed patients. In agreement, a "leptin hypothesis of depression" was formulated as low levels of leptin have been found in association with depression in humans as well as depressive behaviors in rodents. It was suggested that both leptin insufficiency and leptin resistance may contribute depressive status[72]. These findings are translated and further supported by several animal studies[73] including pharmacological experiments where

leptin induced antidepressive-like behaviour in a forced swimming test, a commonly used test for evaluation of depressive-like phenotype in rodents[74].

Blood levels of another adipokine – adiponectin – were found to be reduced in Brazilian patients with major depression before antidepressant treatment. The authors of the study (Leo et al. 2006) conclude that the reduced availability of circulating adiponectin is likely to have an impact on mood state[75]. Similar findings were reported in an Italian population[76] and later in a US population[77]. However, contradictory findings were reported in a Korean study showing higher levels of adiponectin in depressed individuals[78]. The variability could be due to antidepressant treatment which seems to increase adiponectin levels[79]. Yet, studies reporting no difference before and after treatment have also been published[80].

Regarding resistin, a positive association between blood levels and free cortisol concentrations were found in depressed patients. Resistin levels were normalized when patients remitted after pharmacological treatment but not in non-remitters[80].

ROLE OF GENDER IN THE METABOLIC SYNDROME AND PSYCHIATRIC DISORDERS

Schizophrenia and MetS: implications of gender

Sex-specific differences in the epidemiology, onset and course of schizophrenia are repeatedly reported. More specifically, men have approximately 4-times higher tendency to develop schizophrenia and the first symptoms usually appear at a younger age[81]. On the other hand, women tend to suffer more from comorbid depression while men it is drug addiction. In addition, gender differences in response to antipsychotic treatment are reported. However, a clear explanation is yet to be provided. Influences of sex hormones, sexual dimorphism of the brain, metabolic differences and social factors were so far only proposed as partial explanations[82,83]. Gender differences in MetS comorbidity with schizophrenia were found repeatedly before and after the initiation of the antipsychotic treatment with women being approximately 3-times more prone to develop MetS (ref.[8]).

Furthermore, preclinical studies have recorded gender differences suggesting a greater vulnerability (increase in body weight and metabolic changes) in female rats, specifically, the most suitable model for antipsychotic-induced weight gain appears to be the female Sprague-Dawley rat[84]. In contrast, male rats showed no significant changes in body mass yet still exhibited metabolic disturbances such as increased visceral fat mass and hormonal changes[59]. Nevertheless, increased adiposity was reported in both genders and seems to be adequately modeled in rodents with a schizophrenic phenotype. It has not yet been established which experimental paradigms most accurately reflect weight gain and metabolic abnormalities in schizophrenia, known to be increased by antipsychotic treatment in humans[85].

Depression and MetS: implications of gender

In the case of depression, reports of gender differences are only recently emerging. A similar strength of the overall association has been reported between metabolic risk factors in men and women, but in males, several factors were associated with depressive symptoms, while in females the association was confined to waist circumference only[86]. Earlier, a stronger association of MetS with depression was found in the female US population compared to male group[87] similarly in the Israeli population[88]. However, negative findings have been published as well[89]. Furthermore, depressed women showed significantly higher leptin levels than a control group both before and after the response to antidepressant treatment, whereas no difference was found between the male patients and their controls. The improvement of depression with antidepressant treatment was shown to cause a further elevation of leptin levels, in both female and male patients. Therefore, clinical response to antidepressant treatment seems to be linked to leptin metabolism[90]. In addition, adiponectin levels were found to be dysregulated in men with depression while no differences were observed in that of women[78].

CONCLUSION

In summary, there is likely a metabolic predisposition to MetS in both schizophrenia and depression patients which is evidence of underlying pathophysiology in both humans and animal models. This predisposition is enhanced by antipsychotic medication in psychotic patients. In agreement, numerous authors in clinical fields have already suggested screening for MetS in psychiatric patients to combat increased rates of morbidity and mortality from non-psychiatric reasons in these patients with early lifestyle and pharmacotherapeutic interventions. Specifically, in schizophrenic patients, routine consultation with a diabetologist has been suggested[52].

Moreover, development of new anti-inflammatory treatments for the dual pathology of schizophrenia and MetS has been proposed[91], which also could be a useful approach alleviating the cognitive symptoms of schizophrenia[92]. This evidence concerns newly diagnosed patients as well, with chronic treatment with antipsychotics being a well-known risk factor for MetS development. Thus, a follow-up monitoring of metabolic abnormalities in patients on second generation antipsychotics is strongly recommended by the consensus of the American Diabetes Association, American Psychiatric Association, American Association of Clinical Endocrinologists, and North American Association for the Study of Obesity[93].

In depression, a closer monitoring for MetS (ref.[61,63,94]) together with development of disease-modifying therapies[95] and special regards to sex-differences[96] have been repeatedly suggested. Further, the current scientific literature has suggested the validation of existing animal models and the development of newer models to better reflect psychiatric diseases[97,98].

Furthermore, the possible gender differences in clinical findings (suggesting women with schizophrenia/de-

pression are more vulnerable to MetS development) are not yet assessed in animal studies. In the light of evidence on gender differences in MetS development in psychiatric disorders, sex differences should be taken into account in future preclinical and clinical studies. However, the lack of validated animal models for assessment of metabolic disorders comorbid in psychiatric diseases is problematic. Such validation of existing animal models of schizophrenia and depression could provide a useful tool for developing innovative pharmacotherapeutic solutions with "metabolically inert" profile or even improving the metabolic status of psychiatric patients.

The endocannabinoid system targeting drugs are an important source of candidates and have already been proposed for the treatment of schizophrenia[99,100] and mood disorders[101]. Endocannabinoid targeting drugs effective in reducing abdominal obesity have been identified[102]. More specifically, these drugs act through CB1 receptor inverse agonism. Unfortunately, marketing of the first drug, rimonabant, was discontinued for psychiatric side-effects, namely inducing depressive states and suicidal ideas[103,104]. However, newer cannabinoid compounds are emerging and a strong influence on appetite, metabolism and energy homeostasis is consistently reported[105-107]. Most importantly, preclinical studies are constantly widening the range of new candidate molecules[108-110].

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REVIEW

Leading compounds for the validation of animal models of psychopathology

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Abstract Modelling of complex psychiatric disorders, e.g., depression and schizophrenia, in animals is a major challenge, since they are characterized by certain disturbances in functions that are absolutely unique to humans. Furthermore, we still have not identified the genetic and neurobiological mechanisms, nor do we know precisely the circuits in the brain that function abnormally in mood and psychotic disorders. Consequently, the pharmacological treatments used are mostly variations on a theme that was started more than 50 years ago. Thus, progress in novel drug development with improved therapeutic efficacy would benefit greatly from improved animal models. Here, we review the available animal models of depression and schizophrenia and focus on the way that they respond to various types of potential candidate molecules, such as novel antidepressant or antipsychotic drugs, as an index of predictive validity. We conclude that the generation of convincing and useful animal models of mental illnesses could be a bridge to success in drug discovery.

Keywords Depression · Schizophrenia · Animal models · Antipsychotics · Antidepressants

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Introduction

Animal models in neuroscientific research are of irreplaceable value. They are important tools for the assessment of pathological mechanisms, for the testing of hypotheses that cannot be addressed in clinical studies and for the development of novel pharmacological treatment (Nestler et al. 2002). Psychiatric disorders such as depression and schizophrenia (SCZ) are difficult to replicate in a laboratory animal. At the same time, no animal model is able to fully mimic any mental illness, as these are characterized by specific disturbances in functions that are absolutely unique to humans, such as markedly diminished interest, thought disorders and hallucinations (American Psychiatric Association 2000). However, a general approach is to reproduce particular symptoms of psychiatric diseases (i.e., attention/cognitive deficits) in laboratory animals or to develop models (i.e., the forced swim test) to identify novel compounds as potential treatments (Cryan et al. 2002; Meyer et al. 2009). Ideally, an animal model should reflect the human psychiatric disease in terms of face validity (i.e., reproduce the symptoms of the human mental disease), construct validity (i.e., replicate the neurobiological abnormalities) and predictive validity (i.e., response to the pharmacological treatment in a way that predicts the effects of that treatment in humans). Nevertheless, none of the available animal models are able to mimic all the aspects of neuropsychiatric disorders, in terms of neurobiological mechanisms and disease symptoms and most likely never will. Therefore, the lack of knowledge regarding the mechanisms that underlie diseases such as depression and SCZ, their comorbidity and symptomatic overlap between them (i.e., patients with psychotic depression) is associated with the partial efficacy of the present pharmacological armoury. This raises the central question to be addressed in this review: are current animal models reliable tools with a predictive validity for the development of novel therapeutic compounds?

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Table 1 Behavioural effects of clinically prescribed antidepressants in validated animal models of depression (*SSRI* selective serotonin reuptake inhibitor, *SNRI* serotonin and noradreniline reuptake inhibitors)

Rodent model of depression	Behavioural antidepressant-like response	Drug treatment
Olfactory bulbectomy	↓ Hyperactivity in the open field test (after chronic drug administration)	Tianeptine, sertraline (Kelly and Leonard 1994), desipramine (Kelly and Leonard 1996), amitryptiline (Stockert et al. 1988), imipramine (Roche et al. 2008), citalopram (Hasegawa et al. 2005; Nguyen et al. 2009), fluvoxamine (Saitoh et al. 2007), fluoxetine (Freitas et al. 2013; Machado et al. 2012; Roche et al. 2007), buspirone (Sato et al. 2010), agomelatine (Norman et al. 2012), tiagabine (Pistovcakova et al. 2008)
Learned helplessness	↓ Number of failures to escape shock	Imipramine (Besson et al. 1999; Demontis et al. 1993; Gambarana et al. 1995; Geoffroy et al. 1991; Ishida et al. 2005; Iwamoto et al. 2005; Iwata et al. 2006; Joca et al. 2003; Martin and Puech 1991; Martin et al. 1987; Meloni et al. 1993; Takamori et al. 2001), desipramine (Beck and Fibiger 1995; Besson et al. 1999; Centeno and Volosin 1997; Duman et al. 2007; Joca et al. 2006; Martin et al. 1987; Rojas-Corrales et al. 2004; Rusakov and Valdman 1983), chlorimipramine (Rusakov and Valdman 1983), clomipramine (Martin and Puech 1991; Martin et al. 1987; Millan et al. 2001), amitriptyline (Besson et al. 1999; Caldarone et al. 2003; Rusakov and Valdman 1983), trazodone (Rusakov and Valdman 1983), tranylcypromine, mianserine (Takamori et al. 2001), venlafaxine (Millan et al. 2001; Rojas-Corrales et al. 2004), fluvoxamine (Iwamoto et al. 2005; Martin and Puech 1991; Rojas-Corrales et al. 2004; Takamori et al. 2001), mirtazapine (Slattery et al. 2005), fluoxetine (Iwamoto et al. 2005; Marco and Laviola 2012; Marcussen et al. 2008; Page and Abercrombie 1997; Reines et al. 2008; Shumake et al. 2010; Zazpe et al. 2007), paroxetine (Zazpe et al. 2007), sertraline (Duman et al. 2007), St. John's wort extract (Chatterjee et al. 1998), buspirone (Lucki 1991; Martin and Puech 1991); citalopram (Martin and Puech 1991; Millan et al. 2001), escitalopram (Reed et al. 2008), zimelidine (Dabrowska et al. 2008; Joca et al. 2006), lamotrigine (Consoni et al. 2006), agomelatine (Bertaina-Anglade et al. 2006; Dagyte et al. 2011; Popoli 2009; Tardito et al. 2010)
Forced swim test	↓ Time of immobility (↑ swimming or climbing activities) (after acute drug administration)	Amitryptiline (Caldarone et al. 2003), tianeptine (Della et al. 2012; Kelly and Leonard 1994; Solich et al. 2008), imipramine (Bourin et al. 2004; Della et al. 2012; Kulkarni and Dhir 2007; Paulke et al. 2008; Schulte-Herbrueggen et al. 2012; Zanelati et al. 2010), desipramine (Robles-Molina et al. 2012; Simpson and Kelly 2012; Will et al. 2003), venlafaxine (Kulkarni and Dhir 2007), sertraline (Kelly and Leonard 1994; Leggio et al. 2008; Rogoz and Skuza 2006), paroxetine (Akagawa et al. 1999; Leggio et al. 2008), reboxetine (Cryan et al. 2005b; Wong et al. 2000), phenelzine (Bourin et al. 2002; Will et al. 2003), tranylcypromine, agomelatine (Bourin et al. 2002, 2004), fluoxetine (Bourin et al. 2004; Cryan et al. 2005b; Kulkarni and Dhir 2007; Reed et al. 2008; Rogoz and Skuza 2006), paroxetine (Karanges et al. 2011), moclobemide (Cryan et al. 2005b), pramipexol (Rogoz and Skuza 2006; Schulte-Herbrueggen et al. 2012), mirtazapine (Muguruza et al. 2013), St. John's wort extract (Paulke et al. 2008), citalopram (Leggio et al. 2008; Nguyen et al. 2009; Tamburella et al. 2009, 2013), escitalopram (Nguyen et al. 2013; Reed et al. 2008), clomipramine (Consoli et al. 2005, 2007; Leggio et al. 2008; Micale et al. 2006, 2008a, 2008b; Tamburella et al. 2009, 2010, 2013)
	False positive results	Amphetamines (Cryan et al. 2002), caffeine (Slattery and Cryan 2012)
Tail suspension test	↓ Time of immobility (after acute drug administration)	Mianserine, nomifensine, viloxazine (Steru et al. 1985), amitryptiline (Caldarone et al. 2003; Steru et al. 1985), desimipramine (Berrocoso et al. 2013; O'Leary et al. 2007; Steru et al. 1985), imipramine (Berrocoso et al. 2013; Kulkarni and Dhir 2007; Liu and Gershenfeld 2001), reboxetine (O'Leary

Table 1 (continued)

Rodent model of depression		Behavioural antidepressant-like response	Drug treatment
			et al. 2007; Wong et al. 2000), tianeptine (Berrocoso et al. 2013), fluoxetine (Berrocoso et al. 2013; Kulkarni and Dhir 2007; Muguruza et al. 2013; O'Leary et al. 2007), mirtazapine (Muguruza et al. 2013), venlafaxine, duloxetine (Berrocoso et al. 2013; Kulkarni and Dhir 2007), citalopram (Berrocoso et al. 2013)
Chronic mild stress		↑ Responsiveness to rewards (after chronic drug administration)	Fluoxetine (Jindal et al. 2013; Muscat et al. 1992; Mutlu et al. 2012), maprotiline (Muscat et al. 1992), minaserin (Cheeta et al. 1994), imipramine (Marston et al. 2011; Norman et al. 2012; Papp et al. 1996; Przegalinski et al. 1995), buspirone (Papp et al. 1996; Przegalinski et al. 1995), ipsapirone (Przegalinski et al. 1995), agomelatine (Bourin et al. 2004; Dagyte et al. 2011), risperidon (Marston et al. 2011), citalopram (Herrera-Perez et al. 2010; Przegalinski et al. 1995), escitalopram (Christensen et al. 2012), tianeptine (Mutlu et al. 2012)
Social stress—repeated defeat	Resident-intruder	↓ Agressivity, ↑ flight	Acute: SSRIs, SNRIs, tricyclics (Mitchell and Neumaier 2005)
		↑ Aggressivity	Chronic: SSRIs, SNRIs, tricyclics (Mitchell and Neumaier 2005)
		↑ Ambulation in open field test	Fluoxetine, reboxetine (Rygula et al. 2006, 2008)
	Group-housed vs. singly-housed aggressive partner	↑ Ambulation in open field test	Chronic: citalopram, valproate, felbamate (Pistovcakova et al. 2005; Sulcova 1999)

Status of current animal models of depression and their pharmacological validation

Unfortunately, an animal model that perfectly includes the aetiology, pathophysiology and symptoms of depression while allowing an evaluation of the responses to treatments remains difficult to envisage. Although the generation of genetically modified mice could result in animal models mimicking genetic, biochemical or behavioural characteristics of human depression, we have to keep in mind the role of major confounding factors such as background strain, neurodevelopment or interactions between genetic and environmental factors during the interpretation of any findings (Urani et al. 2005). However, various models, each with specific limitations, are able to reproduce most of the aetiological factors and symptoms of the disease or possess a satisfactory predictive value for identifying new compounds. On this basis, we review the validation of rodent models of depression, such as bilateral olfactory bulbectomy (OBX), learned helplessness, the forced swim test (FST) or the tail suspension test (TST) and the chronic mild stress (CMS) or chronic social stress paradigm, according to the effects of pharmacological interventions that have successfully achieved antidepressive-like activities in animals and treatment efficacy in depressive patients.

Olfactory bulbectomy

OBX results in behavioural (i.e., hyperactive response in the open field paradigm) and neurochemical (i.e., changes in the endocrine, immune and neurotransmitter systems) alterations in rats (Cairncross et al. 1975; Jesberger and Richardson 1985; Kelly et al. 1997) and mice (Hellweg et al. 2007; Zanelati et al. 2010; Zueger et al. 2005); the alterations resemble some of those seen in depressed patients and are reversed by chronic treatment with clinically approved or potential antidepressants (Tables 1 and 2). Since the olfactory system in rodents is part of the limbic region in which the amygdala and hippocampus contribute to emotional behaviour, OBX affects the cortical-hippocampal-amygdala circuit, which also seems to be dysfunctional in depressed patients (Song and Leonard 2005). Interestingly, a dysregulation of the functionality of the central reward pathway in bulbectomized rats has also been reported, suggesting that it may have an impact on the development of depression/addiction comorbidity. Thus, OBX could be a useful animal model of these dual diagnosis disorders (Kucerova et al. 2012).

Learned helplessness

Learned helplessness might model in animals a human situation of unpredictable and uncontrollable events leading to consequences: "stress-coping depression". Thus, the animal model is considered to provide specificity towards antidepressant pharmacotherapy (Chourbaji et al. 2005; Christensen 1993; Maier 1984; Miller and Seligman 1976; Seligman and Beagley 1975; Sherman et al. 1982; Vollmayr and Henn 2001). Animals exposed to inescapable and unavoidable electric shocks in one situation later fail to escape shock in a

different situation in which escape is possible. A drug is considered to be effective as an antidepressant if the learned helplessness is reduced (the number of failures to escape is decreased). However, we need to assess a depressive-like phenotype in experimental animals and exclude some subjects from the study. In mice, approximately 30 % of individuals reportedly become helpless after shock exposure. However, the remaining animals show helpless behaviour with high escape latency and thus a low number of failures to escape might be attributable to variable pain sensitivity (Chourbaji et al. 2005). Parameters for inescapable shock and the testing of learned helplessness to minimize artifacts have been stated in a study published elsewhere (Chourbaji et al. 2005). Two rat lines have also been established by selective breeding, namely helpless and non-helpless, which differ in neurochemical and behavioural parameters that are known to be related to depression (Henn and Vollmayr 2005).

Forced swim test and tail suspension test

These two tests are widely used paradigms specifically developed to test new antidepressants. In the FST (also known as Porsolt's test; Porsolt et al. 1977), rodents are forced to swim in an inescapable cylinder and will eventually adopt a characteristic immobile posture that is interpreted as a passive stress-coping strategy or depression-like behaviour (behavioural despair). The FST has shown its ability to detect a broad spectrum of substances that are therapeutically effective in human depression, as these drugs shift passive-stress coping towards active coping, which is detected as reduced immobility (Table 1). Furthermore, the quantity of the different movements, such as climbing or swimming behaviour, has a predictive value for differentiating between noradrenergic (NAergic) and serotonergic (5-HTergic) activity (Cryan et al. 2002). However, care must be taken with regard to the strain (variations have been shown between inbred and outbred mice and rats) used for the test because of differential spontaneous locomotor activity possibly reducing the duration of immobility (Crawley et al. 2007; Petit-Demouliere et al. 2005). False positive results can be obtained when testing drugs with psychostimulant activity, e.g., amphetamines, caffeine (Cryan et al. 2002; Slattery and Cryan 2012).

Similar assumptions and interpretations to those for the FS, can be drawn from the TST (Steru et al. 1985). In this test, mice are suspended by their tails for a defined period of time during which their immobility is decreased by several antidepressants. The percentage of animals showing passive behaviour should be counted and then compared with that after vehicle or active drug treatment, as several mouse strains have been shown to be essentially resistant to tail-suspension-induced immobility (Cryan et al. 2005a). The test however is sensitive to acute treatment only and its validity for non-monoamine antidepressants is uncertain (Berrocoso et al. 2013; Cryan et al. 2005b).

Chronic mild stress

Chronic mild stress procedures (food or water deprivation, 45° cage tilt, intermittent illumination, soiled cage, paired housing or low-intensity stroboscopic illumination), applied for a period of several consecutive weeks decrease the responsiveness to rewards (consumption of a 1 % sucrose solution) in rats or mice; this is reversed by chronic administration of antidepressant drugs. This "chronic mild stress model" is considered to represent anhedonia in depression (Papp et al. 1996; Willner 1984, 1997; Willner et al. 1992). In comparison with other animal models of depression, it has been evaluated as a high perspective research approach, despite its procedural complexity and difficult reproducibility (Porsolt 2000). Chronic treatment with clinically used antidepressants normalizes sucrose drinking (Table 1).

Drug-withdrawal-induced anhedonia

A withdrawal from abuse of psychoactive compounds (e.g., cocaine, amphetamines) is known to be associated with states of depression in humans and depressive-like states in animals (Barr and Phillips 1999; Jang et al. 2013; Renoir et al. 2012). The animal model "drug-withdrawal-induced anhedonia" is based on experimental experience with laboratory rodents; upon their withdrawal from long-term treatment with psychostimulatory agents, they show mild food and water avoidance as depressive-like symptoms (anhedonia) in response to rewards in various paradigms, e.g., place preference, i.v. drug self-administration, electric intracranial self-stimulation or sucrose solution preference (Barr and Phillips 1999; Cryan and Mombereau 2004). Rates of reward responding is increased by subsequent treatment with antidepressants, e.g., imipramine and amitriptyline (Kokkinidis et al. 1980).

Chronic social stress

Repeated social stress was suggested as an aethologically relevant animal model of depression in mice (Keeney and Hogg 1999), rats (Rygula et al. 2005) and tree shrews (Fuchs 2005). Any behaviour indicative of social conflict such as threat, attack, fight or escape, avoidance or subordination is called agonistic behaviour and encompasses the actions of both the instigator and the victim (Scott 1966). Compared with control individuals, the animals that are subjected to repeated agonistic encounters exhibit significantly reduced locomotor activity in the open field test, which, in turn is normalized by previously clinically proven or potential antidepressants, e.g., citalopram or valproate and by potential

antidepressants, e.g., felbamate (Pistovcakova et al. 2005; Sulcova and Pistovcakova 2008; Table 1).

Alterations of hypothalamic-pituitary-adrenal functions have been established in states of depression and stress, including social stress conditions, in both humans and animals (Blanchard et al. 2001; Kubera et al. 2011; Mathews et al. 2006; Morris et al. 2012). In rodents, social defeat and subordination are stressful, especially in males (Blanchard et al. 2001; Martinez et al. 1998). Animals that are subjected to repeated agonistic encounters are used for testing potential antidepressant treatment effects (Mitchell 1994, 2005; Sulcova 1999). The same stress procedure results in increased release of corticosterone and dopamine (DA). Felbamate decreases NA concentrations and inhibits the stress-induced rise in corticosterone and DA. Modulation of stress hormone release has been suggested to be induced by the action of felbamate on glutamate neurotransmission and neuroendocrine changes might contribute to behavioural effects of the drug (Pistovcakova et al. 2005). The mood-stabilizing action of felbamate and other anti-epileptic drugs has been proposed by clinicians for further verification (Cavanna et al. 2010).

Current leading compounds for development of new antidepressants

Pharmacological analyses of action of clinically approved antidepressants support the predictive validity of the animal models presented. However, consideration of the behavioural and molecular phenotypes corresponding to the human disorders suggests that these models are also useful for the improvement of our knowledge of the neuronal mechanisms of the disease, the biomarkers of its specific symptoms and the integration of basic and clinical methodologies (translational medicine) for the development of new antidepressants (Borsini 2012; Cryan et al. 2002; Dzirasa and Covington 2012; Kluge et al. 2011; Neumann et al. 2011; Rupniak 2003). Taking into account that the 5-HT hypothesis of depression has not been abandoned (Albert and Benkelfat 2013), the targets of potential relevance as treatments for mood disorders are also those involved in the regulation of several other neuronal systems in the brain, including the opioid system (Pradhan et al. 2011), the cholinergic system (Drevets et al. 2013), the endocannabinoid system (Marco and Laviola 2012; Micale et al. 2013), the neuropeptidergic signalling system (Griebel and Holsboer 2012), the melatoninergic system (Lanfumey et al. 2013) and the glutamatergic system (Connolly and Thase 2012; Hashimoto 2011; Javitt 2012; Machado-Vieira et al. 2012; Mathews et al. 2012; Serafini et al. 2013; Tokita et al. 2012). Thus, attention should be given to compounds influencing these systems, which have been shown to produce antidepressant-like effects in animal models. Currently, the compounds that modulate glutamatergic neurotransmission are reported to hold the greatest promise for the development of new antidepressants (Serafini et al. 2013). Suggested mechanisms are based on the antagonistic influence on ionotropic N-methyl-D-aspartate (NMDA) receptors, the modulation of metabotropic glutamate receptors, especially the negative modulation of mGlu2/3 and mGlu5 receptors (Chaki et al. 2013) and the positive modulation of mGlu2 and mGlu7 receptors (Sanacora et al. 2012). The animal model studies with leading glutamatergic compounds are cited in Table 2.

Status of current animal models of SCZ and their pharmacological validation

SCZ, described by Kraepelin in 1896 as a dementia praecox, is a unique human disorder for which modelling in animals might prove problematic because of the lack of a uniform set of symptoms in patients and indictions of the heterogeneity of the disorder. Thus, a greater understanding of the disorder might arise from modelling specific signs and symptoms, as opposed to the entire syndrome. In line with this strategy, several efforts have been directed at developing animal models that allow the translation of the symptomatology in SCZ and prediction of antipsychotic activity. Although positive symptoms such as hallucinations and delusions cannot be measured in animals, the most reliable behavioural indices of positive symptoms in animal models are hyperlocomotor activity and behavioural stereotypes that mimic the psychomotor agitation and presence of stereotyped behaviour in acutely psychotic patients (Young et al. 2010). The rationale for the use of these indices is based upon the principle that the hyperfunction of the mesolimbic DAergic system, which seems to be involved in the enhanced locomotor activity and stereotyped behaviours, is consistent with the clinical conditions in which enhanced subcortical DAergic activity plays a pivotal role in precipitating positive symptoms (Murray et al. 2008). The loss of selective associative learning in the form of the disruption of latent inhibition, which is also induced by hyperdopaminergic activity at the subcortical level, seems to be another cross-species translational index relevant to positive symptoms of SCZ (Weiner 2003). Indeed, some behavioural aspects of SCZ can be modelled and objectively assessed in rodents. More specifically, anhedonia and social behaviour as hallmarks of negative symptoms in humans can be assessed in rodents, together with prepulse inhibition, which reflects disrupted sensory gating abilities both in schizophrenic patients and in experimental animal models (Young et al. 2010). Finally, the various cognitive aspects affected in the disease, as identified by the NIH Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative (Marder and Fenton 2004), can be

Table 2 Antidepressant-like effects of glutamatergic leading compounds in animal models of depression (*NMDA* N-methyl-D-aspartate, *AMPA* α-amino-3-hydroxy-5-methylisoxazole-2-proprionic acid)

Pharmacological mechanism	Compound: animal models (references)
NMDA receptor antagonist	Ketamine: learned helplessness, tail suspension test (Koike et al. 2011), forced swim test (Engin et al. 2009; Lindholm et al. 2012), chronic mild stress (Garcia et al. 2009)
mGlu2/3 receptor antagonist	MGS0039: tail suspension test (Koike et al. 2011), learned helplessness (Yoshimizu et al. 2006), olfactory bulbectomy (Palucha-Poniewiera et al. 2010), forced swim test (Kawasaki et al. 2011); LY341495: tail suspension test (Chaki et al. 2004; Koike et al. 2013)
mGlu2/3 receptor allosteric negative modulator	RO4491533: tail suspension test (Campo et al. 2011)
mGlu2 receptor allosteric potentiator	THIIC: forced swim test (Fell et al. 2011)
mGlu5 receptor uncompetitive antagonist	MTEP: tail suspension test, forced swim test (Belozertseva et al. 2007; Li et al. 2006)
mGlu5 receptor negative allosteric modulator	GRN-529: tail suspension test, forced swim test (Hughes et al. 2013)
mGlu7 receptor allosteric agonist	AMN082: tail suspension test, forced swim test (Bradley et al. 2012)
NMDA receptor glycine-site partial agonist	GLYX 13: learned helplessness, forced swim test (Ashton and Moore 2011; Burgdorf et al. 2013)
AMPA receptor potentiator	LY 451646: forced swim test (Lindholm et al. 2012)

experimentally addressed in animal models by the use of specific test batteries (Peleg-Raibstein et al. 2012). Among the several approaches used to create experimental animal models of SCZ, which also include the lesion model (Jones et al. 2011) and genetic-based preparations (Inta et al. 2010), we will examine, in the following discussion, (1) pharmacological models and (2) neurodevelopmental models that are the most used in drug discovery studies (Tables 3, 4)

Pharmacological models

Hyperfunction of the DAergic system in the mesolimbic pathway was the original tenet for the occurrence of SCZ; thus, the first animal models were developed on the basis of the pharmacological manipulation of the DAergic system in an attempt to mimic this dysregulation (Carlsson et al. 2001). In rodents, repeated treatment with the DA-releasing agent amphetamine induced a persistent sensitization exaggerating the hyperactivity caused by an acute amphetamine challenge, which was prevented by antipsychotic pre-treatment. This model is supported by the observation that chronic psychostimulant abuse can lead to psychotic episodes, whereas low doses of amphetamine worsen the symptoms (Featherstone et al. 2007). Amphetamine sensitization is also characterized by deficits in prepulse inhibition or latent inhibition and in prefrontal-cortex-dependent cognitive tasks, whereas hippocampal function is unaltered (Peleg-Raibstein et al. 2012; Russig et al. 2002, 2005; Tenn et al. 2005). Furthermore, it is accompanied by neurochemical (i.e., increase in DA, NA and 5-HT efflux in nucleus accumbens, striatum or prefrontal cortex) and structural changes (i.e., reduction of parvalbumin and brain-derived neurotrophic factor expression in the medial prefrontal cortex and hippocampus, respectively) (Doucet et al. 2013; Morshedi and Meredith 2007; Motawaj and Arrang 2011; Salomon et al. 2006). However, it fails to induce any deficits in social activity as an index of negative symptoms and therefore limits the conformity to available human data (Srisurapanont et al. 2003, 2011). Similarly, the preferential DA receptor agonist apomorphine has induced a SCZ-like phenotype in rodents (Peleg-Raibstein et al. 2012). Overall, behavioural changes induced by DA-stimulating drugs have been employed as models of psychosis or cognitive-related abnormalities but they fail to capture cardinal aspects of negative symptoms.

The glutamate hypothesis of SCZ has been developed from the observation that NMDA receptor antagonists induce, in normal humans, a psychosis-like state (plus negative and cognitive symptoms) that closely resembles SCZ, leading to the establishment of glutamatergic models of SCZ (Javitt 2012). In animals, acute phencyclidine (PCP) treatment induces hyperactivity and disruption of prepulse inhibition; this is reversed by atypical but not typical antipsychotics (Mouri et al. 2007). However, both classes of antipsychotic agents are able to counteract the ketamine-induced deficits, suggesting a different involvement of D2 receptors in the PCP or ketamine effects (Neill et al. 2010). Acute PCP treatment affects social activity and sucrose consumption, as indices of negative symptoms and various different cognitive domains (Mouri et al. 2012; Turgeon and Hulick 2007). More conflicting results have been obtained from repeated PCP treatment, which elicits reduced (Snigdha and Neill 2008) or no effects (Sams-Dodd 2004) on social behaviour and an improvement in negative-like symptoms (Brigman et al. 2009). PCP-induced deficits have also been found in various cognitive domains, which are counteracted by atypical antipsychotics (Amitai et al. 2007; Kunitachi et al. 2009). However, in clinical practice, antipsychotics do not

Table 3 Pharmacological models of schizophrenia (*5-HT* serotonin, *AMY* amygdala, *ARI* aripiprazole, *BDNF* brain-derived neurotrophic factor, *BLA* basolateral amygdala, *CB* cerebellum, *CLZ* clozapine, *DA* dopamine, *DRD2* dopamine D2 receptor, *FC* frontal cortex, *GLU* glutamate, *HPC* hippocampus, *HP* haloperidol, *mPFC* medial prefrontal cortex, *ND* not determined, *NE* noradrenalin, *NOR-1* nuclear orphan receptor 1, *PFC* prefrontal cortex, *PPI* prepulse inhibition, *PV* parvalbumin, *OLA* olanzapine, *RIS* risperidone)

Drug	Positive-like symptoms	Negative-like symptoms	Spatial/working memory	Latent inhibition	Prepulse inhibition	Neurochemical changes	Antipsychotic response	References
Dopamine agonists								
Amphetamine	Yes	No	Deficit	Deficit	Deficit	↑ Mesolimbic DA ↑ NE and 5-HT in the PFC ↓ PV in the mPFC ↓ BDNF in the HPC	Deficits reversed by CLZ and HP	Doucet et al. 2013, Featherstone et al. 2007, Morshedi and Meredith 2007, Motawaj and Arrang 2011, Peleg-Raibstein et al. 2012, Russig et al. 2002, 2005, Salomon et al. 2006, Srisurapanont et al. 2003, 2011, Tenn et al. 2005
Apomorphine	Yes	ND	Deficit	Deficit	Deficit	↓ mGluR5 in the PFC	PPI deficit reversed by CLZ	Geyer and Ellenbroek 2003, Gourgiotis et al. 2012, Leng et al. 2003, Melo et al. 2009, Posch et al. 2012, Shao et al. 2010
NMDA receptor antagonists								
Phencyclidine	Yes	Yes	Deficit	Deficit	Deficit	↓ DA and GLU in the PFC ↓ PV in the FC,HPC and CB ↓ CaMKII in the PFC ↓ ERK in the HPC and AMY ↑ 5-HT in the PFC	Deficits reversed by HP, CLZ, ARI, RIS and OLA	Amitai et al. 2007, Bullock et al. 2009, Kunitachi et al. 2009, Li et al. 2011, Mouri et al. 2007, 2012, Noda et al. 2000, Pollard et al. 2012, Turgeon and Hulick 2007
Ketamine	Yes	Yes	Deficit	Deficit	Deficit	↓ PV in the HPC	Deficits reversed by HP, CLZ and RIS	Enomoto and Floresco 2009, Gama et al. 2012, Gao et al. 2009, Maehara et al. 2011, Neill et al. 2010, Pitsikas et al. 2008, Razoux et al. 2007, Rujescu et al. 2006
Dizocilpine (MK-801)	Yes	Yes	Deficit	Deficit	Deficit	↑ GLU and 5-HT in the mPFC ↓ PV in the mPFC, HPC and BLA	Deficits reversed by HP, CLZ, OLA and RIS	Feinstein and Kritzer 2013, Gaisler-Salomon et al. 2008, Gururajan et al. 2012, Lopez-Gil et al. 2007, 2012, Mutlu et al. 2012, Ozdemir et al. 2012, Romon et al. 2011, Uehara et al. 2012, Wiescholleck and Manahan-Vaughan 2013
5-HT agonist								
Lysergic acid diethylamide (LSD)	Yes	Yes	ND	ND	Deficit	↑ DRD2, 5-HT2c and NOR1 in the mPFC	CLZ reversed positive symptoms. HP has no effects on PPI disruption	Marona-Lewicka et al. 2011, Moreno et al. 2011, 2013, Ouagazzal et al. 2001, Palenicek et al. 2010
Muscarinic acetylcholine receptor antagonist								
Scopolamine	Yes	Yes	Yes	Deficit	Deficit	ND	PPI deficit reversed by CLZ and HP	Barak and Weiner 2010, 2011b, Depoortere et al. 2007, Guan et al. 2010, Harada et al. 2012, Johnson et al. 2005, Shannon and Peters 1990, Singer and Yee 2012

Table 4 Neurodevelopmental models of schizophrenia (*5-HT* serotonin, *AMY* amygdala, *CLZ* clozapine, *DA* dopamine, *dHPC* dorsal hippocampus, *GLU* glutamate, *HPC* hippocampus, *HP* haloperidol, *MAM* methylazoxymethanol, *mPFC* medial prefrontal cortex, *NAc* nucleus accumbens, *PFC* prefrontal cortex, *PV* parvalbumin, *OLA* olanzapine, *RIS* risperidone, *SER* sertindole, *vHPC* ventral hippocampus, *VTA* ventral tegmental area)

Experimental method	Positive-like symptoms	Negative-like symptoms	Spatial/ working memory	Latent inhibition	Prepulse inhibition	Neurochemical changes	Antipsychotic response	References
Prenatal manipulation								
Prenatal MAM exposure	Yes	Yes	Deficit	Deficit	Deficit	↑ DA activity at the VTA ↓ PV and mGlu5 in the mPFC ↔ Reelin in the HPC	Hyperactivity of DA neurons in the VTA reduced by HP and SER	Gastambide et al. 2012, Lodge et al. 2009, Lodge and Grace 2009, Matricon et al. 2010, Moore et al. 2006, Snyder et al. 2012, Valenti et al. 2011, Zimmerman et al. 2013
Prenatal polyinosinic: polycytidylic acid exposure	Yes	Yes	Deficit	Deficit	Deficit	↓ PV in the HIP ↓ DA in the mPFC and vHPC ↑ 5-HT in the AMY and NAc ↓ Reelin in the dHPC ↑ GAD67 in the vHPC	Deficits are reversed by RIS and CLZ	Bitanihirwe et al. 2010, Cardon et al. 2010, Harvey and Boksa 2012, Meyer et al. 2009, 2010, Piontkewitz et al. 2009, 2011, 2012, Vuillermot et al. 2012, Wolff and Bilkey 2010
Postnatal manipulation								
Postweaning isolation rearing	Yes	Yes	Deficit	Deficit	Deficit	↑ Mesolimbic DA ↑ GAD67 in the AMY ↓ PV and reelin in the vHPC ↓ CB1 and GluR1in the PFC ↑ Plasma tryptophan metabolites	Deficits reversed by HP, OLA, RIS and CLZ	Cassidy et al. 2010, Gilabert-Juan et al. 2012, Harte et al. 2007, Hermes et al. 2011, Marsden et al. 2011, Moller et al. 2011, 2013, Zamberletti et al. 2012a, 2012b
Neonatal ventral hippocampal lesion	Yes	Yes	Deficit	Deficit	Deficit	↑ DA in the PFC ↓ PV in the HPC ↓ GAD67 in the mPFC	Deficits reversed by HP, CLZ and RIS	Bringas et al. 2012, Lee et al. 2012, Macedo et al. 2012, Naert et al. 2013, O'Donnell 2012, Richtand et al. 2006, Swerdlow et al. 2012

improve cognition in patients; thus, further studies are necessary to assess the mechanisms underlying the PCP effect on cognition. Interestingly, the recent use of genetically modified mice has revealed that various components of the glutamatergic systems, such as specific glutamate receptor subtypes or various components of their intracellular transduction mechanism, might be involved in the pathophysiology of SCZ (Inta et al. 2010). Hallucinogens such as lysergic acid diethylamide (LSD) or cholinergic receptor antagonists, e.g., scopolamine, have induced, in humans and animals, psychotic-like effects, thus supporting the 5-HTergic or cholinergic hypothesis of SCZ, respectively. Therefore, the full potential of 5-HT or cholinergic manipulations in preclinical research of SCZ needs to be further validated (Barak 2009; Vollenweider et al. 1998).

Neurodevelopmental models

In the last few decades, human epidemiological data have supported the finding that pre-perinatal environmental factors such as malnutrition, infection and obstetric complications increase the risk of the development of SCZ (Brown et al. 2013). This knowledge has stimulated the development of models based on direct pre-perinatal damage of the central nervous system (CNS); such models replicate several behavioural and neurochemical

changes linked to the disease. In agreement with this approach, rats exposed in utero on gestional day 17 to methylazoxymethanol (MAM), an antimitotic agent that methylates DNA, show behavioural (hyperactivity, cognitive and social deficits or prepulse inhibition disruption) and histopathological (decreased parvalbunin expression, hyperdopaminergia) patterns similar to those observed in SCZ (Lodge et al. 2009; Lodge and Grace 2009). Although the MAM model seems to have face validity for SCZ symptoms and construct validity in terms of the structural and DAergic changes observed, only a few recent studies have been performed to detect the antipsychotic activity of current agents (Belujon et al. 2012; Valenti et al. 2011) or novel compounds (Brown et al. 2013; Gastambide et al. 2012, 2013; Gill et al. 2011) and thus the predictive validity of this model is not extensively established. Similarly, maternal administration of the viral mimetic polyinosinic:polycytidylic acid induces, in the offspring, a spectrum of neurochemical and behavioural SCZ-related changes that were partially reversed by antipsychotics (Bitanihirwe et al. 2010; Ozawa et al. 2006). An alternative approach makes use of environmental manipulations during postnatal brain development and maturation, such as maternal separation, isolation rearing, early handling or brain lesions. These procedures are based on the hypothesis that they can deflect the physiological development, within the CNS, of an aberrant maturation process prone to the emergence of psychotic-like behaviour and of social, cognitive or attention/gating deficits that are sensitive to the existing antipsychotics.

The advantage of neurodevelopmental over pharmacological models of SCZ is the ability to perform behavioural and neurochemical investigations in the absence of confounding drugs and to identify new classes of antipsychotics by the use of agents operating on multiple pharmacological mechanisms.

New potential pharmacological targets in the treatment of SCZ: lessons from animal models

Current pharmacological treatment for SCZ is primarily focused on modulating DA and 5-HT signalling, which is generally effective in treating positive symptoms. However, it is less effective in treating the negative and cognitive symptoms and can induce several side effects, such as the extrapyramidal side effect, weight gain and diabetes mellitus. Furthermore, a significant proportion of patients are refractory to all current treatments; thus, the development of new approaches for treating SCZ is urgently needed (Keefe 2007). At the same time, we are becoming increasingly aware that the pathophysiology underlying SCZ cannot merely be explained by simple changes in monoamine signalling but involves more complex alterations in activity through key brain circuits that are critical for sensory, cognitive and emotional processing (Lisman et al. 2008; Marek et al. 2010). These brain circuits are modulated by DA and 5-HT, by the major excitatory and inhibitory neurotransmitters glutamate and GABA, which are critical for signalling through these circuits and by acetylcholine. Thus, all these factors represent potential targets for pharmacological intervention (Table 5). Based on the hypothesis that impaired NMDA function in important cellular compartments of the limbic forebrains might represent a critical feature underlying the pathophysiology of SCZ, the mGlu2/3 receptor agonists (Cartmell et al. 1999; Fabricius et al. 2011; Hackler et al. 2010; Harich et al. 2007; Hikichi et al. 2013; Johnson et al. 2005, 2011; Moghaddam and Adams 1998; Nakazato et al. 2000; Patil et al. 2007; Profaci et al. 2011; Schlumberger et al. 2009; Takamori et al. 2003), the mGlu2- (Galici et al. 2005; Harich et al. 2007; Nikiforuk et al. 2010) and mGlu5-positive allosteric modulators (PAMs; Clifton et al. 2013; Darrah et al. 2008; Gastambide et al. 2013; Gilmour et al. 2013; Horio et al. 2012; Kinney et al. 2005; Kjaerby et al. 2013; Schlumberger et al. 2009, 2010; Stefani and Moghaddam 2010; Vales et al. 2010) and the mGlu group III orthosteric agonists (Palucha-Poniewiera et al. 2008; Wieronska et al. 2012, 2013) have all shown preclinical efficacy in reversing SCZ-like symptoms in several experimental models. Although the positive results have not been fully confirmed by clinical trials, the mGlu receptor ligands seem to represent the first non-dopamine D2 receptor-based antipsychotics (Hashimoto et al. 2013). To obtain a more efficient NMDA receptor activation through an increased synaptic glycine concentration, selective glycine transporter-1 (GlyT-1) inhibitors have been shown to be effective in specific preclinical models of SCZ (Alberati et al. 2012; Hagiwara et al. 2013; Chen et al. 2010; Karasawa et al. 2008; Nagai et al. 2012; Shimazaki et al. 2010; Yang et al. 2010). Although definitive trials remain ongoing, encouraging results to date have been reported (Javitt 2012). Several lines of evidences suggest that alterations in central muscarinic or nicotinic cholinergic neurotransmission are involved in the pathophysiology of SCZ (Jones et al. 2012). Thus, based on the above premise, the M1/M4 muscarinic acetylcholine receptor (mAChR) agonist xanomeline (Barak and Weiner 2011b; Jones et al. 2005; Thomsen et al. 2010; Woolley et al. 2009), the M1 or M4 PAMs (Brady et al. 2008; Chan et al. 2008; Jones et al. 2005; Thomsen et al. 2010; Vanover et al. 2008) and the α7 nAChr agonist/activators (Barak 2009; Feuerbach et al. 2009; Hauser et al. 2009; Rezvani et al. 2010; Roncarati et al. 2009; Wallace and Porter 2011; Wishka et al. 2006) have been shown to be effective in animal studies. Despite the promising preclinical data, additional studies are needed to develop more selective mAChRs subtype compounds (i.e., molecules without agonistic activity at M2 and M3 mAChRs) to avoid undesirable cholinergic side effects (Langmead et al. 2008). Among the phosphodiesterases (PDEs), which are a class of enzymes within the intracellular

Table 5 Leading compounds in experimental models of schizophrenia (*5-HT* serotonin, *mAChR* muscarinic acetylcholine receptor, *MAM* methylazoxymethanol, *nAChR* nicotinic acetylcholine receptor, *ND* not determined, *NMDA* N-methyl-D-aspartate, *PAMs* positive allosteric modulators, *PDE* phosphodiesterase, *PPI* prepulse inhibition)

Drugs	Animal models	Positive-like symptoms	Negative-like symptoms	Cognitive dysfunctions	Sensorimotor gating deficits in PPI	References
mGlu2/3 agonists						
LY354740, LY404039, LY379268, MGS0008 MGS0028 BINA CBiPES	Amphetamine, NMDA antagonist, Neonatal ventral hippocampal lesion	Improvement	ND	Improvement	Improvement	Cartmell et al. 1999, Fabricius et al. 2011, Hackler et al. 2010, Harich et al. 2007, Hikichi et al. 2013, Johnson et al. 2005, 2011, Moghaddam and Adams 1998, Nakazato et al. 2000, Patil et al. 2007, Profaci et al. 2011, Schlumberger et al. 2009, Takamori et al. 2003
mGlu2 PAM						
LY487379	Amphetamine, NMDA antagonist	Improvement	ND	Improvement	Improvement	Galici et al. 2005, Harich et al. 2007, Nikiforuk et al. 2010
mGlu5 PAM						
CDPPB ADX47273 CPPZ LSN2463359 LSN2814617	Amphetamine, NMDA antagonist, MAM	Improvement	Improvement	Improvement	Improvement	Clifton et al. 2013, Darrah et al. 2008, Gastambide et al. 2012, Horio et al. 2012, Kinney et al. 2005, Kjaerby et al. 2013, Schlumberger et al. 2009, 2010, Stefani and Moghaddam 2010, Vales et al. 2010, Vardigan et al. 2010
mGlu group III orthosteric agonists						
LSP1-2111 ACPT-I	Amphetamine, NMDA antagonist,	Improvement	Improvement	Improvement	ND	Palucha-Poniewiera et al. 2008, Wieronska et al. 2012, 2013
Glycine transporter 1 inhibitors						
RG1678 Sarcosine d-Serine	Amphetamine NMDA antagonist Polyinosinic: polycytidylic acid	Improvement	Improvement	Improvement	Improvement	Alberati et al. 2012, Hagiwara et al. 2013, Chen et al. 2010, Karasawa et al. 2008, Nagai et al. 2012, Shimazaki et al. 2010, Yang et al. 2010
M1/M4 mAChR agonists						
Xanomeline	Amphetamine NMDA antagonist Scopolamine	Improvement	Improvement	Improvement	Improvement	Barak and Weiner 2011a, Thomsen et al. 2010, Woolley et al. 2009
M1/M4 mAChR PAMs						
TBPB LY2033298 BQCA AC-260584 VU0152100	Amphetamine Apomorphine Scopolamine	Improvement	ND	Improvement	Improvement	Bradley et al. 2010, Brady et al. 2008, Chan et al. 2008, Jones et al. 2008, Vanover et al. 2008

Table 5 (continued)

Drugs	Animal models	Positive-like symptoms	Negative-like symptoms	Cognitive dysfunctions	Sensorimotor gating deficits in PPI	References
α7 nAChR agonist/activator						
SSR180711 RG3487 SEN12333 TC-5619 MEM3454 JN403	Amphetamine, apomorphine NMDA antagonist	Improvement	Improvement	Improvement	Improvement	Barak 2009, Feuerbach et al. 2009, Hauser et al. 2009, Rezvani et al. 2010, Roncarati et al. 2009, Wallace and Porter 2011, Wishka et al. 2006
PDE4/PDE10A inhibitors						
Rolipram Papaverine TP-10 MP-10 Vp1-15 THPP-1	Amphetamine NMDA antagonist	Improvement	Improvement	Improvement	Improvement	Davis and Gould 2005, Grauer et al. 2009, Kanes et al. 2007, Schmidt et al. 2008, Siuciak et al. 2008, Smith et al. 2013, Weber et al. 2009
H3 antagonists/inverse agonists						
ABT-239 Pitolisant GSK-189254 GSK207040 Irdabisant A-431404	Amphetamine NMDA antagonist MAM	Improvement	ND	Improvement	Improvement	Brown et al. 2013, Fox et al. 2005, Ligneau et al. 2007, Mahmood et al. 2012, Medhurst et al. 2007, Raddatz et al. 2012, Southam et al. 2009
5-HT_6 agonists/antagonists						
EMD386088 E-6801 PRX-07034 GSK-742457	NMDA antagonist Scopolamine	ND	ND	Improvement	No effect	Burnham et al. 2010, de Bruin et al. 2013, Kendall et al. 2011, Mohler et al. 2012, Nikiforuk et al. 2013

signal transduction cascade associated with brain abnormalities in SCZ, PDE4 and PDE10A seem to be novel therapeutic targets (Andreasen et al. 2011). Interestingly, specific PDE4 or PDE10A inhibitors ameliorate positive symptoms and cognitive/attention deficits (Davis and Gould 2005; Grauer et al. 2009; Kanes et al. 2007; Schmidt et al. 2008; Siuciak et al. 2008; Smith et al. 2013; Weber et al. 2009). Several compounds are currently undergoing clinical testing, mostly in clinical phase I trials in which SCZ is the leading indication (Kehler 2013). Studies on histamine function in the CNS have focused largely on the effects mediated via H3 receptor signalling. Hence, H3 receptors antagonists or inverse agonists have advanced into clinical assessment based on their effectiveness as cognition enhancers in experimental models of human diseases such as attention deficit hyperactivity disorder, SCZ and Alzheimer's disease (Brown et al. 2013; Fox et al. 2005; Ligneau et al. 2007; Mahmood et al. 2012; Medhurst et al. 2007; Raddatz et al. 2012; Southam et al. 2009; Vohora and Bhowmik 2012). In addition, the serotonin 5-HT_6 receptors have been identified as a potential target for the treatment of cognitive deficits in various disorders (Mitchell and Neumaier 2005). The 5-HT_6 receptor is almost exclusively expressed in brain areas associated with learning and memory and a large number of studies have shown that 5-HT_6 antagonists (de Bruin et al. 2013; Mitchell et al. 2006; Mohler et al. 2012) and 5-HT_6 agonists (Burnham et al. 2010; Kendall et al. 2011; Nikiforuk et al. 2013) have beneficial effects in several domains of cognition. Although the explanation for their similar pro-cognitive effect is unavailable, they might act on various neuronal subpopulations (Kendall et al. 2011; Schechter et al. 2008) and trigger diverse signalling pathways (Yun et al. 2007).

Conclusive remarks and future prospectives

In conclusion, the development of reliable and predictive animal models for neuropsychiatric disorders is a major challenge for assuring successful drug development. The field desperately needs better animal models of depression

and SCZ because of the partial efficacy of present pharmacological treatment. Without improved models of human disease, we cannot know whether particular molecular and cellular findings in animals are relevant to the clinical situations. Improved animal models of depression could come from various sources, such as mutant mice exhibiting particular depressive symptoms or human genetic studies identifying the genetic abnormalities that increase an individual's risk. Given the complexity of the neurobiological mechanisms involved in the SCZ, the recreation of the diversity of the disease in a single animal model might not be possible. Thus, the development and use of symptom-focused tests is important, whereby the goal is to replicate specific symptoms such as anhedonia or the seven cognitive domains as identified by the NIH-MATRICS consensus committee, which are impacted in SCZ, rather than the entire syndrome. Therefore, novel potential pharmacological targets (see Tables 2, 5) and positive control compounds will probably be needed for each of these domains. Nevertheless, all the findings reviewed above suggest that the identification of candidate compounds and the validation of efficacious treatments that can be used as positive controls in the development of new preclinical paradigms remain to be of paramount importance.

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POHLAVÍ A DROGOVÁ ZÁVISLOST: OD ANIMÁLNÍCH MODELŮ KE KLINICKÉ PRAXI

souborný článek

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SOUHRN

Amchová P, Kučerová J. Pohlaví a drogová závislost: od animálních modelů ke klinické praxi

Drogová závislost je závažný zdravotní i psychosociální problém, který kromě organického poškození organismu může vést až k neschopnosti plnit normální sociální funkce a role ve společnosti a rodině. Z klinických zkušeností je patrné, že závislost má u žen a u mužů odlišné charakteristiky, a přestože absolutní počet žen užívajících návykové látky je nižší než počet mužů, což je dáno především sociálními faktory, míra eskalace, potíže s ukončením a frekvence relapsů po abstinenci jsou u žen výrazně vyšší než u mužů. Tyto odlišnosti mezi pohlavími budou v budoucnu bezpochyby vyžadovat odlišné strategie léčby drogové závislosti u žen a mužů. Preklinické studie látkových závislostí byly dlouho vedeny výhradně na samcích laboratorních zvířat, neboť je známo, že estrální cykly samic výrazně ovlivňují chování i laboratorní výsledky. Ale i zde je v poslední době kladen stále větší důraz právě na identifikaci behaviorálních a neurochemických rozdílů mezi pohlavími za účelem vývoje personalizovaných léčebných řešení. Plazmatické koncentrace ženských a mužských pohlavních hormonů ovlivňují chování jedince, včetně adiktivního, velmi významně, ale byly již zaznamenány i rozdíly v mozkové struktuře závislé na pohlaví u člověka i experimentálních zvířat. Mezi další možné příčiny těchto rozdílů lze zařadit odliš-

SUMMARY

Amchová P, Kučerová J. Gender and drug addiction: from the animal models to human medicine

Drug addiction is a serious medical and psychosocial problem which leads to organic harm of the body as well as distortion of the normal functioning of affected persons within the society and family. There is a large body of clinical evidence suggesting differential characteristics of the disorder in men and women. Despite the absolute number of female drug abusers is lower than the male ones, women usually show higher escalation rate, more frequent relapses and more difficulties when discontinuing the drug use. These gender specific differences will require specific treatment strategies for men and women in the near future. Preclinical studies of drug addiction were carried out with male subject only for a long time because significant influence of the estrous cycle is well known in terms of behavioural and neurochemical effects but recently this approach has been abandoned on order to identify the gender differences and develop new more specific treatments. Levels of male and female gonadal hormones strongly affect the behaviour of people which concerns the addictive one as well. However, there have been reported also gender dependent differences in the brain structure in both humans and animals. Another source of the gender differences comprise metabolic adjustments leading to pharmacokinetic changes of

nosti v metabolismu, které způsobují rozdíly ve farmakokinetice návykových látek včetně rozdílů v množství tuku, vody a svalové hmoty, a to i na úrovni mozku. Dalšími faktory jsou rozdíly farmakodynamické, jejichž podkladem může být odlišná konektivita neuronálních drah a neuromediátorových systémů, které jsou již prenatálně modifikovány pohlavními hormony a chromosomy. Především farmakodynamické rozdíly vedou k subjektivním rozdílům v účinku návykových látek a následně i variabilitě v tendenci k rozvoji drogové závislosti, toleranci či senzitizaci k látce. Cílem této práce je poskytnout přehled současných znalostí z klinických i preklinických studií a mechanismů, které jsou podkladem pro mezipohlavní rozdíly v průběhu drogové závislosti.

Klíčová slova: animální modely, drogová závislost, farmakodynamika, farmakokinetika, geny, mezipohlavní rozdíly, pohlavní hormony, socio-kulturní rozdíly.

the drugs including different fat deposition, amount of water in the body or proportion of skeletal muscles. Pharmacodynamic changes could be under lied by changes in connectivity of the neuronal tracts and neurotransmitter systems which are modified prenatally by gonadal hormones or chromosomes. These pharmacodynamic specificities are the major source of subjective differences in the effects of the abused drugs and to the variable tendency to develop the addiction, tolerance or sensitization to the drug. The aim of this review article is to provide a survey of the current knowledge on the gender differences in drug addiction based on both clinical and preclinical studies preklinických together with the mechanisms responsible for such differences in the course of the drug addiction.

Key words: animal models, drug addiction, gender differences, pharmacodynamics, pharmacokinetics, sex hormones, genes, socio-cultural differences.

ÚVOD

Drogová závislost je zdrojem závažných zdravotních a psychosociálních problémů, které kromě organického poškození organismu mohou vést až k selhání normálního sociálního zařazení jedince. Pohlavní rozdíly v těchto fenoménech vyplývají již z epidemiologických dat. Česká republika dlouhodobě patří mezi země s největší konzumací alkoholu na světě, kde 15 % dospělých pije alkohol pravidelně a velmi často (denně nebo obden). U mužů je výskyt 23 %, u žen 8 %. Pití alkoholických nápojů alespoň jednou týdně uvedla více než polovina mužů a více než čtvrtina žen. Současných kuřáků tabákových výrobků je u nás 31 %, mužů 36,5 %, žen 26,3 %.[1]

Monitoring prevalence ostatních návykových látek se rutinně provádí pouze na základě evidence všech uživatelů drog, kteří za daný rok alespoň jedenkrát navštíví některé ze zařízení, která poskytují péči osobám užívajícím drogy. Tab. 1 uvádí prevalenci těchto osob v závislosti na pohlaví za rok 2013.

Drogová závislost má u žen a mužů odlišné charakteristiky: počínaje volbou návykové látky, její dávky, přes zahájení a průběh, až po tendenci k relapsu v období abstinence. Tyto odlišnosti mezi pohlavími budou v budoucnu bezpochyby vyžadovat odlišné strategie léčby závislosti u žen a mužů. Cílem tohoto článku je identifikovat možné zdroje těchto rozdílů a poskytnout přehled dostupných humánních i animálních dat.

Tab. 1. ***Prevalence uživatelů drog léčených v roce 2013 v ČR (zdroj: Hygienická stanice hl. m. Prahy)***

Návyková látka	Muži (počet)	Muži (%)	Ženy (počet)	Ženy (%)
Opiáty	1232	73,3	443	26,4
Kokain	13	68,4	6	31,6
Stimulancia	4587	66,7	2260	32,9
Hypnotika, sedativa	27	40,9	39	59,1
Halucinogeny	1	33,3	2	66,7
Kanabinoidy	822	76,3	251	23,3

K možným zdrojům lze řadit:

1) genetické odlišnosti související s geny nesenými pohlavními chromosomy,
2) odlišnou farmakokinetiku a farmakodynamiku,
3) odlišné hladiny pohlavních hormonů a
4) socio-kulturní rozdíly.

Animální modely drogové závislosti

Tato práce čerpá z klinických i preklinických dat, proto je nutné alespoň stručně popsat základní animální modely, které se ve výzkumu používají. V těchto modelech lze jasně izolovat jednotlivé fáze rozvoje a průběhu drogové závislosti. Preklinický výzkum tak umožňuje lépe pochopit neurobiologii drogové závislosti a poskytuje možnost studovat jednotlivé proměnné, které mají vliv na adiktivní chování. Takové studie umožňují analýzu změn v chování zvířat po expozici návykovým látkám i sledování hladin neurotransmiterů v příslušných mozkových drahách. Zvířecí modely mají samozřejmě své limity vycházející z rozdílnosti člověka a zvířete na všech úrovních. Rozvoj závislosti na návykové látce je komplexní proces ovlivněný mnoha faktory a animální model nemůže obsáhnout stav člověka ve všech aspektech. Většina animálních modelů vychází z předpokladu, že droga je pozitivní posilovač a stimuluje tak subjekt k vyhledávání další dávky (*positive reinforcement*). Motivace k opakovanému užití drogy může být na druhou stranu i z důvodu jejího averzivního účinku (*negative reinforcement*) za účelem odstranění nepříjemných pocitů (tzv. abstinenční syndrom) po přerušení opakované aplikace látky.

K modelům založeným na pozitivním posilovacím účinku drogy patří operantní intravenózní autoaplikace látek (***IV drug self-administration; IVSA***). Ten slouží ke sledování příjmu drogy, jeho eskalace a motivace zvířete pracovat pro další dávku. Dále sem lze zařadit model intrakraniální autostimulace (***intracranial self-stimulation, ICSS***), který spočívá v implantaci elektrody do mozku zvířete tak, aby elektricky stimulovala oblasti motivace a odměny po příslušném chování.

Stimulem pro vyvolání cravingu může být, kromě samotné drogy, i prostředí (***conditioned place preference, CPP***) nebo podnět (např. injekční stříkačka), který drogu připomíná. Na stejném principu jako CPP, kde má zvíře spojeno prostředí s pozitivním zážitkem (po aplikaci drogy), je založen i test averze k místu (***place aversion***) s očekávaným opačným efektem (jako negativní zážitek se užívá např. mírný elektrický výboj).[2]

GENETICKÉ ODLIŠNOSTI SOUVISEJÍCÍ S GENY NESENÝMI POHLAVNÍMI CHROMOSOMY

Existují klinické i preklinické studie dokazující vliv samotných pohlavních chromosomů (XX, XY), anebo kombinace chromosomů a hormonů na pohlavní rozdíly. Psychická závislost (tzv. habit formation) na kokainu může být ovlivněna nikoliv pouze pohlavními hormony, jak se předpokládá, že tomu je u fyzické závislosti po opakovaném podání látky, či při rozvoji behaviorální senzitizace,[3] ale vlivem pohlavních chromosomů.

Pro tento výzkum se v preklinických studiích stala ideálním modelem transgenní myš, které byl z chromosomu Y odstraněn gen *Sry* určující vznik varlat. U zvířete se pak vyvinuly vaječníky, přestože jeho gonozomy zůstaly XY.[4] Další studie využívající tohoto modelu zkoumala vliv amfetaminu na odměnu vyvolanou umělou elektrickou mozkovou stimulací (ICSS model) a došla k závěru, že amfetamin zesiluje systém odměny u genotypu XY, zatímco u XX nikoliv. Tento výsledek autoři odůvodňují rozdílnou citlivostí dopaminergního systému podmíněného pohlavním dimorfismem mozku, který vzniká v důsledku odlišného vývoje nezávisle na hormonálních hladinách.[5]

ROZDÍLY DANÉ ODLIŠNOU FARMAKODYNAMIKOU A FARMAKOKINETIKOU

Klinické údaje

V závislosti na návykové látce dochází k ovlivnění jednotlivých neuromediátorových systémů (dopaminergní, serotonergní, noradrenergní, opioidní, cholinergní, GABA-ergní, glutamátergní, endokanabinoidní a další) a ke změnám v jednotlivých částech mozku zodpovědných za systém odměny (kortikolimbický systém). Hladiny neurotransmiterů a mediátorů po podání látky jsou jiné u žen než u mužů, mezipohlavní rozdíl se také vyskytuje v objemu šedé a bílé hmoty míšní.

Existuje hypotéza, že k predispozici závislosti na návykových látkách může vést hypertrofie striata, která byla prokázána u lidí užívajících amfetamin.[6] Avšak vysoký objem striata může být také kompenzací toxicity vyvolané vysokými hladinami dopaminu v bazálních gangliích. Dalším společným znakem chronických uživatelů amfetaminu je snížený objem šedé hmoty mozkové.[6]

Také užívání kokainu je spojeno s poškozením neuronů a gliových buněk v šedé i bílé hmotě mozkové předního laloku. U abstinujících jedinců dochází k jejich reparaci, a to v mnohem vyšší míře u žen v porovnání s muži.[7] Dlouhodobé užívání alkoholu taktéž vede k úbytku objemu šedé i bílé hmoty u obou pohlaví, ale u žen v porovnání s muži markantněji.[8] Denier et al. ve své studii se závislými na heroinu dávají do souvislosti úbytek objemu šedé hmoty ve frontální oblasti mozku a snížený průtok krve v téže oblasti. U abstinujících závislých na metamfetaminu byl měřen průtok krve v týlním laloku a ve střední čáře, kdy u obou struktur byl naměřen nižší průtok u mužů ve srovnání se ženami.[9]

Důvodem odlišného vlivu návykové látky v ženském a mužském těle může být specifická farmakokinetika drogy, jak bylo např. popsáno u alkoholu: ženy mají nižší procento celkové tělesné vody, menší vliv prvního průchodu játry („first-pass“ efekt), a pomalejší metabolismus alkoholu v důsledku nižších hladin alkohol-dehydrogenázy v žaludeční sliznici, nebo farmakodynamické rozdíly, podmíněné např. rozdílným počtem, lokalizací či expresí receptorů v centrálním nervovém systému. V porovnání s muži jsou ženy více ohroženy intoxikací alkoholu[10] a mají i horší a rychleji nastupující chronické následky, jako atrofie mozku, onemocnění srdce, kosterních svalů a jater.[11]

Je již dlouho známo, že chronické užívání metamfetaminu působí neurotoxicky na dopaminergní neurony, kdy nejvíce postiženy jsou frontální a subkortikální oblast,[12]

a zdá se, že u mužů se projevují neurodegenerativní změny ve větší míře než u žen. Kromě již zmíněného sníženého průtoku krve a zmenšeného objemu šedé hmoty dochází k poklesu cerebrálního glukózového metabolismu v bílé hmotě ve frontální oblasti, což koreluje se sníženými funkcemi v této oblasti, a to pouze u mužů. U žen k této metabolické změně nedochází, což může být vysvětleno tím, že funkční glukózový metabolismus umožňuje reparaci gliových buněk.[13] Dalším vysvětlením je pak možné neuroprotektivní působení estrogenu.[14]

Preklinické údaje

Mezipohlavní rozdíly mohou být kromě hormonálních vlivů vysvětleny především na základě odlišností v mozkové organizaci. Bylo prokázáno, že samice potkanů po ovariektomii, tzn. nezávisle na hormonech, si aplikují kokain rychleji a ve větším množství než samci (model IVSA).[15] To znamená, že existuje rozdíl v nervových systémech zprostředkujících lokomočně pátrací chování v závislosti na pohlavní diferenciaci mozku v časných stadiích vývoje.[16] Studie zabývající se touto problematikou však ne vždy skutečně používají gonadektomizovaná zvířata, což je hlavním limitem pro interpretaci výsledků ve smyslu nezávislosti na hormonálních hladinách.

ROZDÍLY V HLADINÁCH POHLAVNÍCH HORMONŮ

Pohlavní hormony působí na systém odměny a stresu, čímž ovlivňují dva nejčastější důvody příjmu drogy: pro potěšení a za účelem uvolnění stresu. To se potvrdilo jak v preklinických, tak v klinických studiích, přestože hormonální změny u zvířat přesně nekopírují změny hladin hormonů u lidí.

Klinické údaje

Hormonální odlišnosti se nabízejí jako první vysvětlení mezipohlavních rozdílů v oblasti zneužívání návykových látek. Pohlavní hormony neslouží pouze k regulaci reprodukčních procesů, ale mají také vliv na kognitivní a afektivní funkce. Interpretaci klinických údajů nicméně komplikuje praktická obtížnost sledování jejich aktuálních hladin v průběhu menstruačního cyklu a jejich přímá korelace s adiktivním chováním, což značně limituje i počet publikovaných klinických studií.

Když se pokusíme zjednodušit průběh menstruačního cyklu podle převládajících hladin hormonů, charakterizuje folikulární fázi vysoká hladina estrogenů, zatímco v luteální fázi dominuje progesteron. Byla provedena klinická studie, ve které byly nalezeny rozdíly v subjektivních účincích amfetaminu na ženský organismus v těchto dvou fázích cyklu. Objektivní účinek drogy, hodnocený pomocí krevního tlaku a srdeční frekvence, byl v obou fázích cyklu prakticky stejný. Naproti tomu subjektivní efekt amfetaminu, hodnocený několika dotazníky (míra euforie, bažení, přátelských pocitů, energie apod.), se významně odlišoval – ve folikulární fázi byly tyto účinky signifikantně vyšší než v luteální fázi.[17] Carpenter et al. později publikovali metaanalýzu 13 studií sledujících ženy kuřačky a jejich pokusy přestat kouřit v závislosti na menstruačním cyklu. Ženy inklinovaly ke zvýšenému cravingu (dychtění po droze) a dysforiím v pozdní luteální fázi oproti folikulární fázi.[18] Jedním z vysvětlení je schopnost estradiolu potlačovat úzkostné chování, a tudíž ženy, které přestanou kouřit ve folikulární fázi, mají větší naději na úspěch. Tyto odlišnosti byly zaznamenány i pomocí měření funkčního MRI vyšetření – BOLD fMRI (*blood-oxygen-level dependent functional magnetic resonance imaging*), kde bylo prokázáno, že v průběhu folikulární fáze jsou u žen více aktivovány oblasti mozku spojené s odměnou (střední mozek, striatum, levá frontopolární prefrontální kůra) ve srovnání s luteální fází.[19]

Vliv mají i exogenně podané hormony, např. estradiol zvyšuje pozitivní subjektivní účinek amfetaminu během folikulární fáze.[20] Analogická data byla zaznamenána u kokainu, kde navíc exogenně podaný progesteron snižoval vliv drogy, a to pouze u žen, zatímco u mužů se jeho efekt po akutním podání neprojevil.[21] Progesteron byl použit již v několika klinických studiích jako podpůrná farmakoterapie při odvykání závislosti na kokainu a nikotinu u žen se slibnými výsledky.[22]

Méně pozornosti v souvislosti s drogovou závislostí má testosteron, a to u žen i u mužů. Fluktuace jeho hladin u mužů patrně souvisí především se sklonem k relapsu drogové závislosti, který může být odstartován v souvislosti se sexuální aktivitou, vzhledem k tomu, že muži často subjektivně uvádějí relaps do souvislosti s příjemnými pocity (např. výhra, obecněji jakýkoli druh odměny). To je zvlášť patrné u psychostimulancií.[23] Hladiny testosteronu u žen se mění v závislosti na menstruačním cyklu, ale také v různých sociálních situacích (sport, výhry, prohry, pláč dítěte). Zdá se, že když žena začne užívat psychostimulancia s cílem ulevit depresi či úzkosti, riziko následného rozvoje závislosti se výrazně liší podle fáze menstruačního cyklu.[24]

Muži a ženy se liší i v klinických projevech závislosti. Míra eskalace, potíže s ukončením a frekvence relapsů po abstinenci je u žen výrazně vyšší než u mužů.[25] Tyto výsledky jsou patrné u alkoholu, tabáku, marihuany, kokainu i opioidů.[16,26] Nejprozkoumanější je z tohoto pohledu fumátorství. Ženy kouří v kratších intervalech, tzn. více cigaret za jednotku času a přestat kouřit je pro ně obtížnější než pro muže.[16]

Stejně jako pohlavní hormony ovlivňují adiktivní chování a s tím spojené mezipohlavní rozdíly, tak i naopak, návykové látky mají vliv na uvolňování a metabolismus hormonů. Příkladem je alkohol, kokain a marihuana, jejichž užívání je spojováno se sníženými hladinami luteinizačního hormonu a testosteronu a zvýšenými hladinami progesteronu, adrenokortikotropního hormonu (ACTH) a kortikotropinu, což vede k ohrožením plodnosti žen[27] i mužů.[28] S těmito změnami charakteristickými pro jednotlivé návykové látky bude rovněž nutné počítat při vývoji případných hormonálních terapií drogových závislostí.

Preklinické údaje

U pokusných zvířat (nejčastěji hlodavců) se projevil vliv pohlavních hormonů ve všech fázích experimentálně

navozené drogové závislosti. V souladu s klinickými důkazy samice potkanů v době zvýšených hladin estradiolu a snížených hladin progesteronu (tj. estrus) vyhledávají drogy nejintenzivněji. Tento modulační vliv estradiolu se vysvětluje prostřednictvím zvýšeného vyplavování dopaminu v mezokortikolimbických strukturách, což je dopaminergní dráha spojená s procesy odměny. V případě podání opioidů přistupuje k vyplavení dopaminu další mechanismus, a to aktivace mí a kappa opioidních receptorů v okruhu odměny.

S hladinou estrogenu souvisí i relaps, který je obecně častější u žen, a samice potkanů vykazují v příslušných modelech rovněž vyšší vulnerabilitu. Touha po droze se v animálních modelech měří v testu autoaplikace drogy po určité době abstinence. Potkaní samice v estru vykazují trend zvýšeného bažení po droze.[29]

Analogicky jako u žen, i u zvířat potlačuje progesteron posilující vliv estrogenu. Progesteron jako potenciální léčba závislostí je již klinicky zkoumán.[22] Dalším důkazem vlivu progesteronu na adiktivní chování jsou jeho nízké hladiny (samice potkana v estru) odpovídající vyšší motivaci k autoaplikaci kokainu. U samic s přirozeným estrálním cyklem byly naměřeny nízké hladiny progesteronu ve fázi vyhledávání drogy i při relapsu.[30]

Limitujícím faktorem je v této oblasti léčba závislosti na alkoholu, u kterého se regulující vliv pohlavních hormonů nepotvrdil. Ani ovariektomie, ani exogenně podaný progesteron neovlivnil míru užívání alkoholu u zvířat. Navíc u lidí se projevují mezipohlavní rozdíly v alkoholismu obráceně než u hlodavců (muži pijí více než ženy, samci méně než samice). Alkoholovou závislost však u člověka ovlivňuje mnoho komplexních faktorů (sociální, genetické, hormonální a neurobiologické), které tento fenomén vyvolávají a které je nemožné modelovat u zvířat v jejich úplnosti.[31]

SOCIO-KULTURNÍ ROZDÍLY

Společnost od každého pohlaví očekává jiné chování, tzv. genderové stereotypy, které se projevují také u lidí se syndromem závislosti. Tato genderová specifika se promítají do prevalence, preference užívané látky, motivace k užití, rodinné anamnézy, poskytování sexuálních služeb aj. Obecně lze tvrdit, že muži užívají drogy častěji než ženy. Tento celosvětový fenomén se vysvětluje jak příležitostí a přístupem k droze, což platí zejména v asijských státech (např. v Indii nebo Íránu je ze všech drogově závislých cca 7 % žen), tak vnímáním role ženy v socio-kulturním kontextu (nejen ve východních zemích, ale i v Evropě a USA). Podstatou genderové specifičnosti je tzv. fenomén dvojí stigmatizace či dvojí deviace, kdy žena je stigmatizována primárně za samotné užívání návykové látky, a navíc za to, že zklamala ve své roli matky/pečovatelky.[24] Ženy začínají s užíváním drog v dřívějším věku než muži a také míra eskalace a kvantita je u většiny návykových látek markantnější u žen v porovnání s muži. Muži mají většinou jinou motivaci k experimentování s návykovými látkami než ženy, obecně muži sáhnou primárně po droze z důvodu jejího posilujícího účinku (pro pocit vzrušení nebo posílení jejich sociální pozice), naopak u žen je více pravděpodobné, že využijí drogy k léčbě psychického stavu (většinou úzkost, deprese, sociální izolace). Tato genderová rozdílnost v iniciativě odpovídá i tomu, že více mužů užívá ilegální drogy (cannabis, opiáty, kokain), ženy naopak ve větší míře zneužívají léky na předpis (opiáty, sedativa). Ženy mají také v anamnéze častěji než muži různé formy fyzického zneužívání, jemuž jsou v dětství a/nebo v dospělosti vystavovány, a díky tomu vstupují do procesu vzniku závislosti již s existujícím psychickým zatížením, což může být další důvod, proč se u nich rozvine závislost na látce rychleji než u mužů.[32]

Zahraniční výzkumy potvrzují, že ženy vyhledávají odbornou pomoc v souvislosti s užíváním návykových látek méně často než muži, ale pokud do léčby nastoupí, jejich výsledky a míra úspěšnosti léčby jsou podobné jako u mužů. Důvodů, proč ženy váhají při vyhledávání odborné pomoci, se naskytuje více. V první řadě je to již zmiňované společenské stigma a dále také strach z odebrání dětí, proto mají ženy větší tendenci svou závislost skrývat, jejich okolí reaguje opožděně. Rovněž si častěji obstarávají peníze na nákup drog prostřednictvím sexuálního průmyslu, a pokud zažádají o institucionální pomoc, musejí vyjít alespoň částečně z anonymity. Navíc většina zařízení je zaměřena na řešení problémů spojených s užíváním ilegálních drog, jež častěji užívají muži.[24]

ZÁVĚR

K přiblížení neurobiologických rozdílů mezi pohlavími v rozvoji drogové závislosti nám mohou posloužit následující 3 koncepty:

1) závislost na návykových látkách postihuje u žen jiné mozkové oblasti než u mužů,
2) závislost má mnoho klinických projevů, které vedou k maladaptivnímu chování a
3) závislost může předcházet přítomnost psychopatologie před začátkem užívání drogy.

Společnost do jisté míry určuje mezipohlavní rozdíly v užívání návykových látek, což se odráží na vyšší prevalenci drogově závislých mužů. Naopak u žen hrozí vyšší riziko vzniku závislosti již po akutní aplikaci drogy v souvislosti s fluktuujícími hladinami hormonů (podle fáze menstruačního cyklu), které ovlivní subjektivní náladu a účinky drogy.[24] V mozku žen se více stimuluje dopaminergní systém na počátku expozice drogy, a to ve smyslu většího uvolňování dopaminu i zvýšené inhibice zpětného vychytávání, proto u žen dochází k rychlejší eskalaci příjmu návykové látky a rozvoji závislosti.[24] Chronické užívání drogy vede k adaptačnímu hypodopaminergnímu stavu ve striatu u obou pohlaví, avšak u žen je tento efekt díky vyšší reaktivitě dopaminergního systému výraznější. Snížené hladiny dopaminu v periodách abstinence se projevují silnou dysforií a anhedonií, ke zvýšení hladin dopaminu již nestačí přirozené druhy odměny (jídlo apod.) a dochází k většímu sklonu k relapsu.[5] Droga navíc může být formou samoléčby depresivních stavů charakterizovaných sníženými hladinami monoaminů,[33] jak ukazují i preklinické studie s návykovými látkami z různých skupin.[34,35] Chronické užívání téměř všech návykových látek je spojeno také se zvýšeným vyplavováním noradrenalinu,

který přispívá k negativnímu afektivnímu stavu: úzkost, podrážděnost. U žen je stav horší díky zvýšené noradrenergní a kortikotropní aktivitě.[24]

Léčba závislostí zatím nemá jasný koncept a existuje jen málo účinných postupů. Vzhledem k tomu, že existují mezipohlavní rozdíly ve vzniku, průběhu i následcích závislosti na drogách, měla by se i léčba závislostí zaměřit jednotlivě na obě pohlaví, aby byla co nejúčinnější. Např. naltrexon a disulfiram snižují užívání kokainu u mužů, avšak ne u žen.[36,37] Léčba je často zacílená na zmírnění negativních abstinenčních příznaků, jako dysforie, úzkost, podrážděnost, čímž se sníží frekvence užívání drog a recidivy. K potlačení subjektivních negativních pocitů se osvědčily inhibitory acetylcholinesterázy, avšak účastníci studií byli pouze muži.[24]

Animální experimenty zásadním způsobem obohacují dostupné údaje o neurobiologických podkladech mezipohlavních rozdílů ve všech fázích látkových závislostí. Při interpretaci dat získaných z experimentů na zvířatech je třeba počítat s jejich limity při predikci reaktivity lidského organismu, která se může zásadně lišit v mnoha ohledech. Lidský organismus může odbourávat dané léčivo či návykovou látku odlišně (farmakokinetické rozdíly), může disponovat jinou receptorovou a enzymatickou výbavou a dalšími zvláštnostmi. Přes svá omezení však mohou animální behaviorální modely na tomto poli, obdobně jak je jednoznačně prokazováno i v jiných oblastech medicíny, přinášet validní informace o reaktivitě celého organismu, možných interakcích jednotlivých látek a především o slibných možnostech ovlivnění drogové závislosti.

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ALKOHOLIZMUS A DROGOVÉ ZÁVISLOSTI
(PROTIALKOHOLICKÝ OBZOR)

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PREHĽADOVÉ PRÁCE

SPOLOČNÉ NEUROBIOLOGICKÉ MECHANIZMY DEPRESIE A METAMFETAMÍNOVEJ ZÁVISLOSTI

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S ú h r n

V klinickej praxi je častou komorbiditou drogovej závislosti depresia, hlavne v prípade sekundárnej drogovej závislosti u depresívnych pacientov. Vysoká úroveň komorbidity v populácii (30 – 50 %) sa dá vysvetliť zdieľanými neurobiologickými abnormalitami a aberantnou neuroadaptáciou na akútny efekt drogy vedúcej k neurochemickým zmenám, ktoré majú spoločné prvky s abnormalitami vyskytujúcimi sa pri depresii. Článok poskytuje prehľad neurobiologického podkladu drogovej závislosti a depresie so zameraním na amfetamínové psychostimulanciá.

K ľ ú č o v é s l o v á : závislosť – depresia – psychostimulanciá – metamfetamín

Z. Babinská, J. Kučerová: COMMON NEUROBIOLOGICAL MECHANISMS OF DEPRESSION AND METHAMPHETAMINE ADDICTION

S u m m a r y

Drug addiction and depression is the most common comorbidity, especially in case of secondary drug addiction of patients with depression. High comorbidity in population (30 – 50 %) may reflect shared neurobiogical abnormalities, aberrant neuroadaptation to acute drug effect leading to neurochemical changes, which have common elements with abnormalities connected to depression. Article presents review of neurobiological background of drug addiction and depression focused on amphetamine-like psychostimulants.

K e y w o r d s : addiction – depression – psychostimulants – methamphetamine

Úvod

V západnej Európe sa na psychiatrickú starostlivosť vydáva 6 – 10 % rozpočtu zdravotníctva, zatiaľ čo v Českej a Slovenskej republike tieto náklady tvoria len 3,5 % rozpočtu. Lieky a zdravotnícka starostlivosť predstavujú len malú časť nákladov, neporovnateľne väčšiu časť tvoria nepriame ekonomické straty v dôsledku pracovných absencií a zníženia produktivity. Počas života depresiu zažije 1 zo 7 ľudí, čo poukazuje na vysokú prevalenciu tejto poruchy v populácii. Z tohto hľadiska je skorá psychiatrická intervencia, prevencia vzniku porúch a závislostí komorbídnych s depresiou vysoko aktuálna a žiaduca (EMCDDA, 2013).

V klinickej praxi je častou komorbiditou depresie drogová závislosť (Daniulaityte a kol., 2010), hlavne v prípade sekundárnej drogovej závislosti u pacientov s anamnézou depresie, ale i ďalších psychiatrických porúch (Langas a kol., 2010), kedy sa pacienti snažia uľaviť príznakom svojej poruchy užívaním návykovej látky. Epidemiologické dáta naznačujú, že chronickí užívatelia návykových látok sú až v 30 až 50 % prípadov postihnutí depresiou (Cottencin, 2009; Davis a kol., 2008). Klinická perspektíva svedčí o tom, že liečba antidepresívami znižuje príjem drogy u drogovo závislých pacientov s depresiou v porovnaní s jedincami bez depresie (Carroll a kol., 1995). Všeobecne vysoká úroveň komorbidity v populácii sa dá vysvetliť zdieľanými neurobiologickými abnormalitami a symptomatickými prejavmi.

Ďalším vysvetlením by mohla byť aberantná neuroadaptácia na akútny efekt drogy vedúca k neurochemickým zmenám, ktoré majú spoločné prvky s abnormalitami vyskytujúcimi sa pri depresii.

Cieľom tohto prehľadového článku je zmapovať spoločné neurobiologické podklady drogovej závislosti a depresívnej poruchy, so zameraním hlavne na psychostimulanciá amfetamínového typu.

Závislosť od amfetamínových psychostimulancií a následné neurochemické zmeny

Metamfetamín a jeho farmakodynamika

V rámci Európy je užívanie metamfetamínu najbežnejšie v ČR a v menšej miere na Slovensku a v Poľsku. V Českej republike je aktuálne približne 31 000 problémových užívateľov metamfetamínu (EMCDDA, 2013). V súčasnosti je spájaný so závažnými sociálnymi, zdravotnými a bezpečnostnými problémami aj v USA a Ázii. Od svojej prvotnej syntézy v roku 1919 prekonalo užívanie metamfetamínu veľa zmien. Z pôvodne legálnej pokusnej látky, ktorá sa používala ako liek na depresiu, poruchy pozornosti, alkoholizmus, obezitu či anorexiu sa stalo stimulans zvyšujúce výkon vojakov v druhej svetovej vojne. Od počiatku sedemdesiatych rokov sa nelegálne vyrába v ČR. Surovinou na výrobu bol spočiatku efedrín, v súčasnosti sa metamfetamín nelegálne vyrába z pseudoefedrínu obsiahnutého v liečivých prípravkoch voľne predajných s obmedzením.

Metamfetamín pôsobí ako stimulans centrálneho nervového systému – nepriame sympatomimetikum, zvyšuje činnosť noradrenergických a dopamínergických neurotransmiterových systémov. Podobne ako amfetamín je plným agonistom receptorov asociovaných so stopovými amínmi (TAAR1) a receptorom spriahnutým s G-proteínom, ktorý reguluje katecholamínové systémy v mozgu. TAAR1 receptory sú relatívne nedávno objavené receptory lokalizované na presynaptickej membráne, kde majú úlohu hlavne v regulácii monoamínov. Aktivácia TAAR1 prostredníctvom adenylátcyklázy zvyšuje produkciu cyklického adenozínmonofosfátu.

Metamfetamín po naviazaní na TAAR1 spúšťa fosforyláciu transportéru pomocou proteínkinázy A a proteínkinázy C, čo vyústi do internalizácie alebo spätnej funkcie monoamínových transportérov. Je známe, že metamfetamín inhibuje takisto vezikulárny monoamínový transportér 1 a 2 (VMAT1, VMAT2), rovnako ako SLC22A3 a SLC22A5. SLC22A3 je extraneuronálny monoamínový transportér prítomný v astrocytoch a SLC22A5 je transportér s vysokou afinitou pre karnitín. Interakcia metamfetamínu s VMAT2 spôsobí uvoľnenie monoamínov zo synaptických vezikúl do cytozolu presynaptického neurónu (Xie a Miller, 2009). Metamfetamín je tiež agonista α-2

adrenergných receptorov a monoaminooxidázy B a A. Jeho podanie vedie k eufórii, znižuje únavu, vyvoláva nechutenstvo a stereotypné pohyby. Silne zvyšuje tep, dych a krvný tlak. Po odznení účinku nastáva depresia, celkové psychické a fyzické vyčerpanie. Pri akútnej intoxikácii dráždi sympatikus, môže dôjsť až k delíriu, fyzickému vyčerpaniu a smrti. Chronické užívanie je charakteristické psychózami, podvýživou, trasom, depresiami, halucináciami a samovražednými tendenciami (Nordahl a kol., 2003). Hlavne vďaka prispeniu vedľajších produktov amatérskej syntézy spôsobuje poškodenie ciev v mozgu, kožné abscesy a zápal žíl v oblasti v injekčnej aplikácie. Rizikom je tiež otrava látkami, ktoré sa používajú pri nelegálnej výrobe metamfetamínu, napríklad olovom, ktoré je obsiahnuté v používanom reagente – octane olovnatom. Fetálna expozícia má za následok predčasný pôrod, abnormálne reflexy a extrémnu iritabilitu novorodenca (Scott a kol., 2007).

Za účinkami všetkých psychostimulancií amfetamínového typu stojí na molekulárnej úrovni predovšetkým ovplyvnenie hladín monoamínov. Dopamínergický systém hrá úlohu v posilňovacom a behaviorálne-stimulačnom účinku týchto látok u ľudí aj zvierat. Rovnako aj sérotonínový a noradrenalínový systém modulujú neurochemickú a behaviorálnu odpoveď na psychostimulanciá. Uplatňujú sa tu prídavné neurotransmiterové systémy, napríklad kortikálne glutamátergné systémy zabezpečujú reguláciu funkcie dopamínu a inhibičné systémy GABA modulujú bazálne uvoľnenie dopamínu a glutamátu. Opakované vystavenie účinku psychostimulancií vedie k silným a dlhotrvajúcim zmenám v neurobiológii monoamínov a k citlivosti na efekt drogy, ktorý ovplyvňuje neurochemické parametre a správanie.

V následujúcej časti bude popísaný vplyv podania amfetamínových stimulancií na jednotlivé neurotransmiterové systémy.

Vplyv amfetamínových stimulancií na monoamíny

Dopamínergický systém

Podávanie zneužívaných látok, gambling, sexuálne správanie a konzumácia sladkostí sú spojené so zvýšenými intrasynaptickými hladinami dopamínu v mezokortikolimbickom okruhu odmeny (Hajnal a Norgren, 2001; Cheer a kol., 2004). Dopamín má taktiež úlohu v očakávaní odmeny a v uľahčení konsolidácie spomienok na výnimočné udalosti (Saunders a Robinson, 2012). Existuje 5 dopamínergických receptorov, ktoré sa dajú rozdeliť do dvoch skupín. D_1 a D_5 receptory stimulujú adenylátcyklázu k produkcii cyklického adenozínmonofosfátu (AMP). D_2, D_3, D_4 receptory inhibujú produkciu cyklického AMP. Lokalizácia väčšiny D_1 a D_5 receptorov je postsynaptická, D_2, D_3, D_4 presynaptická (Centonze a kol., 2003). Funkcie D_1, D_2,

D_3 sa týkajú okrem iného odmeny a motivácie, D_4 a D_5 vplýva skôr na behaviorálnu inhibíciu (Avale a kol., 2004; Kramer a kol., 2007).

Aktivácia D_1 receptorov je spájaná s odmenou po požití psychostimulancií, etanolu a jedla (Cooper a Al-Naser, 2006; D 'Souza a kol., 2003). Podávanie D_1 antagonistu vyústilo do nadmernej senzitivity D_1 receptoru, čo zvýšilo posiľňujúce a subjektívne vlastnosti kokaínu. Vplyv D_1 receptoru na nadmernú senzitivitu ako súčasť pocitu odmeny z kokaínu je sprostredkovaný zvýšením intrasynaptického dopamínu po podaní kokaínu. Preto sa predpokladá, že nadmerná senzitivita tohto receptoru bude mať rovnaký vplyv na akúkoľvek látku zvyšujúcu intrasynaptickú dostupnosť dopamínu a prispieva k rozvoju závislosti (Barone a kol., 1988; Parkitna a kol., 2013).

Štúdie pozitrónovej emisnej tomografie (PET) a tomografickej scintigrafie (SPECT) na užívateľoch kokaínu, etanolu, metamfetamínu a heroínu ukázali redukciu denzity D_2 receptorov vo ventrálnom striate, ktorá pretrvávala ešte dlho po detoxikácii (Volkow a kol., 2007). Nízke hladiny D_2 receptorov predisponujú pacienta k vyhľadávaniu psychoaktívnych substancií a kompenzácii zníženej aktivácie dráhy odmeny (Volkow a kol., 1999). Polymorfizmus génu Taq1A D_2 receptoru je taktiež prepojený s užívaním psychostimulancií, alkoholu, fajčením, patologickým gamblerstvom a prehnaným pôžitkom z jedla (Huang a kol., 2007; Noble, 2003).

D_3 receptory sú lokalizované hlavne v limbických oblastiach, preto sa tiež predpokladá ich vplyv na závislosť. Selektívny antagonista D_3 SB-277011-A blokuje opätovné vyhľadávanie kokaínu po jeho vysadení (reinstatement) vyvolané kokaínovým primingom. Priming popisuje mechanizmus, pri ktorom má spracovanie určitej informácie (vnemu, napríklad po podaní drogy u zvieraťa) vplyv na nasledujúcu činnosť. SB-277011-A redukuje orálnu auto-aplikáciu (self-administration) etanolu a nikotínu u potkanov (Gilbert a kol., 2005; Le Foll a kol., 2003; Vengeliene a kol., 2006). SB-277011-A tiež potenciuje farmakologickú odpoveď pacienta na amfetamín na MRI (Schwarz a kol., 2004). Nedávna štúdia ukázala, že depresívni pacienti vykazovali signifikantne vyššie odpovede na odmeňujúci efekt psychostimulancia a pozmenenú aktivitu orbitofrontálneho kortexu a putamenu (Tremblay a kol., 2005). Ďalšie výskumy potvrdili, že zvýšené hladiny glukokortikoidov uľahčujú dopamínergickú transmisiu v *nucleus accumbens* a že zanedbávanie materskej opatery v raných fázach života spojené so psychosociálnym stresom zvyšuje ventrostriatálnu koncentráciu dopamínu. Takáto senzitizácia mezolimbického dopamínergického systému v detskom veku opäť predisponuje pacienta k závislosti v dospelosti (Pruessner a kol., 2004).

Sérotonínergický systém

Aktivita sérotonínu (5-hydroxytryptamín, 5-HT) je spojená s emocionálnou stabilizáciou, moduláciou apetítu, behaviorálnou inhibíciou, zmyslovou reaktivitou, citlivosťou na bolesť, kognitívnymi funkciami, spánkom a sexuálnym správaním (Ressler a Nemeroff, 2000; Stein a kol., 1993). 5-HT_1 receptorová skupina je pre- aj post-synapticky inhibičná a znižuje aktivitu adenylátcyklázy skrz aktiváciu G_i. Excitačná skupina 5-HT_2 je predovšetkým post-synaptická a aktivuje fosfolipázu C prostredníctvom G_0. 5-HT_3 receptor využíva svoju excitačnú aktivitu a uplatňuje sa ako iónový kanál. 5-HT_4,5-HT_5, 5-HT_6 aktivujú adenylátcyklázu skrz G_s (Muller a Huston, 2006).

In vitro aplikácia sérotonínu na dopamínergické neuróny z ventrálnej tegmentálnej oblasti (VTA) zvýšila intenzitu pálenia (firing), ktorá bola prisúdená efektu sérotonínu na 5-HT_2 receptory (Pessia a kol., 1994). Uvažuje sa, že zvýšená citlivosť k sérotonínergickej stimulácii zdieľaná aj ostatnými závislosťami by mohla byť kľúčovým faktorom pri náchylnosti k týmto poruchám. Najdôležitejšia súčasť sérotonínergického systému, ktorá ovplyvňuje odmenu a motiváciu je subtyp 5-HT_1B. Tento receptor spojený s G_i je lokalizovaný na termináloch axónov mnohých typov neurónov. Terminály axónov GABA neurónov, prebiehajúcich od *nucleus accumbens* shell do ventrálnej tegmentálnej oblasti, obsahujú 5-HT_{1B} receptory, ktoré po aktivácii znižujú uvoľňovanie GABA. Toto zníženie uvoľňovania GABA dezinhibuje mezolimbické dopamínergické neuróny a potencuje „odmeňujúce" správanie (Yan a kol., 2004). Up-regulácia 5-HT_{1B} receptorov na axónových termináloch GABAergických neurónov v *nucleus accumbens* shell v oblasti dráhy odmeny môže prispievať k náchylnosti jedinca k závislosti (Miszkiel a Przegalinski, 2013).

Noradrenergický systém

Noradrenalín (NE) je neurotransmiter produkovaný locus coeruleus mozgového kmeňa a zároveň je hormónom drene nadobličiek. Jeho syntéza prebieha z dopamínu za pomoci enzýmu dopamín beta-hydroxylázy (DBH). Noradrenergný systém mozgu pozostáva z dvoch častí: dorzálny noradrenergický zväzok, ktorý začína v *locus coeruleus* a premieta sa do hipokampu, mozočku a predného mozgu a ventrálny noradrenergický zväzok, ktorý vzniká v jadrách mostu a premieta sa do hypotalamu, amygdaly a stredného mozgu (Leonard, 2001). Chronicky zvýšené hladiny NE môžu dysregulovať dopamínergický systém odmeny, pravdepodobne prostredníctvom down-regulácie presynaptickej dopamínergickej transmisie alebo prostred-

níctvom nadmernej senzitivity postsynaptických D2/D3 receptorov (Ferrucci a kol., 2013).

Vplyv amfetamínových stimulancií na glutamátergný systém

Štúdia z roku 1989 ako prvá poukázala na skutočnosť, že glutamát má podiel na psychomotorických stimulačných vlastnostiach amfetamínov (Karler a kol., 1989). Pomocou in vitro release a in vivo mikrodialýzy sa dokázalo, že metamfetamín zvyšuje extracelulárne hladiny glutamátu v dorzálnom striate, kortexe, hipokampe a VTA (Bustamante a kol., 2002; Shoblock a kol., 2003). Akútne podanie metamfetamínu spôsobuje zníženie fosforylácie podjednotky GluR1 na AMPA (kyselina α-amino-3-hydroxy-5-me-tyl-4-izoxazol-propionová) receptore v striate, čo mení vodivosť receptorového kanálu AMPA. Metamfetamín znižuje rýchlosť vedenia vzruchov na dopamínergických neurónoch vo VTA a indukuje somatodendritické uvoľnenie dopamínu.

V porovnaní s akútnym podaním opakované podanie amfetamínov značne mení glutamátom sprostredkovanú neuronálnu aktivitu a vyúsťuje v zvýšenú neurálnu odpoveď na lokálne aplikovaný glutamát vo VTA a frontálnom kortexe (White a kol., 1995). V štúdii z roku 2005, kde bol skupine depresívnych pacientov podaný d-amfetamín autori poukázali na zvýšené odmeňujúce účinky v porovnaní s kontrolnou skupinou. Stupeň zvýšenia koreloval s hĺbkou depresie a závažnosťou anhedonických symptómov u pacientov. Na základe tohto poznatku sa dá vyvodiť spojitosť medzi stupňom závažnosti depresívnej poruchy a rizikom rozvoja závislosti (Tremblay a kol., 2005).

Vplyv amfetamínových stimulancií na GABA-ergný systém

GABA (γ-aminomaslová kyselina) je hlavným inhibičným neurotransmiterom v centrálnom nervovom systéme, ktorý moduluje bazálne uvoľnenie dopamínu a glutamátu. Poznáme tri podskupiny GABA receptorov: $GABA_A$, $GABA_B$, $GABA_C$. $GABA_A$ a $GABA_C$ receptory radíme k ionotropnému typu a $GABA_B$ k metabotropnému typu. Antagonisty $GABA_A$ receptorov ako sú napríklad pikrotoxín alebo bikukulín redukujú operantnú auto-aplikáciu etanolu a kokaínu u zvierat. Rovnako bolo popísané podanie pikrotoxínu do VTA, ktoré následne znížilo množstvo požitého etanolu. Táto neurochemická zvláštnosť môže odrážať úlohu VTA v dopamínergickom systéme odmeny (Czlonkowska a kol., 2000; Michaeli a kol., 2012). Farmakologická in-

hibícia GABA-transaminázy vedie k rýchlemu zvýšeniu extracelulárnej GABA a v rámci správania zvierat sa prejaví útlm operantnej auto-aplikácie kokaínu. Existujú hypotézy o možnej súvislosti medzi hyposenzitivitou $GABA_A$ receptorov vo VTA, prípadne nadmernou senzitivitou $GABA_A$ receptorov v *nucleus raphe dorsalis* a vznikom závislosti. Rovnako sa polemizuje o účasti $GABA_B$ receptorov, ktoré za predpokladu abnormálnej funkčnosti dezinhibujú dopaminergické neuróny, čo spôsobí ich nadmernú odpoveď a intenzifikuje posiľňujúci efekt látok so závislostným potenciálom (Li a kol., 2013).

Vplyv amfetamínových stimulancií na endokanabinoidný systém (ECS)

V rámci kanabinoidného systému poznáme dva základné receptorové podtypy: CB_1, ktoré sa nachádzajú hlavne v centrálnom nervovom systéme a CB_2, ktoré nachádzame v enterálnom nervovom tkanive a v gliových bunkách CNS. Endogénne kanabinoidy fungujú ako retrográdne neurotransmitery. Po uvoľnení z postsynaptických neurónov prostredníctvom depolarizácie membrány migrujú späť do presynaptickej membrány a aktivujú presynaptické CB_1 receptory, čo následne inhibuje uvoľnenie neurotransmiteru. Inaktivácia alebo eliminácia CB_1 receptorov zoslabuje odpoveď dopamínergického systému na podávanie psychoaktívnych látok a na správanie spojené s uvoľnením dopamínu v *nucleus accumbens*. Určité genetické varianty CB_1 receptorov boli identifikované ako faktor zvyšujúci pravdepodobnosť rozvoja závislosti (Alvaro-Bartolome a Garcia-Sevilla, 2013). Rovnako sa predpokladá účasť endokanabinoidného systému na posiľovaní motivácie a odmeňovania v mezolimbickom dopamínergickom systéme a regulácii konzumačného správania. Rovnako sa javí aj zapojenie ECS do modulácie depresie, stresu a anxiety. Pôsobenie chronického stresu down-reguluje expresiu CB_1 receptoru a signifikantne redukuje množstvo 2-arachidonylglycerolu v hipokampe (Campos a kol., 2013), pričom 2-arachidonylglycerol je najrozšírenejším endokanabinoidom v CNS. ECS sa aktivuje ako odpoveď na úzkostné situácie a môže regulovať emocionálne stavy modulovaním výstupov z amygdaly, čo je súčasťou systému negatívnej spätnej väzby, ktorá limituje úzkosť. Primárnou funkciou ECS sa však javí byť regulácia a kontrola chronického stresu. Narušenie ECS pravdepodobne množstvo chronického stresu zvyšuje, čo následne zvyšuje pravdepodobnosť rozvoja závislosti (Somaini a kol., 2012).

Neurobiologický podklad depresívnej poruchy

Presné neurobiologické mechanizmy vyvolávajúce depresiu nie sú doteraz úplne objasnené. Pozornosť sa venuje predovšetkým neuroplasticite a neurotransmiterovým zmenám. Poruchy kognitívnych funkcií vznikajú na podklade narušenej neuroplasticity v mozgovej kôre, hipokampe a amygdale. Toto narušenie neuroplasticity je spôsobené pravdepodobne zmenami neurotransmiterov a hormónov, čo predurčuje ďalší smer výskumu v tejto oblasti. Nasledujúci text sumarizuje hlavné neuropatofyziologické podklady depresie.

Monoamínergická hypotéza

Samotný neurobiologický podklad depresie vysvetľuje viacero teórií. Najstaršou z nich je monoamínergická teória, ktorá je základom hypotéz o vzniku depresie ostatných 60 rokov. Bola postulovaná po pozorovaní hypertenzných pacientov liečených rezerpínom, u ktorých sa na základe tejto liečby vyvinula depresia. Rezerpín je alkaloid rastlinného pôvodu pôsobiaci depresogénne prostredníctvom deplécie monoamínov. Podľa monoamínergickej teórie je depresia následok deficitu sérotonínergickej a noradrenergickej synaptickej neurotransmisie v mozgu (Goldberg, 2006; Millan, 2004). Pôsobením antidepresív sa zvyšuje množstvo monoamínov na synapse, čo je počiatkom celej kaskády procesov prejavujúcich sa antidepresívnym účinkom. Zvýšenie množstva monoamínov na synapse indukuje desenzitizáciu inhibičných auto a heteroreceptorov. Táto desenzitizácia spôsobí vyššiu monoamínergickú aktivitu, ktorá spôsobí terapeutickú odpoveď. Tieto adaptívne zmeny zodpovedné za terapeutický efekt závisia na dostupnosti špecifického monoamínu na synapse a deplécia tohto monoamínu zvráti antidepresívny účinok alebo spôsobí relaps u aktuálne neliečeného depresívneho pacienta. Blokáda somatodendritických a terminálových autoreceptorov zvyšuje odpoveď na liečbu u veľkej a rezistentnej depresie dokazujúc, že antidepresívny účinok vychádza z dlhodobých adaptívnych zmien v monoamínergických auto a heteroregulačných receptoroch. Samotná aktivácia postsynaptických receptorov iniciuje kaskádu biochemických efektov sprostredkovávajúcich transdukciu signálu a zahŕňa stimuláciu cAMP (cyklický adenozín monofosfát) alebo Ca^{2+} kaskády G-proteínom (Kato, 2007; Levinson, 2006). Následná aktivácia CREB (cAMP response element-binding protein) má za následok zvýšenú expresiu BDNF (brain-derived neurotrophic factor – mozgový neurotrofný faktor), ktorý napomáha neurogenéze a čiastočne objasňuje terapeutický účinok antidepresív. V súvislosti s BDNF bolo zistené, že štrukturálne a funkčné abnormality v mozgoch osôb trpiacich de-

presívnou poruchou môžu byť spojené s jeho nízkymi koncentráciami (Munno a kol., 2013), zvýšenou aktivitou hypotalamo-hypofýzo-nadobličkovej osi (HPA) (Schatzberg a kol., 2014) a glutamátergickou toxicitou (Zhou a kol., 2013). Narušenej monoamínergickej neurotransmisii a abnormalitám v chronobiológii sa pripisuje taktiež insomnia, zistená u 60 % prípadov pacientov s depresiou (Ohayon a Roth, 2003). Chronobiologická porucha počas depresie má signifikantný vplyv na architektúru spánkového cyklu, pamäte, učenia a vznik stresu. Narušenie spánku a perzistentná insomnia zvyšuje u liečených pacientov riziko relapsu do ďalšej epizódy depresie (Armitage, 2007; Pigeon a kol., 2008). Meranie melatonínu v slinách a plazme alebo jeho metabolitov v moči ukázalo významné zmeny v koncentrácii melatonínu a jeho cirkadiánnych zmenách u osôb v depresívnej fáze, predovšetkým pri sezónnych afektívnych poruchách (Eyre a Baune, 2012). Vďaka týmto poznatkom sa do terapie depresie zaradili aj liečivá s iným ako monoamínergickým účinkom. Príkladom je agomelatín, ktorý je agonistom melatonergických receptorov a zároveň 5-HT_{2C} antagonistom.

Úloha glutamátergického systému v patofyziológii depresie

Takmer po polstoročí od prvotnej formulácie monoamínergickej hypotézy depresie vieme, že dlhodobé zmeny v mozgových oblastiach a dráhach mediujú komplexný systém kognitívno-emocionálneho správania, ktorý reprezentuje neurobiologický podklad porúch nálady. Kvantum klinických štúdií upriamuje pozornosť na patofyziológiu spojenú s dysfunkciou glutamátergického systému, malfunkciu mechanizmov regulujúcich clearance a metabolizmus glutamátu a morfologické maladaptívne zmeny v množstve mozgových oblastí týkajúcich sa regulácie správania a nálady (Sanacora a kol., 2012). Samotný glutamát je excitačný neurotransmiter, ktorého fyziologická funkcia je sprostredkovaná ionotropnými (iGluR) a metabotropnými receptormi (mGluR). Aktivácia mGlu receptorov vedie k rôznym bunkovým odpovediam vrátane presynaptickej modulácie synaptického prenosu a postsynaptickej interakcie s ionotropnými glutamátergickými receptormi. Nadmerná stimulácia glutamátergických receptorov, konkrétne ionotropného receptoru NMDA (N-metyl-D-aspartát) vedie k zvýšenej intracelulárnej hladine kalcia a následnej bunkovej smrti. Štúdie preukázali, že pri depresívnych poruchách býva narušená homeostáza a neurotransmisia glutamátu (Hashimoto, 2009; Zhou a kol., 2013). So zmenenými hladinami glutamátu boli spojené aj zmeny na astrocytoch, ktoré majú dôležitú úlohu pri modulácii glutamátergického systému a udržujú energetický metabolizmus mozgu. Zvýšené hladiny glutamátu boli nájdené v hipokampe a amygdale

v preklinických animálnych modeloch stresu a rovnako aj *post mortem* vo frontálnom kortexe ľudských pacientov s depresiou (de Diego-Adelino a kol., 2013). Tieto zistenia potvrdili aj MRI štúdie, v ktorých boli nájdené zvýšené hladiny glutamátu a jeho metabolitov v okcipitálnom kortexe pacientov s depresívnou poruchou (Hasler a kol., 2007). Napriek veľkému počtu liečiv účinkujúcich na monoamínovom mechanizme ostáva pomerne vysoké percento pacientov, ktorí napriek liečbe nedosiahnu fázu remisie depresívnych symptómov. Preto existuje priestor pre vývoj nových liečiv s mechanizmom účinku zameraným na relevantné terapeutické ciele v rámci glutamátergickej transmisie.

Úloha GABAergického systému v patofyziológii depresie

Možnosť účasti GABA na vzniku depresie bola prvýkrát zaznamenaná Hinderkom Emrichom v roku 1980 a následne boli formulované ďalšie teórie objasňujúce jej úlohu v rámci patofyziológie depresie. Obecne je známe, že depresia je spojená s GABAergickým deficitom (Rajkowska a kol., 2007) a bolo preukázané, že niektoré liečivá zo skupiny benzodiazepínov majú u pacientov antidepresívny efekt (Petty a kol., 1995). Vystavenie chronickému stresu aktivuje GABAergické oblasti predného mozgu vrátane dorzomediálneho hypotalamu a hipokampu, ktoré sú súčasťou dráh zapojených do depresie (Herman a kol., 2003). Zmeny morfológie mozgu počas depresie boli pozorované v ďalších dvoch štruktúrach bohatých na GABAergické neuróny – v amygdale a *tectum mesencephali* (Brandao a kol., 2003; Guilloux a kol., 2012). Vyšetrenie magnetickou rezonanciou ukázalo signifikantne nižšie koncentrácie GABA v okcipitálnom kortexe u depresívnych pacientov v porovnaní so zdravými kontrolami (Sanacora a kol., 2004). Ďalšie *post-mortem* a zobrazovacie štúdie preukázali zníženú hustotu GABAergických neurónov v prefrontálnom a okcipitálnom kortexe u depresívnych pacientov (Maciag a kol., 2010). Celkovo získané neuroanatomické dáta naznačujú existenciu prelínajúcich sa GABAergických nervových dráh, ktoré by sa dali považovať za jeden integrálny okruh, pozostávajúci z troch domén: úzkosť, pamäť a depresia.

Úloha endokanabinoidného systému v patofyziológii depresie

Výskum biologických mechanizmov zapájajúcich sa do pôsobenia marihuany na náladu viedol k objavu endokanabinoidného systému u ľudí a zvierat. Sú známe dva hlavné endokanabinoidy: anandamid

a 2-arachidonylglycerol, ktoré súvisia okrem iného aj s hlavným psychoaktívnym komponentom marihuany Δ-9-tetrahydrokanabinolom. Endokanabinoidy účinkujú prostredníctvom rozličných receptorov: CB_1 a CB_2, čiastočne TRPV1 (vaniloidný receptor) a niektorých receptorov spražených s G-proteínmi. Endokanabinoidný systém sa podieľa na neuromodulácii nálady prostredníctvom pôsobenia na emocionálnu odmenu a kognitívne procesy, je spájaný aj s odpoveďou na stres (Micale a kol., 2013).

Typicky sa depresia prejavuje patofyziologickými zmenami v signalizačných dráhach spojených práve s emocionálnou reguláciou. CB_1 receptory sa vyskytujú v mozgových oblastiach zahrnutých do spracovania stresových podnetov a depresie, ktoré sa nachádzajú hlavne na GABAergických a glutamátergických synapsiách. Konkrétne boli popísané na glutamátergických neurónoch v paraventrikulárnom jadre hypotalamu, ktoré je súčasťou modulácie stresovej odpovede prostredníctvom HPA osi (Hill a Tasker, 2012). CB_2 receptory boli detekované v mozgu a periférnom imunitnom systéme, ktorý je pri depresii rovnako ako HPA os dysregulovaný (Steiner a Wotjak, 2008). Narušenie tejto homeostázy vedie k zmenám v nálade a správaní (Micale a kol., 2013). Farmakologická blokáda CB_1 receptoru u ľudí sa spája s prejavmi depresie. Toto zistenie vyplynulo z humánnych štúdií CB_1 receptorového inverzného agonistu rimonabantu, ktorý bol pôvodne vyvinutý na terapiu obezity a následne stiahnutý z trhu pre signifikantne zvýšenú prevalenciu samovrážd a exacerbáciu depresie (Van Gaal a kol., 2008). Zmeny v hladinách endokanabinoidov, v denzite a párovacej schopnosti CB_1 receptorov boli popísané v prefrontálnom kortexe pacientov s depresiou a alkoholikov so suicidálnymi tendenciami (Hungund a Basavarajappa, 2004; Kirilly a kol., 2013) V rámci preklinických štúdií vysoko účinný CB_1 agonista HU 210 a WIN 55212,2 už v nízkych dávkach obmedzovali depresívny fenotyp hodnotený ako zkrátenie času imobility potkanov počas testu núteného plávania (Hill a Gorzalka, 2005). Tak isto v tomto teste vykazovali účinnosť aj nepriami agonisti CB_1 AM 404 a inhibítor FAAH (hydroláza amidu mastných kyselín) URB 597 (Gobbi a kol., 2005; Hill a Gorzalka, 2005). Tieto zistenia podporujú fakt, že sa exogénna aktivácia CB_1 receptoru podieľa na behaviorálnom depresívnom fenotype v testoch imobility v preklinických animálnych štúdiách.

Napriek rôznym dôkazom rekreačných užívateľov marihuany o jej „antidepresívnom a anxiolytickom“ efekte, vedecké dôkazy týchto účinkov sú rozporuplné. Klinické štúdie ukázali, že užitie dronabinolu (syntetický Δ-9-tetrahydrokanabinol) urýchlilo vymiznutie pocitu strachu (Rabinak a kol., 2013). Na druhej strane akútne užitie Δ-9-tetrahydrokanabinolu alebo chronické užívanie THC u ľudí zvyšuje riziko psychóz, panických atakov a bipolárnej poruchy (Piomelli, 2003). Výskum v tejto oblasti sa preto sú-

streďuje hlavne na úlohu endokanabinoidného systému v patogenéze a liečbe porúch vyvolaných stresom, keďže endokanabinoidný systém je spojený s reguláciou hypotalamo-hypofýzo-nadobličkovej osi, čo zahŕňa účinok na bežné podmienky, rovnako ako na situácie akútnej a opakovanej odpovede na stres (Crowe a kol., 2014).

Úloha HPA osi a imunitného systému v patofyziológii depresie

HPA os reguluje stresovú odpoveď kontrolou sekrécie kortikotropín uvoľňujúceho hormónu (CRH), adrenokortikotropného hormónu (ACTH) a glukokortikoidov. Táto os je kontrolovaná mineralokortikoidným a glukokortikoidným receptorom. V prípade depresie sa glukokortikoidy nenaviažu na receptory, čo vedie k hyperaktivite HPA osi a zvýšenej hladine cirkulujúcich glukokortikoidov (Zhu a kol., 2014). U potkanov viedla opakovaná expozícia stresu k down-regulácii CB_1 receptorov v glutamátergických synapsiách (Wamsteeker a kol., 2010). U ľudí sú biologické dôsledky stresu spostredkované sekréciou uvoľňovacieho faktoru pre kortikotropín (CRF) v cerebrospinálnom likvore a vedú ku zvýšenej sekrécii adrenokortikotropného hormónu a uvoľneniu glukokortikoidov (Kendler a kol., 2005). Chronický stres má za následok hypersenzitivitu HPA osi a následná depresia býva spojená so zvýšenými koncentráciami CRF v cerebrospinálnom likvore, zvýšenou imunoreaktivitou, génovou expresiou CRF v *nucleus paraventricularis* hypotalamu a down-reguláciou CRF-R1 receptorov vo frontálnom kortexe. Protrahovaná sekrécia glukokortikoidov má neurotoxické účinky hlavne na neurogenézu v hipokampe (Kelly a kol., 2012). Aktivácia osi HPA je charakteristickým znakom chronického stresu a rady neuropsychiatrických porúch (Holsen a kol., 2013). Rovnako boli počas spánkovo-endokrinných štúdií u pacientov s depresiou a insomniou namerané podobné hodnoty – zvýšený ACTH a zvýšená sekrécia kortizolu (Kunugi a kol., 2006). Existuje rada dôkazov o súbežnej depresii a imunitnej odozve organizmu. Na základe interleukínovej hypotézy depresie sa predpokladajú zmeny v bunkovej a humorálnej imunite a v sekrécii prostaglandínov. Indukcia depresie cytokínmi by mohla byť daná ich vplyvom na glutamátergický, sérotonínergický, noradrenergický a HPA systém. Rovnako je známe, že HPA os sa aktivuje zápalovými cytokínmi ako sú IL-6, IL-1α, IL-1ß a TNF-α. Tieto zápalové cytokíny znižujú neuroplasticitu svojim neurotoxickým pôsobením, ktoré vedie k apoptóze a môže ďalej prispievať k depresii (Hayley a kol., 2005). Zvýšenie koncentrácie IL-6 signifikantne koreluje s narušením HPA osi, ktoré sa prejavuje u depresívnych pacientov zvýšením kortizolu (Jehn a kol., 2010). Príkladom z praxe je použitie antide-

presíva imipramínu, ktorý signifikantne down-reguluje plazmatické hladiny adrenokortikotropného hormónu a kortikosterónu, čo naznačuje vhodnosť HPA osi ako potenciálneho smeru vývoja ďalších skupín antidepresív (Frost a kol., 2003).

Komorbidita depresie a drogovej závislosti

Psychiatrickú komorbiditu definujeme ako spoločný výskyt dvoch a viacerých psychických porúch u jedného jedinca (DSM V, 2013), niekedy označovanú ako duálna diagnóza. Opakovane sa potvrdzuje, že drogová závislosť a depresia majú spoločné neurobiologické mechanizmy, ktoré zahŕňajú hlavne alterácie mezokortikolimbickej dopamínergickej dráhy – dráhy odmeny (Markou a kol., 1998; Nestler a Carlezon, 2006). Jedným z vysvetlení tejto situácie je teória samoliečenia („self-medication theory"), ktorá objasňuje komorbiditu depresie a drogovej závislosti na podklade samoliečby závislého drogou, ktorá zvýšením monoamínergickej neurotransmisie uľaví monoamínergickému deficitu manifestujúcemu sa ako depresia (Hall a Queener, 2007; Khantzian, 1985). Z klinickej a teoretickej perspektívy vidíme podobnosti medzi symptomatológiou depresie a symptomatológiou z odňatia niektorých zneužívaných látok. Táto symptomatológia je primárne charakterizovaná zmenami v procesoch odmeny a motivácie, čo je jedným z hlavných charakteristík oboch týchto stavov. Aj napriek spoločnej symptomatológii sa nedá jednoznačne určiť, ktorá diagnóza bola primárna (objavenie sa depresie pred začatím užívania drog) alebo sekundárna (objavenie sa depresie po začatí užívania drog alebo po ich náhlom odňatí). Napriek tomu sa zdá, že drogovo závislí jedinci s depresiou môžu profitovať z antidepresívnej liečby po stránke úľavy od depresívnej symptomatológie rovnako ako po stránke zníženého užívania drog.

Dôkazy zo štúdií na ľuďoch

Sérotonín je jedným z kľúčových neuromediátorov pri depresii aj odňatí niektorých látok so závislostným potenciálom (psychostimulanciá, etanol, benzodiazepíny). Znížená sérotonínergická neurotransmisia mediuje depresiu a v prípade odňatia psychostimulancií a etanolu bola taktiež zistená nižšia hladina sérotonínu v *nucleus accumbens* (Burattini a kol., 2014). Ďalším dôležitým neuromediátorom zúčastňujúcim sa tejto komorbidity je dopamín. Hypofunkcia mezolimbickej dopamínergickej dráhy je zodpovedná za anhedóniu, ktorá je jedným zo základných príznakov depresie, ktorý je antidepresívami z kategórie SSRI (selektívny inhibítor spätného vychytávania sérotonínu) ovplyvnený len minimálne (Kulkarni a Dhir, 2009). Nízka trans-

misia sérotonínu zvyšuje dopamínergickú a apetitívnu odpoveď na kokaín a je spoločným znakom pre závislosť a depresiu (Cox a kol., 2011). Znížené množstvo dopamínergických receptorov bolo rovnako zistené aj pomocou zobrazovacích štúdií závislých pacientov. Pri závislosti dopamín mediuje odmeňujúci efekt drogy a jeho deficit spôsobuje stratu kontroly a impulzívne konanie (Volkow a kol., 2003). Ďalším spoločným faktorom tejto komorbidity je zvýšená transmisia CRF a oslabená neurotransmisia NPY (neuropeptid Y). NPY je endogénny pufer pôsobiaci proti stresom indukovanému uvoľneniu CRF, ktorý je kľúčovou súčasťou osi HPA (Kormos a Gaszner, 2013). Na reguláciu stresu pôsobí aj ECS, ktorého dysregulácia potenciálne môže vyvolať príznaky depresie a rovnako ovplyvnenie CB_1 receptoru je zodpovedné za neurofyziologické a behaviorálne prejavy závislosti. U pacientov liečených antagonistom CB_1 receptoru rimonabantom sa podarilo odvyknúť od závislosti na nikotíne (Wilcox a kol., 2011). U pacientov s depresiou je pozorovaná dlhodobejšia a silnejšia závislosť od nikotínu ako v bežnej populácii, čo bolo potvrdené aj zobrazovacími štúdiami mezokortikolimbického systému (Kushnir a kol., 2013). Spojnicou depresie a drogovej závislosti je aj lokalizácia zmien v mozgu pozorovaných počas depresie a odňatia drogy. Zmeny vznikajú v oblasti limbických štruktúr ako je *nucleus accumbens*, hipokampus, amygdala, hypotalamus a olfaktorický bulbus (Baicy a kol., 2005). Množstvo štúdií na dvojčatách ukázalo, že zdieľané genetické faktory prispievajú k depresii komorbídnej s fajčením a obecne závislosťou od nikotínu (Lyons a kol., 2008; Tsuang a kol., 2012). Štúdie rodín potvrdili túto hypotézu, keď Lyons a kol. demonštrovali, že jedinci s rodinnou vulnerabilitou k depresii vykazujú zvýšené užívanie tabaku (Lyons a kol., 2008). Depresívni jedinci sú rovnako náchylnejší k závislosti od marihuany (Diehl a kol., 2010). Depresiu a komorbídne užívanie návykových látok môžu ovplyvniť aj environmentálne faktory ako sú nízke vzdelanie, limitované ekonomické zdroje, narušené interpersonálne vzťahy, sociálna izolácia alebo stresové situácie (Diehl a kol., 2010). Spoločné neuroanatomické a neurochemické podklady podporujú hypotézu zdieľanej neurobiológie depresie a drogovej závislosti.

Dôkazy z animálnych štúdií

Animálne modely založené na hypotéze drogovej závislosti ako sekundárnej komplikácie depresie poskytujú údaje, ktoré súhrnne poukazujú na vyššiu vulnerabilitu zvierat s depresívnym fenotypom k rozvoju drogovej závislosti. V transgénnom modeli CB_1 knockout myši vykazovali zmeny v odmeňovacom správaní a pri vyhľadávaní drogy ako napríklad nikotín, etanol, kokaín, amfetamín a iné psychostimulanciá, čo potvrdzuje nálezy u ľudí

(Forget a kol., 2005; Maldonado a kol., 2006). V štúdiách na potkaňom modeli depresie navodenej chronickým miernym stresom bol dokázaný vyšší efekt odmeny u stresovaných zvierat ako u kontrolných skupín. To môže znamenať, že vystavenie stresu zvyšuje potenciál odmeny z drogy a následne tak zvyšuje vulnerabilitu jedinca k drogovej závislosti (Lin a kol., 2002). Operantná autoaplikácia psychostimulancií ako sú kokaín a amfetamíny zvyšuje v animálnych modeloch monoamínergickú neurotransmisiu, ktorá následne sprostredkuje posilnené efekty týchto látok v mezokortikolimbikkom systéme (Calipari a kol., 2014). V potkaňom modeli komorbidity depresie a závislosti sa u bulbektomovaných zvierat prejavila vyššia náchylnosť k auto-aplikácii metamfetamínu (Kucerova a kol., 2012). Olfaktorická bulbektómia spočíva v odsatí čuchových bulbov potkana a je animálnym modelom depresívneho fenotypu, ktorý navodzuje behaviorálne, neurochemické, imunitné a štruktúrne zmeny podobné ľudským symptómom depresie. Tieto poznatky potvrdili aj ďalší autori pre amfetamín (Holmes a kol., 2002), CB_1 receptorového agonistu WIN 55,212-2 (Amchova a kol., 2014) a kokaín (Frankowska a kol., 2014).

Preklinické štúdie operujúce opačnou hypotézou, čiže sekundárnym rozvojom depresívneho fenotypu po chronickej aplikácii návykových látok rovnako poskytujú užitočný vhľad do patofyziológie tejto komorbidity. Príkladom je depresogénny efekt chronickej aplikácie alkoholu, ktorý sa v animálnom modeli prejavil v signifikantnom zvýšení času imobility v teste núteného plávania a zníženej hladiny BDNF v hipokampe (Hauser a kol., 2011).

Záver

Symptómy duševnej poruchy a drogovej závislosti interagujú a navzájom sa ovplyvňujú. Užívanie drog sa tiež dá považovať za súčasť alebo symptóm duševnej poruchy a pokus pacienta naordinovať si liečbu sám – teória samoliečenia, pri ktorej úľavový účinok drog pri depresii potencuje vznik závislosti. Zároveň však môže byť ťažké odlíšiť symptómy vyvolané drogovou intoxikáciou od psychotických epizód, ktoré s užívaním drog nesúvisia.

Existencia spoločných neurobiologických podkladov depresie a drogovej závislosti bola jasne dokázaná. Súčasné neurobiologické štúdie za prispenia zobrazovacích techník umožňujúcich vizualizáciu mozgových procesov u človeka vedú k formulácii hypotéz o interakcii medzi fyzickou a psychickou traumou, vývoji mozgu, účinkoch návykových látok, stresu a systému odmeny. Tento systém hrá pri vzniku závislosti podstatnú úlohu a spája sa so štruktúrnymi zmenami v mozgu a adaptácii na mikro a makro úrovni. Poznávanie spoločných neurobiologických podkladov oboch porúch je nutné pre vývoj inovatívnych terapeutických riešení pre liečbu drogovej závislosti

u depresívnych pacientov založené napríklad na farmakologickom ovplyvnení glutamátergický (ketamín, momentálne vo fáze klinického skúšania) alebo endokanabinoidného systému.

Je zrejmé, že bez ohľadu na to, či je depresia primárnou príčinou alebo sekundárnym dôsledkom drogovej závislosti, jej liečba je nutným predpokladom pre zlepšenie prognózy závislosti. Diagnostika pacientov s komorbídnou depresiou a drogovou závislosťou je obtiažna, ale jedine týmto spôsobom je možné izolovať pacientov, ktorí môžu mať prospech z liečby antidepresívami. Závislosť a z nej vyplývajúce deštruktívne konanie sú väčšinou tými hlavnými faktormi, ktoré určujú klinický obraz pacienta a zastierajú pritom symptóm duševnej poruchy a depresie. Problémom je taktiež abstinenčný syndróm alebo akútna intoxikácia, ktoré sa dajú ľahko zameniť za symptómy duševnej poruchy. Prekážkou z hľadiska stanovenia duálnej diagnózy je tiež rozdielna špecializácia pracovníkov v psychiatrických zariadeniach a v liečebniach drogovo závislých. Compliance pacientov s komorbiditou býva nízka a všeobecne je ich liečba časovo a finančne náročná. K tomu, aby liečba prebehla úspešne sú potrebné integrované a starostlivo koordinované služby a následná sociálna reintegrácia, aby sa zabránilo relapsu pacienta.

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Therapeutic Potential of Cannabinoids in Schizophrenia

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Abstract: Increasing evidence suggests a close relationship between the endocannabinoid system and schizophrenia. The endocannabinoid system comprises of two G protein-coupled receptors (the cannabinoid receptors 1 and 2 [CB1 and CB2] for marijuana's psychoactive principle Δ^9-tetrahydrocannabinol), their endogenous small lipid ligands (namely anandamide [AEA] and 2-arachidonoylglycerol [2-AG], also known as endocannabinoids), and proteins for endocannabinoid biosynthesis and degradation. It has been suggested to be a pro-homeostatic and pleiotropic signalling system activated in a time- and tissue-specific manner during pathophysiological conditions. In the brain, activation of this system impacts the release of numerous neurotransmitters in various systems and cytokines from glial cells. Hence, the endocannabinoid system is strongly involved in neuropsychiatric disorders, such as schizophrenia. Therefore, adolescence use of *Cannabis* may alter the endocannabinoid signalling and pose a potential environmental risk to develop psychosis. Consistently, preclinical and clinical studies have found a dysregulation in the endocannabinoid system such as changed expression of CB1 and CB2 receptors or altered levels of AEA and 2-AG . Thus, due to the partial efficacy of actual antipsychotics, compounds which modulate this system may provide a novel therapeutic target for the treatment of schizophrenia. The present article reviews current available knowledge on herbal, synthetic and endogenous cannabinoids with respect to the modulation of schizophrenic symptomatology. Furthermore, this review will be highlighting the therapeutic potential of cannabinoid-related compounds and presenting some promising patents targeting potential treatment options for schizophrenia.

Keywords: Δ^9-tetrahydrocannabinol, animal models, antipsychotics, cannabidiol, cannabis, CB receptors, endocannabinoid system, schizophrenia.

1. INTRODUCTION

1.1. Current Pharmacological Approach for the Treatment of Schizophrenia

Schizophrenia (SCZ) is a chronic mental disorder affecting about 1 % of the population worldwide. It is characterized by three broad clusters of symptoms which result in enormous personal suffering, as well as social and economic burden. These symptom domains include positive symptoms such as delusions, hallucinations, disorganized speech and behaviour; negative symptoms including anhedonia and social withdrawal; and cognitive impairments in sensory information processing, attention, working memory and executive functions [1]. They occur in different combinations, differing degrees of severity and in a changing pattern over time in each patient. Thus, SCZ is regarded as a complex and highly heterogeneous disorder. Hyperfunction of dopaminergic (DAergic) system in the mesolimbic pathway was the original tenet of the theory underlying the basis of SCZ because antipsychotic drugs blocked dopamine D2 receptors (D2Rs) and amphetamine which indirectly increases the release of dopamine (DA) exacerbated positive symptoms and thus led to the *dopamine hypothesis of schizophrenia* [2]. The treatment of SCZ was revolutionized more than 50 years ago with the discovery - by serendipity rather than design - that chlorpromazine and haloperidol (called today typical neuroleptics or the first generation antipsychotics) alleviate the psychotic manifestations such as hallucinations and delusions by blocking the D2Rs. From the 1970's the second generation or atypical antipsychotics (including clozapine, olanzapine, risperidone and aripiprazole) were developed. These drugs still act mainly by DA antagonism in the central nervous system (CNS) but their effects are mediated by serotonin receptor subtypes (5-HT_{2A}/5-HT_{2c}), D3R and/or D4R in addition to D2Rs. This class is also known as Multi-acting Receptor Targeted Antipsychotics (MARTA) and has less tendency to produce unwanted extrapyramidal side effects and hyperprolactinemia [3]. Although current pharmacological armamentarium is generally effective treating positive symptoms, it is less effective in treating the negative and cognitive symptoms. In addition, it can induce several side effects resembling Parkinson's disease (known as extrapyramidal side effects) and metabolic syndrome. Furthermore, a significant proportion of patients are refractory to the available drugs. Thus, there is a need to develop new approaches for treating SCZ and appropriate animal models for preclinical testing [4, 5]. It is well accepted that the pathophysiological mechanisms underlying

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SCZ cannot be explained by simple changes in monoamine signalling such as DA and 5-HT but involves more complex alterations in brain circuits including glutamate, GABA and acetylcholine [6]. Thus, all these neurotransmitters could represent potential targets for pharmacological intervention [4]. In accordance, a driven focus on rational discovery of highly selective drugs with new mechanisms such as the glutamatergic, cholinergic neurotransmission or neuropeptidergic signalling affecting intracellular signal transduction pathways appeared in the past decade. Unfortunately, none of these drugs have reached the market yet [7]. Therefore, the partial efficacy of current pharmacological armamentarium, since approximately one third of psychotic patients are non-responders raises the central question to be addressed in this review: Should the pharmacological exploitation of the endocannabinoid system (ECS) be a promising therapeutic approach for treatment of the behavioural dimensions which are dysregulated in SCZ?

1.2. Cannabis and Schizophrenia: Clinical Evidence

Cannabis (or marijuana) is the most frequently abused illicit "recreational" substance in the Western society. Its popularity is due to its capacity to alter sensory perception, to induce euphoria and to increase sociability. Although the association between *Cannabis sativa* and psychopathologic conditions had been known for thousands of years, only in the last 50 years the identification of the chemical structure of marijuana components, cloning of specific cannabinoid receptors and discovery of the ECS in the brain has triggered an exponential growth of studies to explore its real effects on mental health [8, 9]. The *Cannabis* plant contains over 100 terpenophenolic pharmacologically active compounds, known as cannabinoids. Of these, Δ^9-tetrahydrocannabinol (THC), characterized in 1964 [10], was identified as the main psychoactive component of *Cannabis* and later shown to act as a direct agonist on cannabinoid CB1 and CB2 receptors. Other cannabinoids include cannabidiol (CBD), cannabichromene and cannabigerol which do not induce any THC-like psychoactivity. They act via several mechanisms, including modulation of endocannabinoid system tone [11-13], interaction with transient receptor potential vanilloid 1 (TRPV1) channels [11] and serotonin 5-HT_{1A} receptors [14], and enhancement of adenosine signalling [15, 16]. As recently reviewed, the above mentioned mechanisms could underlie the positive effects induced by CBD treatment in preclinical and clinical studies of several disorders [17, 18].

In addition, accumulating evidence suggests that the recreational use of *Cannabis* during adolescence increases the relative risk for psychotic disorders. However, it is still unknown whether *Cannabis* use is an independent risk factor for SCZ or simply that the high prevalence of *Cannabis* use in SCZ patients as an attempt of self-medication due to *Cannabis's* euphoric effects and increased sociability to relieve negative symptoms [19, 20]. Furthermore its use may instead contribute as an environmental risk factor in vulnerable individuals with genetic mutation of COMT (Catechol-O-methyltransferase) enzymes [21] given that the majority of *Cannabis* users do not develop SCZ. Multiple lines of evidence have shown that frequent *Cannabis* consumption could down regulate anandamide (AEA) signalling in schizophrenic but not in healthy individuals. Also, it is associated to brain abnormalities in regions which are known to be rich in CB1 receptors such as the *anterior* and *posterior cingulated cortex*, as suggested by magnetic resonance imaging studies [22-25]. Although the exact relationship between *Cannabis* and SCZ is not fully elucidated, alterations of ECS elements as receptors and their endogenous activators seem to be involved in pathophysiology of SCZ. More specifically, previous studies have reported an increase in CB1 receptor binding in prefrontal area of brains from schizophrenic patients [26-31]. However, other studies failed to demonstrate any alteration [32] or reduction of CB1 density on the neuronal surface [33] and CB1 mRNA expression [34]. This contradiction might result from other neuroplastic alterations which further complicate the situation as another study detected lower CB1 receptor density but no differences on the level of CB1 mRNA expression [35]. Although several confounding factors such as *Cannabis* consumption, treatment with antipsychotics or different biochemical techniques used for the determination of CB1 receptors density and proteosynthesis might explain the apparent opposite results; in general, the presence of a dysfunction in CB1 receptors in selected brain regions of patients is supported. Furthermore, polymorphisms in the CB1 receptor gene CNR1, which could be correlated with an increased probability to develop psychosis, have also been described. Yet, the data are still controversial [23, 36-39].

Recently, the potential involvement of CB2 receptors in the pathogenesis of SCZ has been also supported by clinical findings. Patients with first-episode psychosis have a decreased expression of peripheral CB2 receptors in comparison to healthy controls [40, 41], which is in accordance with preclinical studies [42]. Thus, the altered expression of both receptors in SCZ patients confirms that they possess a certain homeostatic role.

Besides CB receptor dysfunctions, alteration in endocannabinoid levels seems to be implicated in the pathophysiology of SCZ as well. AEA levels have been found elevated in cerebrospinal fluid which were negatively correlated with psychotic symptoms and normalized by treatments with typical antipsychotics [43, 44]. In contrast, Muguruza *et al.* showed in cerebellum, hippocampus and prefrontal cortex of schizophrenic subjects lower AEA and higher 2-arachidonylglycerol (2-AG) levels [45]. Considering the glutamate hypothesis of SCZ and the role of 2-AG in the modulation of glutamatergic neurotransmission, this could represent an adaptive response to reduce glutamatergic hyperactivity in schizophrenics. Yet, it must be taken in to account that these alterations in opposite directions may be due to the different regulation of 2-AG and AEA levels under both physiological and pathological conditions [46]. Moreover, the difference of endocannabinoid levels in cerebrospinal fluid may be related to alterations in peripheral amounts of endocannabinoids, so the neuronal origin of the AEA and 2-AG in the cerebrospinal fluid remain conjectural [45]. Evidence of potential endocannabinoid signalling dysregulation in SCZ is also supported by the decreased expression of endocannabinoid synthesizing enzymes NAPE (N-acyl-phosphatidylethanolamine phospholipase) and DAGL (diacylglycerol lipase) in the peripheral blood mononuclear cells of patients with first episode of psychosis [40].

Based on the evidence presented above, functional abnormalities in the endocannabinoid system could be involved in the pathophysiology of SCZ; thus there is increasing interest to explore potential antipsychotic properties of compounds modulating the endocannabinoid signalling.

2. THE ENDOCANNABINOID SYSTEM (ECS)

The endogenous cannabinoid system (ECS) is a neuromodulatory system which is involved in a variety of physiological processes both in the brain and in the periphery. Within the CNS, it acts at the level of inhibitory and excitatory synapses in brain regions involved in emotional or non-emotional processes, and mediates the effects of THC, the main psychoactive constituent of *Cannabis* [47]. Increasing evidence suggest that altered EC signalling could play a role in the pathophysiology of several diseases such as pain and inflammation [48]; immunological disorders [49, 50]; neurodegenerative [9] and stress-related conditions [51]; obesity, metabolic [52, 53] and cardiovascular [54] diseases; cancer [55], gastrointestinal [53, 56] and hepatic [57] disorders. However, the exact pathophysiological mechanisms through which the ECS plays are not clearly understood at present.

The ECS consists of: (1) the cannabinoid receptors CB1 and CB2 [58-60], (2) the endogenous cannabinoid CB receptor agonists, AEA and 2-AG [61, 62], (3) a specific and not yet identified cellular uptake mechanism and [63, 64], (4) the enzymes for endocannabinoid biosynthesis: *N*-acyl-phosphatidylethanolamine-selective phosphodiesterase or glycerophosphodiesterase E1 and diacylglycerol lipase α or β [65, 66]; or degradation: fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) [67, 68], respectively for AEA and 2-AG. Despite it is well accepted that AEA is an endogenous agonist for cannabinoid CB1 receptors in the brain, some of the typical cannabimimetic effects of AEA are still present in transgenic mice lacking CB1 receptors. These effects may be due to AEA capability to act as a full agonist for the TRPV1 channels [69] resulting in mechanisms distinct from CB1 and CB2 receptors activation. However, additional "players" which target TRPV1 and/or CB1 receptors, including putative CB1 antagonist peptides like hemopressins, peroxisome proliferator-activated receptor-α (PPAR-α) and γ (PPAR-γ) ligands, such as oleoylethanolamide (OEA) or palmitoylethanolamide (PEA), and N-arachidonoyl-dopamine (NADA) are described as potential members of this signalling system. Although the existence of a third cannabinoid receptor subtype has been also suggested [70], to date only CB1 and CB2 receptors are recognized as G protein coupled receptors for endocannabinoids [71].

The cannabinoid CB1 and CB2 receptors which are encoded by two different genes on human chromosomes: 6q14-q15 (CNR1) and 1p36.11 (CNR2), are 7 transmembrane Gi/o coupled receptors that share 44 % protein identity but they display different pharmacological profiles and patterns of expression, a dichotomy that provides a unique opportunity to develop pharmaceutical approaches. The cannabinoid CB1 receptors are highly expressed in the *basal ganglia*, *frontal cortex*, *hippocampus* and *cerebellum*. They are expressed with a moderate/low density in the *amygdala*, *nucleus accumbens*, *medulla*, *periaqueductal gray* and *thalamus* [72]; as well as they are also described in non-neuronal cells of the brain such as microglia, oligodendrocytes and astrocytes [73]. Within these cortical areas, they are expressed at the GABAergic interneurons and glutamatergic neurons, which are the two major neuronal subpopulations expressing the CB1 receptors [74]. These neurotransmitter systems represent the two major opposing players regulating the excitation state of the brain; GABAergic interneurons being inhibitory and glutamatergic neurons being excitatory. Recent studies have demonstrated that CB1 receptors are also located in neurons of the *dorsal raphe nucleus* and in the *nucleus coeruleus* which are the major source of serotonin and noradrenalin in the brain [75, 76]. Thus, the direct or indirect modulation by monoamine activity on GABA and glutamate neurons could underlie the psychotropic and non-psychotropic effects of CB1 activation, respectively.

The cannabinoid CB2 receptors, also activated by AEA and 2-AG, are mostly peripherally located on immunological tissues. CB2 receptors are also detected in glia cells and in neurons of several brain regions such as *cerebral cortex*, *amygdala*, *hippocampus*, *hypothalamus* and *cerebellum* but in a much lesser extent [77, 78]. They play an important part in regulation of pain and inflammation even though recent data also suggest their involvement in emotional and non-emotional processes [79]. The observation that the elements of such neuromodulator system are prevalent throughout the neuroanatomical structures and circuits implicated in emotionality provides a rationale for the preclinical development of agents targeting the ECS to treat multiple psychiatric disorders including SCZ.

3. EFFECTS OF PHARMACOLOGICAL MANIPULATION OF THE ENDOCANNABINOID SIGNALLING IN PRECLINICAL AND CLINICAL STUDIES OF SCHIZOPHRENIA

Schizophrenia is a unique human disorder characterized by specific clinical manifestations such as delusions, thought disorders and hallucinations, which was described in 1896 by Kraepelin as *dementia praecox*. Due to the nature of the disease it is impossible to develop an animal model which would fully mimic its symptoms [1]. Thus, a greater understanding of the disorder might arise from modelling specific signs and symptoms, rather than mimicking the entire syndrome. In accordance with this strategy, the most reliable behavioural indices of positive symptoms in experimental models are hyperlocomotor activity and behavioural stereotypes which mimic the psychomotor agitation and presence of stereotyped behaviours in acutely psychotic patients since positive symptoms such as hallucinations and delusions cannot be measured in animals [80]. These are based on the rationale that the hyperfunctioning of the mesolimbic DAergic system, which seems to underlie the enhanced locomotor activity and stereotyped behaviour, is consistent with the human conditions where an enhanced subcortical DAergic activity plays a pivotal role to precipitate positive symptoms [81]. However, some behavioural aspects of SCZ seem to be modelled and objectively assessed in rodents. More specifically, hallmarks of negative symptoms, deficits in social behaviour and anhedonia, can be assessed both in humans and rodents with the pre-pulse inhibition (PPI) as an index of disrupted sensory gating abilities both in schizophrenic pa-

tients and in experimental animal models [82]. Interestingly, the various cognitive deficits in SCZ, as identified by the NIH (National Institute of Health) Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative, could be experimentally assessed by the use of specific rodent behavioural tasks [83]. As recently estimated, more than 20 different rodent models of SCZ have been developed, which fit into four different categories depending on the type of manipulation, namely 1) pharmacological, 2) genetic, 3) lesion based and 4) neurodevelopmental models. First experimental models were developed on the basis of the theory that SCZ is a disorder related to an excessive DAergic activity; accordingly, the DAergic agents such as amphetamine and apomorphine attempt to mimic this feature. However, due to the increased understanding of the genetic basis and potential involvement of glutamate and adverse environmental insults, different experimental manipulations for animal models of SCZ have also been developed [84].

Since the identification of cannabinoid CB1 and CB2 receptors and their endogenous ligands (AEA and 2-AG), a key aspect to assess the function and therapeutic potential of the ECS for SCZ treatment has been the availability of selective pharmacological tools. They vary from directly acting compounds, such as agonists and inverse agonists, to agents that enhance indirectly endocannabinoid signalling by affecting the cellular reuptake of endocannabinoids (experimental agents: AM404 or VDM11) or by inhibiting the hydrolytic enzymes FAAH and MAGL (experimental agents: URB597, AA-5-HT or JZL184). However, several additional elements which can be described as potential members of the ECS such as ligands (i.e. noladin, virodhamide), receptors (GPR55, PPARγ, TRPV1) and synthetic or degradative pathways, could participate in the mechanism of action of the compounds described above [85].

3.1. Studies on Positive- and Negative-like Symptoms

Different substances modulating the endocannabinoid signalling have been evaluated in several animal (mostly pharmacological) models for affecting positive- and/or negative-like symptoms of SCZ, as summarized in Table **1**. In a recent study, Spano *et al.* have shown that chronic exposure to the CB1 agonist WIN55,212-2 reduces phencyclidine (PCP)-induced hyperlocomotion [86], in agreement with previous studies, showing a reduction of cocaine- or quinpirole- induced hyperactive behaviour by direct CB1 activation [87, 88]. Interestingly, stereotyped and hyperlocomotor behaviours, an index of positive-like symptoms, were also reduced by the non-psychoactive component of *Cannabis sativa* cannabidiol (CBD) [89-92]. Although in a recent study CBD failed to reverse the amphetamine-induced hyperactivity, it elicited certain neuroprotective effects [93]. In additional, CBD prevented human experimental psychosis. More specifically, it was effective in open case reports and clinical trials in psychotics with a remarkable safety profile, [94-97] as well as it and others phytocannabinoids such as cannabidiolic acid, tetrahydrocannabivarin, tetrahydrocannabivarin acid, cannabichromene, cannabichromenic acid, cannabigerol, and cannabigerolic acid were patented for their use in combination with one or more anti-psychotic medicaments to prevent or treat psychosis and psychotic disorders [98]. Yet, it is still unknown the exact mechanism(s) of action underlying its antipsychotic effects, but it is clear that CBD does not only act through ECS (as weak partial antagonist at CB1/CB2 receptors or inhibitor of AEA hydrolysis and reuptake), but also activates serotonin 5-HT_{1A} or adenosine receptors or targets nuclear receptors of the PPAR family as well as modulates ion channels including TRPV1 [18]. Regardless the exact mechanism of action, attention has been focused on the potential therapeutic use of CBD in further mental diseases such as mood (i.e. anxiety and depression) and neurodegenerative (Alzheimer's or Parkinson's disease) disorders [17].

In the last years, selective antagonist/inverse agonists of CB1 receptors were some of the most promising molecules in pharmacological research for the treatment of obesity and addictive disorders. The first such compound was rimonabant (SR141716) [99] introduced into clinical practice as antiobesity agent in several countries. However, due to the higher incidence of psychiatric side effects such as anxiety, depression and suicidal tendencies, rimonabant was very soon withdrawn from the market [100]. In contrast to CBD, the ability of CB1 antagonists on positive-like symptoms is still under debate due to the contradictory results. In 1999, Poncelet *et al.* reported that rimonabant as well as clozapine or haloperidol antagonized the hyperlocomotor activity induced by d-amphetamine, cocaine and morphine in gerbils [101]. Potential therapeutic effects on positive symptoms were then also confirmed by Tzavara and colleagues in the PCP animal model [102]. However, in other studies, it failed to ameliorate the hyperlocomotor activity [103-105] or instead increased stereotype behaviour [106]. Although the discrepancies among these studies could be due to interspecies differences, or physiochemical differences between drugs or experimental models, the preclinical data described above suggest that the CB1 blockade might have a limited potential to treat positive symptoms. In line with this concept, AVE1625 (drinabant, so far reported as the CB1 antagonist) has partially reversed the positive-like symptoms in experimental models with an improved side effects profile [107].

Recently, attention has been drawn to the expression of CB2 receptor in the CNS [77, 78]. Further supporting that cannabinoid CB2 receptors may play a role in psychiatric disorder, it has been seen that pharmacological or genetic CB2 receptor blockade increased susceptibility to develop positive-like symptoms [41]. As a result, the CB2 agonist beta-caryophyllene has been recently patented for potential efficacy for SCZ treatment [108]. The ECS seems to play a role in the social behaviour of rodents and the resistance of negative symptoms to pharmacological interventions; therefore, the effects of pharmacological modulation of endocannabinoid signalling on the social deficits of experimental models of schizophrenia have been recently examined. Direct activation of CB1 receptors through the use of CB1 agonists WIN55,212-2 or CP55,940 reversed the PCP-induced social deficits [86, 109]. Interestingly, the pharmacological enhancement of endocannabinoid levels via systemic treatment with the FAAH inhibitor URB597 also reversed the social deficits in the PCP model, but at the same time elicited, as well as the cannabinoid CB1 blockade, harmful effects in the social behaviour of control animals, maybe by

Table 1. Effects of pharmacological modulation of the endocannabinoid system on schizophrenia-like symptoms.

a) Positive-like symptoms

Mechanism	Drug: Effective Dose (Range tested)	Animals	Models	Behavioral Response	Positive Control	Ref.
CB1/CB2 receptor agonists:	WIN: 6 (3-6) mg/kg, i.p.	Wistar rats	cocaine (10 mg/kg, i.p.)	↓ hyperlocomotion	not determined	[88]
	CP: (0.0025-0.01 mg/kg, s.c.)	*Cebus* monkeys	d-amphetamine (0.25 mg/kg, s.c.)	no effect on arousal and stereotypy	not determined	[103]
	CP: 0.1/0.25 (0.01-0.25) mg/kg, i.p.	Wistar rats	quinpirole (0.5 mg/kg, i.p.)	↓ hyperlocomotion	not determined	[87]
	WIN: 0.3 mg/kg/day, i.v. for 14 days	Lister Hooded rats	PCP (2.5 mg/kg, i.p.)	↓ hyperlocomotion	not determined	[86]
CB1 antagonists:	RIM: 1/3 (0.3-3) mg/kg, i.p.	Gerbils	cocaine (5-15 mg/kg, i.p.) d-amphetamine (2.5 mg/kg, i.p.) morphine (4 mg/kg, i.p.) WIN-55,212-2 (1 mg/kg, i.p.)	↓ hyperlocomotion in habituated gerbils	clozapine (3 mg/kg, i.p.) haloperidol (0.1 mg/kg, i.p.)	[101]
	RIM: 3-10 mg/kg, i.p.	Bl6 mice	PCP (4 mg/kg, i.p.) d-amphetamine (2.5 mg/kg, i.p.)	↓ hyperlocomotion	not determined	[102]
	RIM: (0.0005-5) mg/kg, i.p.	Wistar rats	d-amphetamine (3 mg/kg, i.p.)	no effect on hyperlocomotion and stereotypy	haloperidol (0.25 mg/kg, i.p.)	[104]
	RIM: 0.1-0.5 (0.1-0.75) mg/kg, s.c.	*Cebus* monkeys	d-amphetamine (0.25 mg/kg, s.c.)	↓ arousal no effect on stereotypy	not determined	[103]
	RIM: 1 (0.1-1) mg/kg, i.p.	Wistar rats	SKF38393 (0.05-1 mg/kg, s.c.) quinpirole (0.25 mg/kg, s.c.)	↑ stereotypy	not determined	[106]
	RIM: 1 mg/kg, i.p.	Sprague-Dawley rats	amphetamine (1 mg/kg, i.p.)	no effect on hyperlocomotion	not determined	[105]
	AVE: 1- 3-10 mg/kg, i.p.	Wistar rats	MK-801 (0.05 mg/kg, i.p.)	↓ disrupted LI	risperidone (0.01-1 mg/kg, i.p.)	[107]
CB2 antagonist:	AM630: 3-30 mg/kg, i.p.	Bl6/JJ mice	MK-801 (0.5 mg/kg, i.p.) methamphetamine (2 mg/kg, i.p.)	↑ hyperlocomotion	not determined	[41]
AEA reuptake inhibitor:	AM404: 10 µg/rat, i.c.v.	Wistar rats	quinpirole (0.25 mg/kg, i.p.)	↓ hyperlocomotion	not determined	[156]
Non-psychotropic cannabinoid:	CBD: 30/60 (15-60) mg/kg, i.p.	Swiss mice	d-amphetamine (5 mg/kg, i.p.) ketamine (60 mg/kg, i.p.)	↓ hyperlocomotion	haloperidol (0.15-0.6 mg/kg, i.p.) clozapine (1.25-5 mg/kg, s.c.)	[91]
	CBD: 20 (5-20) mg/kg, i.p.	Sprague-Dawley rats	THC (1 mg/kg, i.p.)	↓ hyperlocomotion	not determined	[90]
	CBD: 50 (1-50) mg/kg/day, i.p. for 3 weeks	Bl6/Jarc mice	d-amphetamine (5 mg/kg, i.p.)	↓ hyperlocomotion	not determined	[89]
	CBD: (15-60) mg/kg, i.p. for 7 days	Wistar rats	d-amphetamine (2 mg/kg, i.p.)	no effect on hyperlocomotion	not determined	[93]

(Table 1) contd…..

b) Negative-like Symptoms

Mechanism	Drug: Effective Dose (Range tested)	Animals	Models	Behavioral Response	Positive Control	Ref.
CB1/CB2 agonists:	WIN: 0.3 mg/kg/day, i.v. for 14 days	Lister Hooded rats	intermittent PCP (2.5 mg/kg/day, i.p.)	↓ social deficit in PCP ↑ social deficit in control	not determined	[145]
	CP: 0.01 mg/kg, i.p.	Wistar rats	PCP (5 mg/kg/day, i.p.) twice a day for 7 days	↓ social deficit in PCP	not determined	[109]
	THC: 2.5 mg/kg/day, i.p. (PND37-39); 5 mg/kg/day, i.p. (PND40-43); 10 mg/kg/day, i.p. (PND44-47)	Sprague-Dawley rats	maternal deprivation	↓ aggressive behavior of female in the SI no effect in the FST	not determined	[157]
CB1 antagonists/invesre agonists:	AM251: 0.5 mg/kg/day, i.p. for 3 weeks	Lister Hooded rats	PCP (2.58 mg/kg/day, i.p.) for 4 weeks	↓ immobility time in the FST	clozapine (5 mg/kg/day, i.p.) for 3 weeks	[115]
	AM251: 0.5 mg/kg/day, i.p. for 3 weeks	Lister Hooded rats	social isolation	↓ aggressive behavior in the SI	not determined	[129]
	AM251: 3 (0.3-3) mg/kg, i.p. RIM: 0.3/1 (0.1-1) mg/kg, i.p.	Wistar rats	PCP (5 mg/kg/day, i.p.) twice a day for 7 days	↑ social withdrawal in control rats	not determined	[109]
TRPV1 antagonist:	capsazepine: 1 (1-10) mg/kg, i.p.	Wistar rats	PCP (5 mg/kg/day, i.p.) twice a day for 7 days	↓ social withdrawal in control rats	not determined	[109]
FAAH inhibitor:	URB597: 0.1/0.3/1 mg/kg, i.p.	Wistar rats	PCP (5 mg/kg/day, i.p.) twice a day for 7 days	↓ social withdrawal in PCP rats ↑ social withdrawal in control rats	not determined	[109]

Table 1. The table summarizes the effects of direct pharmacological manipulation of the endocannabinoid signalling on positive and negative-like symptoms in rodent models of schizophrenia. Acronyms: THC: Δ^9-tetrahydrocannabinol, AEA: anandamide, CBD: cannabidiol, CP: CP55940, FAAH: fatty acid amide hydrolase, FST: forced swim test, i.c.v.: intracerebroventricular, i.p.: intraperitoneal, i.v.: intravenous, LI: latent inhibition, PCP: phencyclidine, PND: postnatal day, RIM: rimonabant (SR141716), s.c.: subcutaneous, SI: social interaction, TRPV1: transient receptor potential vanilloid 1 channels, WIN: WIN55,212-2.

disturbing the ECS tone through the activation of TRPV1 channels [109, 110]. In accordance, it has been seen that chronic Cannabis consumption improves negative symptoms in schizophrenic subjects [111, 112], as well as it also induces an amotivational syndrome, which mimics negative symptoms in non schizophrenics [113]. This suggests different effects of cannabinoids on healthy or schizophrenic subjects. Chronic treatment with the CB1 receptor antagonist AM251 counteracted the aggressive behaviour and reversed the PCP-induced immobility in the forced swim test which was accompanied by the rescue of CB1 receptor functionality in a neurodevelopmental animal model based on a social isolation procedure [114, 115]. Although the genetic CB1 disruption in mice was also able to counteract the PCP-induced social deficit [116] further supporting the potential antipsychotic properties of the CB1 blockade, human experimental studies have so far shown controversial results. More specifically, Meltzer *et al*. have not seen a clinical improvement in schizophrenic patients after rimonabant treatment. In contrast, Kelly *et al*. found a significant reduction of psychotic symptomatology in obese patients with SCZ [117, 118]. Thus, further clinical studies are necessary to elucidate the therapeutic potential of CB1 antagonists. To date, several compounds of this pharmacodynamic profile have been patented for potential efficacy for treating SCZ symptoms [119-123].

3.2. Studies on Cognitive/Attention Deficits

It has become clear that SCZ cannot be reduced to its psychotic symptoms and the cognitive deficits of these patients are the most debilitating and remain resistant to treatment. Thus, the development of new drugs has been hampered by the lack of existing drugs for treating the cognitive impairment in schizophrenic patients, since there is not gold standard positive control drug that can be used in cognitive assays. Thus, in light of the high density of cannabinoid CB1 receptors in cortical regions involved in cognition and memory processes, the cognitive effects of the modulation of the endocannabinoid signalling could be one of the potential pharmacological targets for the SCZ treatment. The existing evidence of involvement of ESC in the cognitive/attention processes in animal models of SCZ is presented in the Table **2**.

Table 2. Effects of pharmacological modulation of the endocannabinoid system on cognitive/attention deficits of a schizophrenia-like phenotype.

Mechanism	Drug: Effective Dose (Range tested)	Animals	Models	Behavioral Response	Positive Control	Ref.
CB1/CB2 receptor agonists:	THC: 0.5 mg/kg/day, i.p. for 3 weeks	Lister Hooded rats	PCP (2.58 mg/kg/day, i.p.) for 4 weeks	↑ cognitive deficit	clozapine (5 mg/kg/day, i.p.) for 3 weeks	[126]
	THC: 0.3/1/3 mg/kg, i.v.	Sprague-Dawley rats	social isolation	↑ disruption of PPI	not determined	[144]
	WIN: 3 mg/kg, i.p.	Bl6/J	psychosocial stress	↓ disruption of PPI	not determined	[143]
	THC: 2.5 mg/kg/day, i.p. (PND35-37); 5 mg/kg/day, i.p. (PND38-41); 10 mg/kg/day, i.p. (PND42-45)	Sprague-Dawley rats	maternal deprivation	↑ cognitive deficit in control female	not determined	[157]
	WIN: 0.3 mg/kg/day, i.v. for 14 days	Lister Hooded rats	chronic PCP (2.5 mg/kg/day, i.p.)	↓ disruption of PPI ↓ cognitive deficit	not determined	[145]
CB1 antagonists/inverse agonists:	RIM: (0.3-5) mg/kg, i.p.	Sprague-Dawley rats	apomorphine (0.5 mg/kg, s.c.) MK-801 (0.1 mg/kg, s.c.) d-amphetamine (5 mg/kg, s.c.)	no effect on PPI	clozapine (10 mg/kg, i.p.); olanzapine (10 mg/kg, i.p.); haloperidol (0.3 mg/kg,1 i.p.)	[104]
	RIM: 3 (0.3-3) mg/kg, i.p.	Swiss mice	apomorphine (3 mg/kg, i.p.)	↓ disruption of PPI	not determined	[150]
	AM251: 0.5 mg/kg/day, i.p. for 3 weeks	Lister Hooded rats	PCP (2.58 mg/kg/day, i.p.) for 4 weeks	↓ cognitive deficit	clozapine (5 mg/kg/day, i.p.) for 3 weeks	[130]
	AM251: 0.5 mg/kg/day, i.p. for 3 weeks	Lister Hooded rats	social isolation	↓ cognitive deficit	not determined	[129]
	AM251: 0.5 mg/kg/day, i.p. for 3 weeks	Lister Hooded rats	social isolation	↓ disruption of PPI	not determined	[114]
	AM251: 1 mg/kg, i.p.	Wistar rats	PCP (5 mg/kg/day, i.p.) twice a day for 7 days	↓ cognitive deficit in PCP ↑ cognitive deficit in control	not determined	[110]
	RIM: 0.75/1/3 mg/kg, s.c. AM251: 1.4/1.8 mg/kg, s.c.	Sprague-Dawley rats	PCP (1.25 mg/kg, s.c.)	↓ disruption of PPI	clozapine (7.5 mg/kg, i.p.)	[149]
CB2 antagonist:	AM630: 30 (3-30) mg/kg, i.p.	Bl6/6JJmsSlc mice	MK-801 (0.5 mg/kg, i.p.) methamphetamine (2 mg/kg, i.p.)	↑ disruption of PPI in MK-801 mice no effect on PPI in methamphetamine pretreated mice	not determined	[41]
FAAH Inhibitor:	URB597: 0.3 mg/kg, i.p.	Wistar rats	PCP (5 mg/kg/day, i.p.) twice a day for 7 days	↓ cognitive deficit	not determined	[110]
Non-psychotropic cannabinoid:	CBD: 0.5 mg/kg, i.m.	Rhesus monkeys	THC (0.2-0.5 mg/kg, i.m.)	↓ cognitive deficit	not determined	[138]
	CBD: 5 (1-15) mg/kg, i.p	Swiss mice	MK-801 (1 mg/kg, i.p.)	↓ disruption of PPI	clozapine (4 mg/kg, i.p.)	[154]

Table 2. The table summarizes the effects of direct pharmacological manipulation of the endocannabinoid signalling on cognitive/attention deficits in rodent models of schizophrenia. Acronyms: THC: Δ^9-tetrahydrocannabinol, CBD: cannabidiol, FAAH: fatty acid amide hydrolase, FST: forced swim test, i.c.v.: intracerebroventricular, i.m.: intramuscular, i.p.: intraperitoneal, i.v.: intravenous, LI: latent inhibition, PCP: phencyclidine, PND: postnatal day, PPI: prepulse inhibition, RIM: rimonabant (SR141716), s.c.: subcutaneous, SI: social interaction, WIN: WIN55,212-2.

Acute administration of the main pharmacologically active principle of the *Cannabis sativa*, THC, as well as the CB1 agonists such as WIN55,212-2, CP55,940 or AEA induce in animals and healthy humans memory deficits similar to those seen in SCZ, which could be mediated through a disruption of prefrontal and hippocampal functions [124, 125]. However, in the PCP-induced animal model controversial data have been obtained following CB1 activation. While Vigano and colleagues found that chronic THC treatment in juvenile rats worsened cognitive impairment [126], by contrast the CB1 agonist WIN55, 212-2 attenuated the PCP cognitive deficits in adult rats [86]. Although the authors used the same experimental model, the discrepancies between these studies could be due to either physiochemical differences between CB1 agonists or the different age of pharmacological treatment (juvenile vs. adult). Furthermore, Seillier and colleagues found that indirect activation of CB1 receptors through the use of FAAH inhibitor URB597 caused working memory deficits in saline treated rats comparable to those after PCP treatment, which may arise due to perturbing the endocannabinoid tone [110]. In contrast, other evidence from animal studies suggests that pharmacological CB1 receptor blockade could exert promnesic effects. In this context, it has been seen that the memory disruptive effects induced by CB1 agonists such as THC, AEA or WIN55,212-2 were counteracted by rimonabant treatment [124, 127, 128]. Furthermore, the CB1 receptor antagonist/inverse agonist AM251 reversed memory impairment in pharmacological and neurodevelopmental models of SCZ [110, 114, 129, 130]. Despite potential pro-cognitive effects of CB1 antagonists described above, in the few clinical studies assessing its role on cognitive functioning in human, rimonabant worsened ketamine induced deficits [131] or did not improve global cognitive functioning. In this later study just a specific learning deficit in schizophrenic patients based on response to positive feedback was recorded [132]. In a recent clinical trial assessing the potent and selective CB1 antagonist AVE1625 for improving cognitive deficits in schizophrenic, there was an insufficient efficacy of the treatment (Clinical Trials.gov identifier: NTC00439634). The withdrawal of rimonabant, due to the psychiatric side effects in the metabolic syndrome treatment, interrupted the entire industrial development of CB1 antagonists/inverse agonists. However, several CB1 receptor inverse agonist compounds have been patented for the treatment of cognitive impairment associated with SCZ [133, 134]. Thus, a possible solution for the safe use of this class of compounds could be to determinate which patients are at high risk of psychiatric side effects through detailed phenotypic assessment and genetic testing [135] or change to the use of neutral CB1 antagonist [136, 137].

Clinical and preclinical data suggest that CBD which is the most extensively investigated phytocannabinoid for potential use in psychiatric disorders, is also able to ameliorate cognitive deficits. More specifically, it was able to reverse the THC-induced deficits in rhesus monkeys [138], as well as THC induced cognitive impairment in human [139]. Moreover, CBD effects on cognitive function in schizophrenic patients are currently under investigation in a phase II clinical trial (Clinical Trials.gov identifier: NCT00588731). Patients with SCZ exhibit deficits in an operational measure of sensorimotor gating: pre-pulse inhibition (PPI) of startle reaction. Similar deficits in PPI are produced in animals by pharmacological or developmental manipulations. These experimentally induced PPI deficits in rats clearly do not represent animal models of schizophrenia per se, but provide us an investigative tool with high face, predictive, and construct validity for sensorimotor gating deficits in SCZ patients [140]. To confirm that younger animals have different vulnerability to cannabinoid treatment in development of SCZ-like symptoms, rats treated at adulthood with CB1 receptor agonist WIN55,212-2 have not shown disruption of PPI [141]; in comparison, the prepubertal CB1 agonist treatment induced PPI deficits in adult age [142]. However, at adulthood, WIN55,212-2 and THC improved and impaired the PPI of the startle response in psychosocially stressed rodents, respectively [143, 144]. Discrepancies between these studies could be due to interspecies (rat vs. mice) dissimilarities in response to treatments (e.g. pharmacokinetic issues), to physiochemical characteristics of the specific compounds (THC vs. WIN) and to the different experimental procedures. Nevertheless, in an experimental model of SCZ, CB1 agonist WIN 55,212-2 was able to attenuate the PCP-induced deficit in PPI [145]. Controversial data were obtained following enhancement of AEA level through the use of AM404, an AEA reuptake inhibitor. While in mice AM404 disrupts sensorimotor gating [146]; in contrast it was ineffective in the PPI test in rats [147], suggesting an interspecies (rats vs. mice) difference in the response to the pharmacological modulation of AEA levels.

The potential antipsychotic properties of CB1 antagonists have also been explored in the impaired sensorimotor gating, as a model of perceptual distortion [148]. It has been seen that the antagonists/inverse agonists rimonabant and AM251 reversed the disrupted PPI in several experimental models of SCZ, similarly as the conventional neuroleptics [114, 149, 150]. On the other hand, the genetic blockade of CB1 signalling resulted in unaltered PPI response, as shown by the phenotype of mice with a complete deletion of CB1 receptors; however, they have shown a decreased parvalbumin immunoreactivity in the cortex and striatum, which is typical in schizophrenic human subjects [151, 152]. Again it is still unknown the exact mechanisms underling these discrepancies, but compensatory mechanisms in knock out mice could be involved. Given recent attention has been drawn to the role of CB2 receptors in psychiatric disorders, preclinical and clinical data indicate that a reduced CB2 signalling elicited a sensorimotor gating and an increased risk of SCZ in human, respectively [41, 42]. The potential antipsychotic-like property of CBD have also been supported by its ability to reverse the sensorimotor gating deficits in different experimental models, similarly to that induced by clozapine [153, 154].

4. CURRENT & FUTURE DEVELOPMENTS

As outlined above, preclinical and clinical evidence strongly suggest a dysregulation of the ECS in schizophrenia, such as abnormalities in cannabinoid (CB1 and/or CB2) receptor function and endocannabinoid (AEA and/or 2-AG) levels in different cerebral areas. However, so far, the full picture on the role of the endocannabinoid system in this

pathology has yet to emerge. To date, the pharmacotherapy of negative symptoms and cognitive deficits of SCZ has been disappointing; as antipsychotics have not met the expectations and the development of more effective therapies have been inadequate [155]. Thus, the ability of cannabinoids to modulate schizophrenia-like symptoms is extremely attractive for the development of novel antipsychotics agents. Although use of CB1 antagonists/inverse agonists is hampered by unwanted psychiatric side effects and that the possibly safer direct modulation of CB2 receptors still lacks sufficient experimental evidence to justify its use, the use of CBD has produced very promising results in animal models with a pharmacological profile resembling that of atypical antipsychotics. Clinical evidence also suggests that CBD, being devoid of psychotropic activity, could represent a reliable compound for psychosis in schizophrenia especially in view of its lack of extrapyramidal side effects [96]. Further clinical studies will determine if CBD treatment would be the novel pharmacotherapy for the disturbances in the social and cognitive functions in schizophrenic patients.

AUTHOR CONTRIBUTIONS

Jana Kucerova has collected the literature sources, was involved in the discussion of the manuscript structure and wrote a substantial part of the text.

Katarina Tabiova was responsible for cross-checking the literature, preparation of the tables and reference collection.

Filippo Drago was involved in the discussion of the structure and revised both the draft and final version of the manuscript.

Vincenzo Micale has organized the structure of the whole text and wrote a substantial part of the manuscript.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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ORIGINAL ARTICLE

Aripiprazole does not influence methamphetamine I.V. self-administration in rats

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Abstract

OBJECTIVE: Effects of an atypical antipsychotic and antidepressant aripiprazole on methamphetamine (MET) I.V. self-administration (IVSA) under conditions of behavioural sensitization to MET in rats was investigated in the present study.

METHODS: Adult male Wistar rats were randomly divided into 2 groups: the first group received MET (0.05 mg/kg, intraperitoneally for 14 days); the other received vehicle as a control. During following 14 days of wash-out period the surgery required for IVSA including recovery was realized. The IVSA of MET paradigm was performed in operant chambers under fixed ratio (FR) schedule of reinforcement. When a defined stable intake of MET at FR3 was reached water was given to the animals orally until they achieved stable intake again. Since then aripiprazole (3 mg/kg/day) was administered orally by tube 30 minutes before each self-administration session. For statistic analysis the nonparametric ANOVA Kruskal-Wallis test with Dunn's Multiple Comparisons post test was applied.

RESULTS: A significant decrease of the drug intake was recorded in animals sensitized by MET (MET pretreated) comparing to non-sensitized corresponding group. Aripiprazole did not significantly affect the baseline of MET intake.

CONCLUSION: The behavioural sensitization to MET induced by 14 day intraperitoneal administration of MET decreased the number of the drug doses self-administered per session. This finding might result from increasing efficacy of the drug after sensitization caused by repeated MET intermittent administration. However, aripiprazole did not influence MET intake neither in the behaviourally MET sensitized nor in the non-sensitized rats.

Abbreviations:
MET – methamphetamine; IVSA – IV self-administration

Introduction

The atypical antipsychotic aripiprazole acts as a partial agonist on dopamine (D2 and D3) receptors. At the same time aripiprazole influences serotonin receptors (agonism at 5-HT1A and antagonism at 5-HT2A receptors). So far it has been approved for therapy of schizophrenia (so called 3rd generation antipsychotic) (Mailman & Murthy 2010) and manic episodes in bipolar disorder (De Fazio *et al* 2010). Moreover, it has also been studied and recommended for treatment of resistant depressions (Pae *et al* 2011; Pistovcakova & Sulcova 2008) and anxiety disorders (Pae *et al* 2008; Patkar *et al* 2006). As a partial D2/D3 agonist aripiprazole is believed to be able to normalize dopaminergic tone by acting similarly as antagonists during high

dopaminergic firing and as agonists in dopaminergic deficit during withdrawal (Rung *et al* 2008). Due to its mechanism of action aripiprazole seems to be a promising drug in addiction treatment (Backstrom *et al* 2011) as the partial agonism is considered to be useful in treatment of dependence. There is a number of other partial agonists e.g. selective partial nicotine receptor agonist varenicline in nicotine addiction (Vasic *et al* 2011; Walter & Wiesbeck 2009) or partial opioid agonist buprenorphine in substitution therapy of opioid addiction (Frei 2010).

Behavioural sensitization to drugs of abuse and the related adaptations in striatal neurotransmission are thought to play an important role in certain aspects of addiction such as tendency to relaps to drug use in abstaining individuals (Ohmori *et al* 2000). The aim of this study was to utilize the intravenous drug self-administration (IVSA) model, known as a reliable model for testing dependence potential and abuse liability of drugs (Collins *et al* 1984), for quantitative comparison of the methamphetamine (MET) intake of rat males subjected to a repeated drug pretreatment proven (Landa *et al* 2005) to induce behavioural sensitization and drug naïve animals. We expected a decrease of the total number of MET doses self-administered by rats per experimental session based on the increasing response to MET administered due to behavioural sensitization recorded earlier in our laboratory (Kucerova *et al* 2009).

Aripiprazole is able to attenuate locomotor activity induced by behavioural sensitization probably by the antagonistic action on 5-HT1A serotonin receptors (Futamura *et al* 2011). This pharmacological mechanism could be responsible for the decrease of MET intake induced by behavioural sensitization (Kucerova *et al* 2009). Therefore, the rats were treated repeatedly with aripiprazole before IVSA sessions to evaluate its possible impact on spontaneous MET intake.

The working hypotheses on results of aripiprazole effects in the experimental model used were the following: a) decrease of MET intake in non-sensitized rat group due to D2 partial agonism; b) drug induced suppression of behavioural sensitization in pre-treated (sensitized) animals by normalizing (increasing) MET intake.

Material and methods

Animals

Adult male albino Wistar rats weighting 180-220 g at the beginning of the study were purchased from Biotest Ltd. (Konarovice, Czech Republic). The animals were housed in groups of five in standardized rat plastic cages. After the catheter implantation surgery was performed, the rats were housed individually. Environmental conditions during the whole study were constant: relative humidity 50-60 %, temperature 23 °C ± 1 °C, inverted 12-hour light-dark cycle (5 a.m. to 5 p.m. darkness). Food and water were available *ad libitum*. All experiments were conducted in accordance with relevant laws and regulations of animal care and welfare. The experimental protocol was approved by the Animal Care Committee of the Masaryk University Faculty of Medicine, Czech Republic and carried out under the European Community guidelines for the use of experimental animals.

Drugs and treatments

Methamphetamine from Sigma Chemical, Co., St Louis, MO, USA was used for both initial drug pretreatment and the IVSA model. The administration of MET prior to IVSA was according to the following dosing regimen, which was successfully used in previous studies carried out at our laboratory (Kucerova *et al* 2006; Landa *et al* 2005, 2008) to induce behavioural sensitization: 0.5 mg/kg/day, intraperitoneally, for 14 days, administered in home cages. The identical volume and route of administration of saline solution (SAL) was used for all control treatments. MET dose available in the operant cage for IVSA was 0.08 mg per infusion with the maximum number of infusions obtainable in one session set to 50 which was a procedure producing reinforcing effects in the same model of IVSA in our laboratory (Vinklerova *et al* 2002).

Aripiprazole was used in a dissolved tablet form (ABILIFY 15 mg oral tablets, Bristol-Meyers Squibb S.r.L., Anagni, Italy) at the dose of 3 mg/kg/day administered orally by tube 45 minutes before the self-administration session.

I.V. self-administration surgery and procedures

Under the general anaesthesia with ketamine 50 mg/kg and xylazine 8 mg/kg given intraperitoneally (NARKAMON 5% and ROMETAR 2%, Bioveta a.s., Czech Republic, in combination with isoflurane inhalation for induction to anesthesia) a permanent intracardiac silastic catheter was implanted through the external jugular vein to the right atrium. The outer part of the catheter exited the skin in the midscapular area. A small nylon bolt was fixed on the skull with dental acrylic to stainless-steel screws embedded in the skull; this served as a tether to prevent the catheter from being pulled out while the rat was in the self-administration chamber. The catheters were flushed daily before all the sessions with 0.2 ml of heparinized cephalosporine (VULMIZOLIN 1.0 inj sicc, Biotika a.s., Slovak Republic) solution (0.05 mg/kg in saline with 2.5 I.U./kg) and 0.05 ml of heparin (HEPARIN LECIVA inj. sol. 1x10ml/50 I.U., Zentiva a.s., Czech Republic) solution (5 I.U.) to prevent infection and occlusion of the catheter. During this procedure the blood was aspired daily to assess the patency of the catheter, and changes in general behaviour, weight and other circumstances were recorded. When a catheter was found blocked the animal was excluded from the analysis.

IV self-administration protocol

Standard experimental cages with two nose-poke holes allocated on one side of the cage were programmed by software L2T2 (Coulbourn Instruments, USA) and the IVSA sessions were conducted under the fixed ratio (FR) schedule of reinforcement starting at FR1 (each correct response reinforced). Fixed-ratio requirements were raised (e.g. FR2 - two correct responses required, etc.); when the animal fulfilled the following conditions for three consecutive sessions:

a) at least 70% preference of the drug-active nose-poke;
b) minimum intake of 10 infusions per session;
c) stable intake of the drug (maximum 10 % deviation) in three consecutive sessions.

Active nose-pokes led to the activation of the infusion pump and administration of a single infusion followed by 30 sec time-out, while the other nose-pokes were recorded but not rewarded. The cage was illuminated by a house light which was twinkling when administering infusion and off in the time-out. The daily IVSA sessions lasted 90 minutes and took place regularly between 7 a.m. and 4 p.m. during the dark period of the inverted light cycle.

After reaching stable baseline intake each animal was subjected to water administration (control) orally by tube 45 minutes before the self-administration session and the possible effect on the MET intake was recorded. When the rat developed stable MET intake on oral water administration, it was replaced by aripiprazole solution (3 mg/kg/day) administered by the same manner as water before.

Experimental groups

There were 14 rats at the beginning of the experiment. However, due to complicated surgical procedures 1 of the subjects was lost.

The final groups as introduced to the first IVSA session follow:

d. SAL group (n=7): 14 days of saline (SAL) intraperitoneal pretreatment, the experiment was completed by 7 animals
e. MET group (n=7): 14 days of MET (0.5 mg/kg/day) intraperitoneal pretreatment, the experiment was completed by 6 animals

Statistical Data analysis

The means of MET intake were compared when the animals reached a stable intake of MET at FR2 and FR3 protocol. For statistical analysis of differences in MET IVSA the nonparametric ANOVA - Kruskal-Wallis test with Dunn's Multiple Comparisons post test - was used. Level of statistical significance was determined to $p<0.05$.

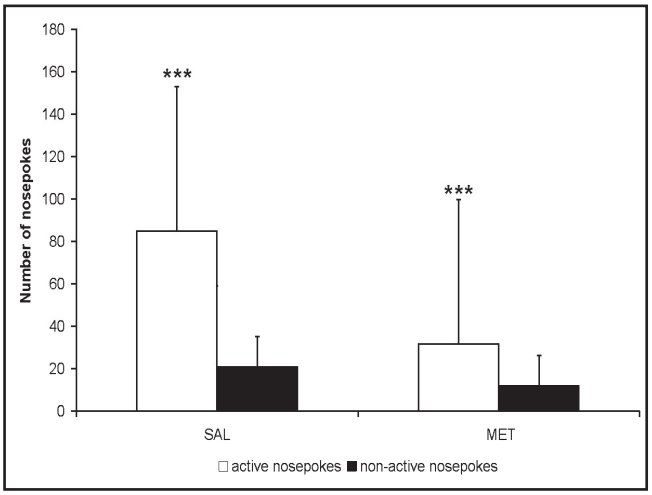

Figure 1: Graph displays mean (± SEM) numbers of nosepokes during baseline intake. Both, methamphetamine (MET) and saline solution (SAL) pretreated rats performed significantly higher number of the nosepokes associated with methamphetamine ($p<0.001$).

Results

The ***Figure 1*** shows that both groups of rats, pretreated with saline or MET, demonstrated significant ($p<0.001$) prevalence of active nosepokes, i.e. those associated with MET reward.

Spontaneous intake of MET defined as a mean number of infusions significantly differed between groups. Chronic intermittent MET pretreatment led to decrease of MET intake in the IVSA model as expected due to behavioural sensitization (***Figure 2***). This effect was statistically significant in comparison to non-treated baseline intake ($p<0.001$) as well as in comparison to intake during oral water pretreatment administration ($p<0.05$).

Figure 3 exhibits the effect of aripiprazole oral administration on the MET intake in IVSA. The significant difference between experimental groups induced by behavioural sensitization was abolished by aripiprazole administration. There was no significant effect of oral pretreatment with water/aripiprazole on MET intake in neither previously drug naïve group (SAL) nor behaviourally sensitized group (MET).

Discussion

According to results of our earlier studies in the same paradigm (Kucerova *et al* 2009) in the present study the significantly higher responding to nosepokes associated with drug administration in all animals tested confirmed the rewarding effect of methamphetamine as expected.

This study correlates with our previous findings that Wistar male rats repeatedly pre-treated with MET ("MET" group) self-administer significantly lower

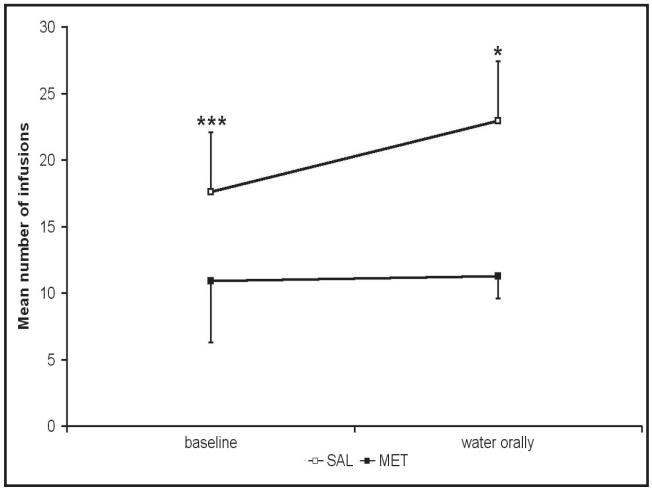

Figure 2: Graph displays mean (± SEM) numbers of MET infusions self-administered by the animals after reaching stable baseline intake and influence of water pretreatment 45 minutes before session orally. There is a statistically significant difference between the sensitized (MET group) and non-sensitized (SAL group) experimental groups in the baseline MET intake ($p<0.001$) as well as in stable intake with control oral water administration before the session ($p<0.05$). However, there was not recorded any significant alteration of MET intake induced by oral administration procedure (water).

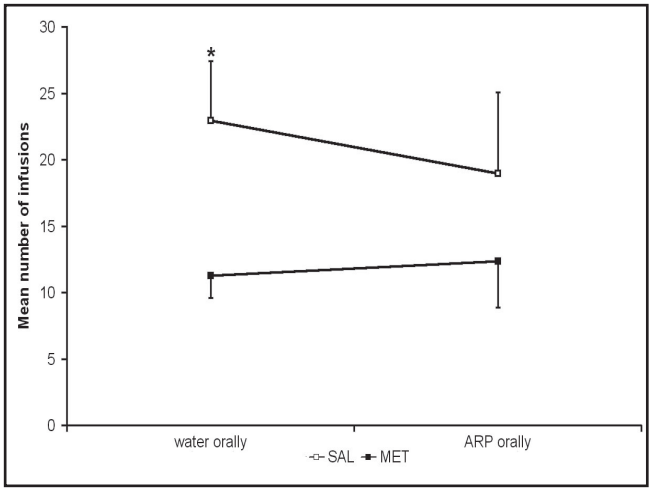

Figure 3: Graph displays influence of aripiprazole pretreatment on the mean (± SEM) numbers of MET infusions self-administered by the animals when stable intake was reached. There is apparent significant difference between the experimental groups at conditions of oral water administration ($p<0.05$). When aripiprazole (3 mg/kg/day) was administered 45 minutes before the session orally, there was neither statistically significant difference between the experimental groups anymore nor significant alteration of MET intake in each group.

number of MET infusions under a fixed ratio schedule compared to animals pretreated with saline ("SAL" group) (Kucerova *et al* 2009). Higher MET rewarding effects decreased drug-seeking behavioural signs in previously sensitized animals what can be considered as behavioural sensitization in this model. At the dose which did not influence rat locomotor activities in the open field test (Pistovcakova & Sulcova 2008) aripiprazole did not elicit any influence on the MET intake in drug naïve nor in sensitized rats. The observed significant difference in drug intake between experimental groups induced by behavioural sensitization was abolished by aripiprazole treatment. However, this cannot be interpreted as a suppression of behavioural sensitization, because MET intake was not significantly increased in sensitized animals by aripiprazole administration.

Clinically, aripiprazole was examined for possible attenuation of drug intake in MET addict volunteers, where it seemed to increase rewarding effects of MET. However, the main limitation of this study was a small sample size (8 in each arm) and authors themselves recognize a low probability of aripiprazole potential to increase MET rewarding effects (Newton *et al* 2008). Clinical studies with alcohol addicts show inconsistent results but in some cases they demonstrate ability of aripiprazole to reduce chronic alcohol consumption in co-administration (Vergne & Anton 2010).

In the preclinical study by Mavrikaki *et al* 2010 a completely opposite result was shown as aripiprazole attenuated rewarding effects of amphetamine in the intracranial self-stimulation model and hyperlocomotion paradigm induced by amphetamine. This result was recorded after acute drug administration as well as after chronic intermittent treatment leading to behavioural sensitization to amphetamine which indicates the possible intensified firing in the dopaminergic reward pathway (Mavrikaki *et al* 2010). However, in IV self-administration paradigm 3 mg/kg dose aripiprazole (equal dose as in our experiment) was recorded to decrease intake of d-amphetamine. This suggests an attenuation of reinforcing effects of d-amphetamine in Wistar rats (identical strain as in our experiment). The opposite effect was recorded in the same paradigm when aripiprazole increased d-amphetamine intake at the dose of 1 mg/kg (Backstrom *et al* 2011). In preclinical experimental paradigm of relapse to drug of abuse intake, aripiprazole exhibited capacity to reduce cocaine relapse rate in two different rat strains (Feltenstein *et al* 2007; Roman & Gyertyan 2009).

Present experiments did not confirm the efficacy of aripiprazole in management of MET intake in the rat model of IVSA of methamphetamine. Nevertheless, the literature available refers a certain potential of aripiprazole in treatment of psychostimulant addiction such as amphetamine, MET or cocaine dependency. Summarizing the currently available data on aripiprazole, we can speculate that aripiprazole might be useful in reduction of the relapse rate in addicted individuals, rather than influencing the overall MET intake. Efficacy of aripiprazole seems to be largely influenced by the choice of appropriate (probably rather high) dose and the treatment paradigm itself. Thus, despite of the contradictory data obtained, it is too early to discard

aripiprazole from suggested indication. Further studies are needed to evaluate its benefit in treating drug addiction.

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Conflict of interest statement

The authors have no financial interest in this manuscript and no affiliations (relationships) to disclose.

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