MUNI
FACULTY
OF MEDICINE

THE EFFECT OF SELECTED PSYCHOTROPIC SUBSTANCES ON BEHAVIOURAL SENSITIZATION TO METHAMPHETAMINE IN ANIMAL MODELS

Habilitation thesis

Annotated publications

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Originality and conflict of interest declaration

The presented experimental data comes from original research that was carried out by the author himself and in collaboration with other colleagues.

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CONTENTS

Annotation

The presented habilitation thesis consists of 9 annotated original articles and appendices containing 2 original and 3 review papers. All these articles were published in journals with an impact factor. The annotated part of the thesis is thematically very consistent and deals with my main research interest, behavioural sensitization to the psychostimulant drug methampetamine and the possible effects of other psychotropic drugs on this phenomenon. These substances include in particular cannabinoid receptor ligands with different intrinsic activity (cannabinoids), as well as the antagonists of NMDA receptor felbamate and memantine, and finally the second-generation antipsychotic (neuroleptic) agent sertindole. The experiments were performed using the mouse open field test, the mouse model of agonistic behaviour, and, in collaboration with the Department of Pathological Physiology, a real-time polymerase chain reaction (real-time PCR).

Behavioural sensitization is believed to play an important role in the processes of drug dependency and it has been suggested that it may contribute to relapse incidence in ex-addicts. The main features of sensitization, including its neurobiological bases, are described in the introduction and subsequent part of the thesis.

These sections are followed by a brief discussion of the characteristics of the substances, methods used in the particular experiments (open field test, mouse model of agonistic behaviour, and real-time polymerase chain reaction) and the most important resuts. The next, fundamental part of the thesis consists of nine experimental papers with short annotations explaining the main aims and results of the individual articles.

The appendices involve two original papers also dealing with dependency-producing substances or behavioural sensitization, however the topics are slightly different from the very consistent thematic scope of the annotated part. The three concluding papers are a review concerning behavioural sensitization with respect to the glutamatergic system and two review papers dealing with the use of cannabinoids/medical cannabis in veterinary and human medicine.

List of abbreviations

CB_1 receptor = cannabinoid receptor subtype 1

cDNA = complementary DNA

CNS = central nervous system

D_1 receptor = dopamine receptor subtype 1

D_2 receptor = dopamine receptor, subtype 2

DAT = dopamine transporter

DNA = deoxyribonucleic acid

GABA = gamma-aminobutyric acid

I.V. = intravenous

5-HT_{2A}, 5-HT_{2C} receptors = subtypes of serotonin receptors

MDMA = 3,4-methylenedioxymethamphetamine

mRNA = messenger RNA

NMDA receptor = N-methyl-D-aspartate receptor

NAc = nucleus accumbens

PCR = polymerase chain reaction

qPCR = quantitative polymerase chain reaction

RNA = ribonucleic acid

VTA = ventral tegmental area

Behavioural elements in the mouse model of agonistic behaviour

Al = alert, At = attack, Cl = climbing over the partner, De = defensive posture, Es = escape, Fo = following the partner, Re = rearing, Ss = sociable sniffing, Tr = tail rattling, Ur = aggressive unrest, Wa = walking

1. Introduction

Behavioural sensitization is a relatively new concept that was consistently described in the last decade of the twentieth century (Robinson and Berridge 1993) and soon become an important target of researchers dealing with the study of dependence-producing substances. The most typical feature of behavioural sensitization is a progressive increase in locomotor activity following repeated administration of several drugs of abuse (Nona and Nobrega 2018).

From the point of view of experimental pharmacology, the ability to elicit sensitization is a very important property of drugs with an addictive potential, enabling the observation of some of their characteristic features that were hidden in the classical "tolerance-dependence" model.

The present habilitation thesis is focused on the most important signs of behavioural sensitization to the psychostimulant drug methamphetamine following its repeated administration, and also following pretreatment with various cannabinoid receptor ligands in the open field test and in the mouse model of agonistic behaviour. Furthermore, by using the open field test it compares the phenomenon of sensitization in the two most frequently used species of laboratory animals (mice and rats). The thesis also describes changes that occurred after the development of sensitization following repeated administration of methamphetamine and the cannabinoid CB_1 receptor agonist methanandamide, specifically at the level of cannabinoid CB_1 and dopamine $D_{1,2}$ receptors studied by means of a real-time polymerase chain reaction (PCR). Finally, it investigates how sensitization to methamphetamine can be influenced by the administration of selected psychotropic substances (felbamate, memantine and sertindole).

2. Main features of behavioural sensitization

Although the first reference describing an increase in locomotor activity after intermittent repeated administration of cocaine (i.e., the main symptom of sensitization) dates back to the 1930s (Downs and Eddy 1932), and other reports suggesting heightened locomotor responses following repeated exposure to psychostimulant drugs followed later on (e.g. Segal and Mandel 1974; Post and Rose 1976), the phenomenon of behavioural sensitization was for the first time comprehensively described only in the last decade of the twentieth century by

Robinson and Berridge (1993). It has been shown that sensitization can be elicited by repeated administration of many dependence-producing substances, and is typically manifested by an increased response to the effects of these substances (Robinson and Berridge 1993; Ohmori et al. 2000; Nona and Nobrega 2018). Progressively increased locomotion is a result of neural changes that were caused by repeated drug administration and reflects a hypersensitive reward system (Nona and Nobrega 2018).

Behavioural sensitization is also sometimes called "reverse tolerance" (Demontis et al. 2015) in contrast to "classical" tolerance, which is a phenomenon characterised by the decreasing response of an organism following repeated drug administration. It has been shown that tolerance typically develops following continuous administration of a drug, whereas sensitization occurs after intermittent application (King et al. 1998). Behavioural sensitization is usually manifested after both repeated doses and an application of a dose administered after a certain period of withdrawal (wash-out period). Interestingly, there are also reports for some substances (amphetamine, cocaine and morphine) that sensitization in rats was conditioned by pre-treatment with just a single dose (Kalivas and Alesdatter 1993; Vanderschuren et al. 1999; Vanderschuren et al. 2001).

Sensitization has been observed after repeated administration of both legal and illegal drugs, and is well-documented for ethanol (Broadbent 2013; Kim and Souza-Formigoni 2013; Linsenbardt and Boehm 2013; Xu and Kang 2017), nicotine (Hamilton et al. 2012; Lenoir et al. 2013; Perna and Brown 2013; Thompson et al. 2018), caffeine (Zancheta et al. 2012; Kumar et al. 2018), cannabinoids (Rubino et al. 2003; Cadoni et al. 2008), psychostimulants (Landa et al. 2006a, b; 2011; 2012a, b; Wang et al. 2010; Ball et al. 2011; Kameda et al. 2011; Kang et al. 2017), and opioids (Bailey et al. 2010; Liang et al. 2010; Farahmandfar et al. 2011; Hofford et al. 2012; Rezayof et al. 2013; Perreau-Lenz et al. 2017).

An increased response to a certain drug can also be elicited by previous repeated administration of a different drug: this phenomenon is called cross-sensitization. Cross-sensitization has been recorded, for example, after repeated pre-treatment with tetrahydrocannabinol to heroin (Singh et al. 2005), between methylphenidate and amphetamine (Yang et al. 2011), after pretreatment with the

cannabinoid agonist WIN 55,2122 to morphine (Manzanedo et al. 2004), between nicotine and amphetamine (Santos et al. 2009), between ethanol and cocaine (Xu and Kang 2017), and between cannabinoids and cocaine (Melas et al. 2018). Development of sensitization to methamphetamine effects in mice has been induced by pre-treatment with the cannabinoid CB_1 receptor agonist methanandamide (an analogue to the endogenous cannabinoid anandamide), and suppressed by the cannabinoid CB_1 receptor antagonist AM 251(Landa et al. 2006a, b).

It has been described that processes involved in sensitization/cross-sensitization can play a key role in certain aspects of drug addiction, such as compulsive drug-seeking behaviour (De Vries et al. 1998; De Vries et al. 2002), and are related to the phenomenon referred to as "craving" (a psychological urge to use a drug again) (Robinson and Berridge 2001), and thus sensitization probably represents one of the main causes for relapses in ex-addicts. Sensitization has already for a quarter of a century been considered a useful model for determining the neural basis of addiction, and its original principles still seem well supported (Steketee and Kalivas 2011; Berridge and Robinson 2016).

2.1 Behavioural sensitization in animals and human beings

The most typical features of behavioural sensitization involve the stimulatory effects of drugs, and in laboratory rodents an increase in locomotor/exploratory activities is considered the most common symptom. Besides this, sensitization can manifest as various other types of behaviour, such as stereotypic sniffing, head movements or rearing (Laviola et al. 1999), as well as defensive-escape activities (Votava and Krsiak 2003). However there are also reports on sensitization to inhibitory drug actions such as catalepsy (Schmidt et al. 1999; Lanis and Schmidt 2001) or an antiaggressive effect during social conflict in mice after repeated administration of methamphetamine (Landa et al. 2006b).

Although it is difficult to demonstrate behavioural sensitization in human subjects, and most research has focused on the characterization of sensitization to behavioural effects in laboratory rodents, some studies carried out on healthy human beings, as well as drug users, have suggested that behavioural sensitization also occurrs in humans (Strakowski et al. 1996; Bartlett et al. 1997). Strakowski et al. (1996) for example described behavioural sensitization after repeated

administration of amphetamine in human volunteers that was manifested by increased activity and energy and elevated mood, rate of speech, and eye-blink rates.

Furthermore, there are reports showing enhanced responses to drugs of abuse after chronic consumption. Progression of responses was reported after repeated administration of D-amphetamine in healthy human volunteers, who reported higher subjective ratings of vigour and euphoria with a greater impact in women (Strakowski and Sax 1998; Steketee and Kalivas 2011). In addition, an open-label clinical study with a 1-year follow-up of repeated amphetamine administration in healthy volunteers confirmed behavioural sensitization to psychomotor and alertness responses, accompanied by an increase in dopamine release as measured by the [11C] raclopride PET method (Boileauetal., 2006).

2.2 Neural bases of behavioural sensitization

The process of behavioural sensitization is very complex and results from drug-induced neuroadaptive changes in a neural circuit consisting namely of dopaminergic, glutamatergic, and GABAergic interconnections between the ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex, and amygdala (Tzschentke 2001; Kalivas 2004; Steketee and Kalivas 2011; Miyazaki et al. 2013; Scofield et al. 2016). The early research concerning sensitization carried out in the lab of T.E. Robinson focused particularly on dopamine neurons and increases in the release of dopamine. It is however presently clear that mesolimbic sensitization alters also other neurotransmitters and neurons (Berridge and Robinson 2016).

Furthermore, behavioural sensitization can be separated into two temporally and anatomically defined domains, called development (or initiation) and expression. Development is associated particularly with VTA, whereas expression mainly with NAc (Kalivas et al., 1993). Development of behavioural sensitization to psychostimulant drugs occurs in the ventral tegmental area and substantia nigra, which are the loci of the dopamine cells in the ventral midbrain that give rise to the mesocorticolimbic and nigrostriatal pathways.

In contrast, the neuronal events associated with expression are distributed among several interconnected limbic nuclei centred on the nucleus accumbens (Pierce and Kalivas 1997). “Development” or “initiation” involves increasing

changes at the molecular and cellular levels that lead to altered processing of environmental and pharmacological stimuli by the CNS; these changes are, however, only temporal and are not detected after longer abstinence. The term "expression" refers to persistent neural changes originating from the process of the sensitization development; i.e., expression is the long-term consequence (Pierce and Kalivas, 1997).

Originally, substantial experimental data supporting the theory of mesolimbic sensitization by drugs was obtained from animal studies; however today sensitization is well documented also in humans (Boileau et al. 2006; Vezina and Leyton 2009).

3. Aims of the studies

Previous research completed at the Department of Pharmacology suggested an interaction between the endocannabinoid system and methamphetamine brain mechanisms in the rat I.V. drug self-administration model (Vinklerova et al. 2002). According to the most recent paper, the endocannabinoid system consists of cannabinoid receptors, endocannabinoids, their synthesizing and degrading enzymes, intracellular signalling pathways and transport systems, and has been found to play a key role in the neurobiological substrate underlying drug addiction (Manzanares et al. 2018), which was however largely discussed already at the time of our research.

Therefore, the first aim of the studies was to investigate whether this interaction can also be found in the model of behavioural sensitization/cross-sensitization. The second aim was to investigate whether cannabinoid receptor agonists may facilitate the effects of other abused drugs and, on the other hand, whether cannabinoid receptor antagonists could block this phenomenon. The third aim was to compare the impact of cannabinoid receptor ligands on sensitization to methamphetamine effects between two species of laboratory animals (mice and rats). For a detailed description, see sections 7.1, 7.2, 7.3.

Since the promising results we obtained in previous behavioural studies, we decided to extend our studies to possible neuroplastic changes at the genomic level. The fourth aim of our studies was to determine whether there are changes in

the relative expression of the cannabinoid CB_1 receptor and the dopamine D_1 and D_2 receptor mRNA in the mesencephalon of mice sensitized by repeated treatments to methamphetamine stimulatory effects and cross-sensitized to methamphetamine by the cannabinoid CB_1 receptor agonist methanandamide pre-treatment. For a detailed description, see sections 7.4 and 7.5.

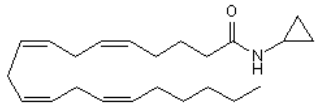
As neurobiological changes underlying behavioural sensitization also concern the glutamatergic system, further studies were oriented towards effects involving NMDA receptor ligands. Thus, the fifth aim of our research was to investigate, whether the NMDA receptor antagonists felbamate and memantine would influence behavioural sensitization to methamphetamine effects. For detailed description, see sections 7.6 and 7.7.

Dopaminergic transmission plays a substantial role in the process of behavioural sensitization. We therefore also decided to select a drug affecting this system to see how it would interfere with sensitizing processes. The sixth aim was to explore the possible effects of sertindole (an antagonist of dopamine D_2 receptors) on the development of behavioural sensitization to methamphetamine. For a detailed description, see section 7.8.

At the end of this experimental set we returned to cannabinoid receptor ligands and investigated the effects of another cannabinoid receptor agonist on behavioural sensitization to methamphetamine. The last (seventh) aim of the study was to determine whether the cannabinoid CB_1 receptor agonist ACPA (a substance with similar properties similar to the cannabinoid CB_1 receptor agonist methanandamide used in the first three experiments) would affect behavioural sensitization to methamphetamine. For a detailed description, see section 7.9.

4. Brief characteristics of the substances used

4.1 ACPA

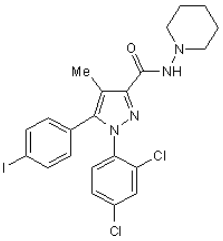


IUPAC name: (5Z,8Z,11Z,14Z)-N-cyclopropylicosa-5,8,11,14-tetraenamide

Tocris Catalogue (https://www.tocris.com/products/acpa_1318]

ACPA (arachidonylcyclopropylamide) is a selective and potent cannabinoid CB_1 receptor agonist (Kumar et al. 2016).

4.2 AM 251

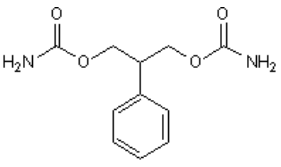


IUPAC name: 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-piperidin-1-ylpyrazole-3-carboxamide

Tocris Catalogue (https://www.tocris.com/products/am-251_1117]

AM 251 is a potent antagonist/inverse agonist of cannabinoid CB_1 receptors and moreover it acts as a GPR55 receptor agonist (Carpi et al. 2015; Kapur et al. 2009).

4.3 Felbamate



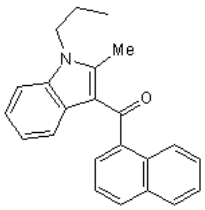
IUPAC name: 3-carbamoyloxy-2-phenylpropyl) carbamate

Tocris Catalogue (https://www.tocris.com/products/felbamate_0869)

Felbamate is an activating antiepileptic drug of the newer second generation (Vohora et al. 2010). It is characterised as an NMDA receptor antagonist

(Germano et al. 2007) that blocks NMDA receptor-mediated currents (Kuo et al. 2004). Generally, antiepileptic drugs from this generation invoke psychotropic effects. They may exert attention-enhancing and antidepressant effects, and cause anxiety, insomnia, and agitation (Nadkarni and Devinsky 2005).

4.4 JWH 015

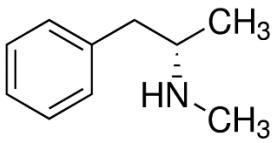


IUPAC name: (2-methyl-1-propylindol-3-yl)-naphthalen-1-ylmethanone

Tocris Catalogue (https://www.tocris.com/products/jwh-015_1341)

JWH 015 is a synthetic cannabinoid CB_2 receptor selective agonist (Lombard et al. 2007).

4.5 Methamphetamine (Pervitin)

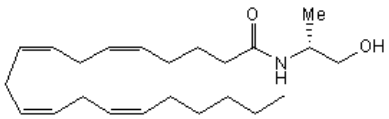


IUPAC name: (2S)-N-methyl-1-phenylpropan-2-amine

Sigma Aldrich (Merck) Catalogue
(https://www.sigmaaldrich.com/catalog/substance/methamphetaminehydrochloride185695157011?lang=en&region=CZ)

Methamphetamine (synonym Pervitin) is closely related chemically to the psychostimulant substance amphetamine, however its stimulatory effects in the CNS are stronger. The main mechanism of action is based on an increase in dopamine release. Methamphetamine binds to the dopamine transporter (DAT), thereby blocking reuptake of dopamine, and furthermore causes dopamine release through the reversal of DAT transport followed by dopamine efflux into the synapse (Sulzer et al. 2005). It was reported that methamphetamine also increased levels of other neurotransmitters, particularly of norepinephrine and serotonin (Rothman and Baumann 2003).

4.6 Methanandamide

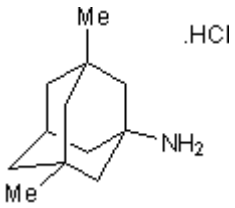


IUPAC name: (5Z,8Z,11Z,14Z)-N-[(2R)-1-hydroxypropan-2-yl]icosa-5,8,11,14-tetraenamide

Tocris Catalogue (https://www.tocris.com/products/r-methanandamide_1121)

Methanandamide is a synthetic analogue of the endogenous cannabinoid anandamide, which is a substance that was isolated from a pig's brain in 1992 as the first natural ligand binding to cannabinoid CB receptors (Devane et al. 1992). Methanandamide is a potent and selective agonist of cannabinoid CB_1 receptors (Abadji et al. 1994). It is also capable of agonistic activity at vanilloid receptors (Malinowska et al. 2001).

4.7 Memantine

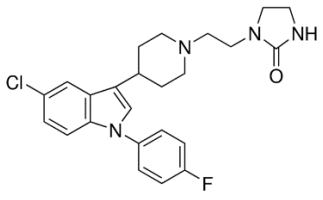


IUPAC name: 3,5-dimethyladamantan-1-amine

Tocris Catalogue (https://www.tocris.com/products/memantine-hydrochloride_0773)

Memantine was found to inhibit N-methyl-D-aspartate (NMDA) glutamate receptors (Johnson and Kotermansi 2006), and it widely used as a medication for Alzheimer's disease (Cummings et al. 2006).

4.8 Sertindole



IUPAC name: 1-[2-[4-[5-chloro-1-(4-fluorophenyl)indol-3-yl]piperidin-1-yl]ethyl]imidazolidin-2-one

Sigma Aldrich (Merck) Catalogue
https://www.sigmaaldrich.com/catalog/product/sigma/s8072?lang=en&region=CZ

Sertindole is a second-generation antipsychotic (neuroleptic) agent, intended for the treatment of schizophrenia (Spina and Zoccali 2008). It acts as an antagonist of dopamine D_2, serotonin 5-HT_{2A}, 5-HT_{2C}, and α_1-adrenergic receptors (Muscatello et al. 2010).

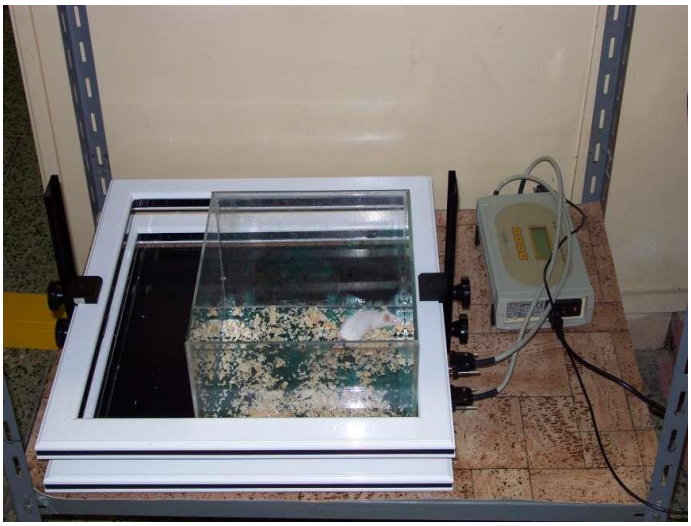
5. Methods used in the studies

5.1 Open field test

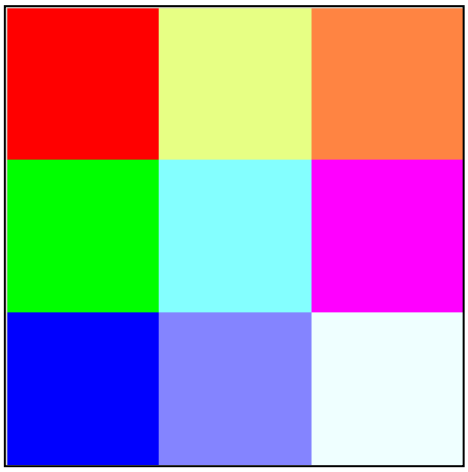
The open field test is a well-known and widely used method of behavioural pharmacology, particularly suitable for mice and rats. Animal behaviour can be observed in real time directly by an experimenter in the open field test and the individual patterns of locomotor/exploratory activity are recorded in an ethogram. In our experiments, locomotor/exploratory activities in the open field test were monitored using an Actitrack apparatus (Panlab, S. L., Spain; see Picture 1).

This instrument consists of two square-shaped frames that deliver beams of infrared rays into the space inside the square. A plastic box is located in this square and serves as an open-field arena (base 30 x 30 cm, height 20 cm for mice and base 45 x 45 cm, height 25 cm for rats), in which the animal can move freely. The apparatu's software can record and evaluate both horizontal and vertical behavioural activities of the animal by registering the beam interruptions caused by movements of its body. It is possible with this apparatus to record various behavioural parameters (e.g. distance run = distance in cm during the 3 minute session, fast movements = time in seconds when the animal moves faster than 5 cm/s, resting time = time in seconds spent without ambulation or rearing). In order to detect possible stereotypic ambulation only in a specific part of the open

field, the bottom of the plastic box was divided into 9 equal squares, a separate evaluation of each of which was available (see Picture 2).



Picture 1: Actitrack Device (Panlab, S. L., Spain)



Picture 2: Open field divided into 9 squares

5.2 Model of agonistic behaviour

The model of agonistic behaviour used in this thesis was based on intraspecies social conflict in adult male mice (Krsiak 1975; Donat 1992). It consists in the observation of the behaviour of individually-housed mice in dyadic interactions with group-housed partners in the neutral environment of a plastic box (base 30 x 20 cm, height 20 cm).

Whereas the group-housed partner does not show aggressiveness, individually-housed mice can, according to their behaviour in control interactions (vehicle treatment), be divided into three groups: a) aggressive mice (displaying at least one attack towards opponents in control interactions); b) timid mice (showing a majority of defensive-escape behaviour and no attack); and c) sociable mice (animals without aggressive or defensive-escape behaviour, showing nevertheless a high frequency of approaches to the partner and sniffing or climbing over the partner - acts thought to be sociable).

Four-minute dyadic behavioural interactions of singly-housed mice with non-aggressive group-housed partners were video-taped and the analysis was processed using the computer-compatible system OBSERVER 3.1 (Noldus Information Technology b.v., Holland).

Behavioural elements in four categories were recorded: **sociable** - social sniffing [Ss], following the partner [Fo], climbing over the partner [Cl]; **timid** - defensive posture (upright) [De], escape [Es], alert posture [Al]; **aggressive** - attack [At], aggressive unrest (threat) [Ur], tail rattling [Tr]; **locomotor** - walking [Wa], rearing [Re]. Only aggressive singly-housed mice served as subjects in the present study.

5.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is intended to produce numerous copies of a defined DNA or RNA segment using two primers that are utilized during repeating cycles of primer extension and DNA synthesis by DNA-polymerase. DNA amplification runs in cycles, which are repeated, and consist of three steps. During the first step (denaturation) DNA is heated to temperatures of about 95° C, which leads to the breaking of hydrogen bonds between the strands of DNA, while double stranded DNA denatures to single stranded DNA. During the next phase, called annealing, DNA primers attach to the template DNA. The primer is a DNA chain, which represents the initial site of DNA replication. In this step, the temperature is reduced to about 50°-60° C so that the primers can hybridize to the template. Molecules of single stranded DNA renature again after cooling. The last step is called extension. The temperature is increased to about 72° C and the DNA polymerase starts adding nucleotides onto the ends of the annealed primers. Heat-stable DNA polymerase from the bacterium *Thermus aquaticus* is usually used to

produce a new DNA strand; it is therefore called Taq polymerase. The number of copies doubles following each cycle. The new fragments of DNA that are produced during PCR also serve as templates to which the DNA polymerase enzyme attaches and begins to synthetize, making DNA. Thus, the increase in the number of DNA molecules has an exponential pattern, which makes possible carrying out analyses of DNA using even very small amounts of sample material (Vejrazka 2006).

Quantitative polymerase chain reaction in the real time (real-time qPCR) is considered the most accurate method of the quantification of template DNA or RNA, and it was used in this work for an analysis of gene expression at the mRNA level. In the process of mRNA quantification (i. e., the assessment of gene expression), reverse transcription is carried out prior to the actual PCR, which means the transcription of mRNA to complementary DNA (cDNA), which is then amplified (mRNA is thus determined indirectly as cDNA). Quantification of amplicone (the product of amplification) in real-time qPCR is realised by means of the detection and quantification of a fluorescence signal in devices that, besides cyclic temperature changes, can also detect fluorescence (Smarda et al. 2005). There are some variants of this quantification reaction. One possibility is to use hydrolysis probes. Hydrolysis probes are dual-labelled oligonucleotides. One end of the oligonucleotide is labelled with a fluorescent reporter molecule (fluorophore), whereas the other is labelled with a quencher molecule. The fluorescent reporter and quencher are in close proximity, and the quencher absorbs (quenches) the fluorescence emission. If no product of amplification complementary to the probe is present, the probe is intact and low fluorescence is found. If a complementary amplicon occurs, the probe binds to it during each annealing phase of the PCR. The double-strand-specific 5'-3' exonuclease activity of the Taq polymerase displaces the 5' end of the probe and then decomposes it. This process results in the release of the fluorophore and quencher into the solution; they are spatially separated, and this leads to an increase in fluorescence from the reporter. Fluorescence is directly detected by a real-time PCR instrument during amplification. The intensity of fluorescence corresponds to the amount of synthetized PCR product following each cycle (Vejrazka 2006; Brookman-Amissah et al. 2011). The quantity of the DNA segment of interest (gene

expression) is usually expressed as a relative amount related to an internal standard, which is most frequently represented by so-called housekeeping genes.

The aforementioned method was carried out in our studies using the Sequence Detection System ABI 7000 (Life Technologies).

6. Results

In this section, the most important results are summarized. For a more detailed description, please see the individual commented papers.

- We confirmed in all experiments that repeated administration of methamphetamine can, under a certain dosage regimen, produce a robust behavioural sensitization to its stimulatory effects.
- Repeated pretreatment with the agonist of the cannabinoid CB_1 receptor methanandamide prior to the methamphetamine challenge dose elicited cross-sensitization to methamphetamine.
- On the other hand, repeated pretreatment with the cannabinoid CB_1 receptor antagonist/inverse agonist AM 251 supressed the phenomenon.
- It has been shown that repeated administration of methamphetamine can under certain circumstances elicit behavioural sensitization to its stimulatory effects not only in mice but also in rats. However unlike in mice, in rats we were not able to provoke cross-sensitization following repeated pretreatment with methanandamide, and similarly we did not demonstrate suppression of the cross-sensitisation after repeated application of AM 251.
- Real-time PCR suggested that stimulation of CB_1 receptor activity may increase the expression of CB_1 receptor mRNA in the mouse mesencephalon.
- Real-time PCR showed an increase in D_1 receptor mRNA expression after the first dose of methamphetamine (persisting also after the last dose of methamphetamine) and after the first dose of methanandamide (which also persisted after the methamphetamine challenge dose).
- Real-time PCR moreover revealed a significant decrease in D_2 receptor mRNA expression both after the first dose of methamphetamine and

methanandamide (persisting also after the methamphetamine challenge doses).

- Combined pre-treatment with methamphetamine and the NMDA receptor antagonist felbamate facilitated the development of sensitization to methamphetamine stimulatory effects. On the other hand, repeated pretreatment with methamphetamine and another NMDA receptor agonist, memantine, did not elicited sensitization following the methamphetamine challenge dose.
- Mice pre-treated with methamphetamine and the dopamine D_2 receptor antagonist sertindole showed an increased response in locomotor activity following the methamphetamine challenge dose, however this increase did not fulfil the criteria to be recognised as behavioural sensitization.
- Repeated pretreatment with the selective cannabinoid CB_1 receptor agonist ACPA produced increased locomotion in mice after the methamphetamine challenge dose, nevertheless the cross-sensitisation was not fully confirmed because there was no significant difference between the stimulatory effects of a single methamphetamine dose administered after the vehicle and a methamphetamine challenge dose after repeated ACPA pretreatment.

Taken together, the most important findings of our studies speak in favour of the belief, that a cannabinoid CB_1 receptor agonist could under certain conditions facilitate consumption of other drugs of abuse and act as so-called gateway drugs.

On the other hand, the effects of cannabinoid CB_1 receptor antagonist/inverse agonist suggested the use of these substances as possibly promising treatment approaches including for patients suffering from drug dependence. Our results from the earlier papers were confirmed by later findings and substances targeting the endocannabinoid system remain in centre of interest for potential use in the treatment of opioid, psychostimulant, nicotine and alcohol addiction (Gamaleddin et al. 2015; Wang et al. 2015; Sloan et al. 2017; Su and Zhao 2017; Di Marzo 2018; Gonzalez-Cuevas et al. 2018; Manzanares et al. 2018).

7. Annotated publications

7.1 Involvement of cannabinoid CB_1 and CB_2 receptor activity in the development of behavioural sensitization to methamphetamine effects in mice

Earlier studies realised at the Department of Pharmacology suggested an interaction between the endocannabinoid system and methamphetamine brain mechanisms (Vinklerova et al. 2002). Thus, the presented experiment was aimed at the influence of pre-treatments with methanandamide (a cannabinoid CB_1 receptor agonist), JWH 015 (a cannabinoid CB_2 receptor agonist), and AM 251 (a cannabinoid CB_1 receptor antagonist/inverse agonist) on behavioural sensitization to methamphetamine effects in the mouse open field test in order to explore further possible connections.

The results of this study confirmed the fact that repeated administration of the psychostimulant drug methamphetamine produces behavioural sensitization to its stimulatory effects, which was in accordance with our earlier research (Landa et al. 2003).

The main finding was that, repeated pre-treatment with the cannabinoid CB_1 receptor agonist methanandamide prior to the methamphetamine challenge dose produced an increase in locomotor response to methaphetamine, too. In other words, stimulation of the cannabinoid CB_1 receptor by its agonist methanandamide elicited cross-sensitization to methamphetamine and on the contrary, blocking the CB_1 receptor with the antagonist/inverse agonist AM 251 suppressed this phenomenon in the same animal model. Stimulation of the cannabinoid CB_2 receptor by agonist JWH 015 did not produce cross-sensitization to methamphetamine in this study.

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Involvement of cannabinoid CB_1 and CB_2 receptor activity in the development of behavioural sensitization to methamphetamine effects in mice

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Abstract

OBJECTIVES: An increased behavioural response ("behavioural sensitization") to drugs of abuse occurs after repeated treatment. In the present study the possibility of cross-sensitization existence between various cannabinoid receptor ligands – CB_1 agonist methanandamide, CB_2 agonist JWH 015, and CB_1 antagonist AM 251 with methamphetamine was explored.

METHODS: Locomotion in the open field was measured in naive mice and in those pre-treated acutely and repeatedly (for 8 days), respectively, with either vehicle or tested drugs.

RESULTS: Methamphetamine produced significant sensitization to its stimulatory effect on locomotion. Methanandamide pre-treatment elicited cross-sensitization to methamphetamine effect, whereas pre-treatment with JWH 015 did not. Combined pre-treatment with methamphetamine+AM 251 suppressed sensitization to methamphetamine.

CONCLUSIONS: These results suggest that the activity of the endocannabinoid system is involved in the neuronal circuitry underlying the development of sensitization to methamphetamine.

Abbreviations:

AM 251 = CB_1 receptor antagonist,
AM+M = mice after the 1st dose of the combination of AM 251+methamphetamine (5.0 mg/kg+2.5 mg/kg),
AM/M = mice sensitized with AM 251+methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg),
CAN = mice after the 1st dose of methanandamide (0.5 mg/kg),
CAN/M = mice sensitized with methanandamide after the challenge dose of methamphetamine (2.5 mg/kg),
JWH 015 = CB_2 receptor agonist,
JWH = mice after the 1st dose of JWH 015 (5.0 mg/kg),
JWH/M = mice sensitized with JWH 015 after the challenge dose of methamphetamine (2.5 mg/kg),
M = mice after the 1st dose of methamphetamine (2.5 mg/kg),
M/M = mice sensitized with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg),
N = naive mice,
V = mice after the 1st dose of vehicle,
V/M = mice sensitized with vehicle after the challenge dose of methamphetamine (2.5 mg/kg)

ORIGINAL ARTICLE

Introduction

The repeated administration of various drugs of abuse – amphetamines [1], cocaine [2], opioids [3], cannabinoids [4] – may result in an increased behavioural response to these substances; in rodents, mainly to stimulated locomotion and the occurrence of various types of stereotypic behaviour such as sniffing, head movements and rearing [5]. This phenomenon, termed behavioural sensitization, is well known [6, 7] and occurs in both laboratory animals and man [8].

It is also known that an increased response to a drug may be elicited by previous repeated administration of another drug, a phenomenon known as cross-sensitization. In the case of cannabinoids this has already been observed, for example, after repeated treatment with tetrahydrocannabinol, which produces sensitization to opioids, morphine [4] and heroin [9]. The pharmacological mechanisms are not fully elucidated yet; however, available data show that these functional alterations might be underlined by neuroplasticity in brain regions and neuronal cell types commonly involved in the action of drugs of abuse. Behavioural sensitization is the consequence of drug-induced neuroadaptive changes in a circuit involving dopaminergic and glutamatergic interconnections between the ventral tegmental area, nucleus accumbens, prefrontal cortex and amygdala [10, 11, 12]. In regards to endocannabinoid activities, it was reported that these underlie a morphological remodelling of neuronal cells and synaptic actions e.g. of amphetamine in the brain [13, 14].

Both behavioural sensitization and cross-sensitization are considered to reinstate drug-seeking behaviour and thus both phenomena could contribute to the relapse of drug behaviour [15], therefore it is worthwhile to elucidate their neurobiology.

In laboratory rodents, effects of drugs on locomotor activities are measured as the most common symptom of behavioural sensitization. Data have shown that the most frequently observed feature of sensitization is the stimulatory effect of drugs. However, there are also reports on sensitization to inhibitory drug actions such as catalepsy [16] or antiaggressive effect during social conflict in mice following the repeated administration of methamphetamine [17].

Following the results obtained in our previous study, showing an interaction between the endocannabinoid system and methamphetamine brain mechanisms in the rat I.V. drug self-administration model [18], the present study investigated whether repeated pre-treatment with cannabinoid receptor ligands in mice would affect their response to acute methamphetamine challenge dose in the open-field test. The attention was paid not only to drugs acting at CB_1 receptor but also at CB_2 receptor which localization in the brain was confirmed too, at least in granule and Purkinje mouse cells [19], rat microglial cells [20], and recently also in the human brain [21]. Glial cells may serve as components of the cannabinoid signalling system for communication with neighbouring neuronal cells [22]. In our previous experiments CB_2 receptor agonist JWH 015 produced significant antiaggressive effect in mouse model of agonistic behaviour [23]. Therefore, in the present study, ligands with different intrinsic activities at both CB_1 and CB_2 cannabinoid receptors were selected: methanandamide (CB_1 receptor agonist), AM 251 (CB_1 receptor antagonist) and JWH 015 (CB_2 receptor agonist). Thus, the working hypothesis of the present study was to verify a development of sensitization to the stimulatory effects of methamphetamine on mouse locomotor behaviour in the novel environment of the open field. Additionally, this study aimed to determine whether: a) the cannabinoid CB_1 and CB_2 receptor agonists develop the potential cross-sensitization to methamphetamine effects; b) the CB_1 receptor antagonist antagonizes the sensitization to methamphetamine.

Material and methods

Animals

Male mice (strain ICR, TOP-VELAZ s. r. o., Prague, Czech Republic) with an initial weight of 18–21g were used. They were randomly allocated into the different treatment groups. Mice were housed with free access to water and food in a room with controlled humidity and temperature, that was maintained under a 12-h phase lighting cycle. Experimental sessions were always performed in the same light period between 1:00 p. m. and 3:00 p. m. in order to minimise possible variability due to circadian rhythms.

Apparatus

Locomotor activity was measured using an open-field equipped with Actitrack (Panlab, S. L., Spain). This device consists of two square-shaped frames that deliver beams of infrared rays into the space inside the square. A plastic box is placed in this square to act as an open-field arena (base 30 x 30 cm, height 20 cm), in which the animal can move freely. The apparatus software records and evaluates the locomotor activity of the animal by registering the beam interruptions caused by movements of the body. Using this equipment we have determined the Distance Travelled.

Drugs

Vehicle and all drugs were always given in a volume adequate to drug solutions (10 ml/kg).

(+)Methamphetamine, (d-N,α-Dimethylphenylethyl amine;d-Desoxyephedrine), (Sigma Chemical Co.) dissolved in saline.

(R)-(+)-Methanandamide, (R)-N-(2-hydroxy-1-methylethyl)-5Z,8Z,11Z-eicosotetraenamide) supplied pre-dissolved in anhydrous ethanol 5 mg/ml (Tocris Cookson Ltd., UK) was diluted in saline to the concentration giving the chosen dose to be administered to animals in a volume of 10 ml/kg; vehicle therefore contained an adequate part of ethanol (a final concentration in the injection below 1%) to make effects of placebo and the drug comparable.

JWH 015, (1 propyl-2-methyl-3-(1-naphthoyl)indole), (Tocris Cookson Ltd., UK), dissolved in ethanol+saline – 1:19; vehicle treatment as a control in this case contained an adequate part of ethanol to make effects of placebo and the drug comparable.

AM 251, (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), (Tocris Cookson Ltd., UK), ultrasonically suspended in Tween 80 (1 drop in 10 ml saline); vehicle treatment as a control in this case contained an adequate part of Tween 80.

Procedure

The experimental design was kept consistent across the three consecutive experiments. In each experiment animals were randomly divided into three groups and their ambulation in the open field recorded using the Actitrack apparatus (1st record) on Day 1 (naive mice). No observations or drug applications were made from Day 2 to Day 6. During this period, animals were kept in their home cages and were not placed into the open field as to avoid the phenomenon of habituation. On Day 7, mice were given the initial dose of the drug treatment or vehicle (I. P.), followed, after 15 minutes, by the open field test (2nd record). Between Day 8 and Day 13 the animals in all groups were given once a day the same drugs at the same doses. On Day 14, all mice in all groups received a challenge dose of methamphetamine at a dose of 2.5 mg/kg. Locomotor activity was then recorded in the Actitrack apparatus (3rd record) 15 minutes after application. The drug treatments for Days 7 – 13 were provided in the following design: the Experiment A: 1) vehicle (n_1=10), 2) methamphetamine at the dose of 2.5 mg/kg (n_2=10), 3) methanandamide at the dose of 0.5 mg/kg (n_3=10); the Experiment B: 1) vehicle (n_1=8), 2) methamphetamine at the dose of 2.5 mg/kg (n_2=9), 3) JWH 015 at the dose 5.0 mg/kg (n_3=10); the Experiment C: 1) vehicle (n_1=12), 2) methamphetamine at the dose of 2.5 mg/kg (n_2= 12), 3) methamphetamine+AM 251 at the doses of 2.5 mg/kg and 5.0 mg/kg, respectively (n_3=12).

The adjustment of all drug doses was based on both literature data and our results received in our earlier behavioural experiments.

The experimental protocols of all three experiments comply with the European Community guidelines for the use of experimental animals and were approved by the Animal Care Committee of the Masaryk University Brno, Faculty of Medicine, Czech Republic.

Data analysis

As the data was not normally distributed (according to preliminary evaluation in the Kolmogorov-Smirnov test of normality), non-parametric statistics were used: Mann-Whitney U test, two-tailed.

Results

In all 3 Experiments – A, B, C (Figures 1, 2, 3) always involving 3 experimental subgroups of experimental mice, no significant differences were found in Distance Travelled when measured for the first time (= naive animals).

In Experiment A (Figure 1), the first dose of treatments resulted in a) no significant behavioural changes in the vehicle (V) treated animals, b) significant ($p<0.01$) stimulation of locomotion after methamphetamine (M), and c) no significant difference between V and methanandamide (CAN) treated animals. The challenge dose of M produced a significant increase in Distance Travelled ($p<0.05$) in animals pre-treated repeatedly with M when compared to animals pre-treated with V which were given in this session M for the first time

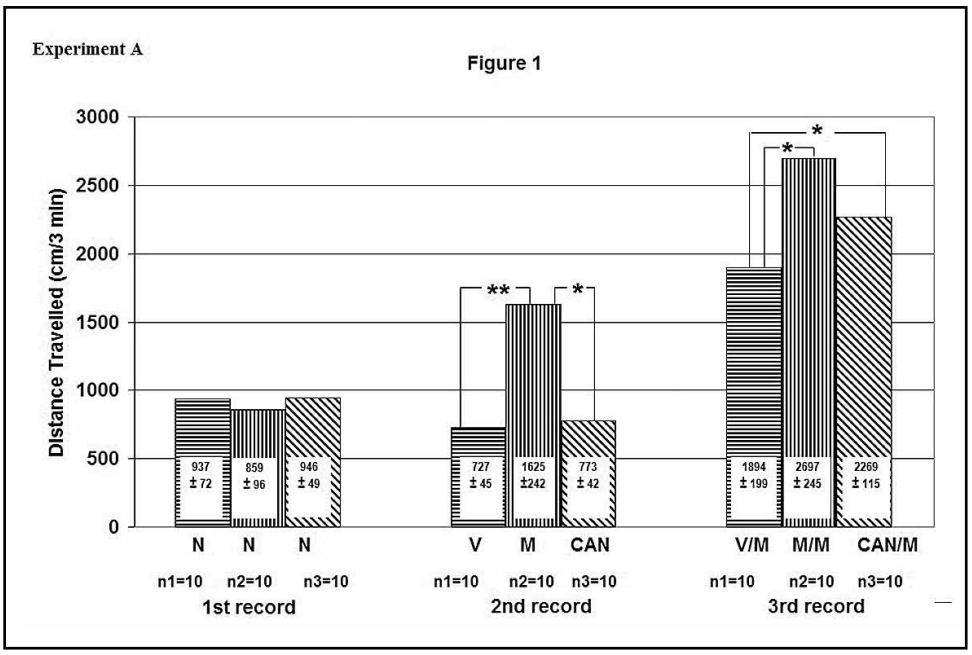

Figure 1. Effects of drug treatments in Experiment A on Distance Travelled (cm/3 min) in the mouse open field test shown as mean ± SEM: N = naive mice, V = mice after the 1st dose of vehicle, M = mice after the 1st dose of methamphetamine (2.5 mg/kg), CAN = mice after the 1st dose of methanandamide (0.5 mg/kg), V/M = mice sensitized with vehicle after the challenge dose of methamphetamine (2.5 mg/kg), M/M = mice sensitized with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg), CAN/M = mice sensitized with methanandamide after the challenge dose of methamphetamine (2.5 mg/kg) * = $p < 0.05$, ** = $p < 0.01$ - the nonparametric Mann-Whitney U test, two tailed.

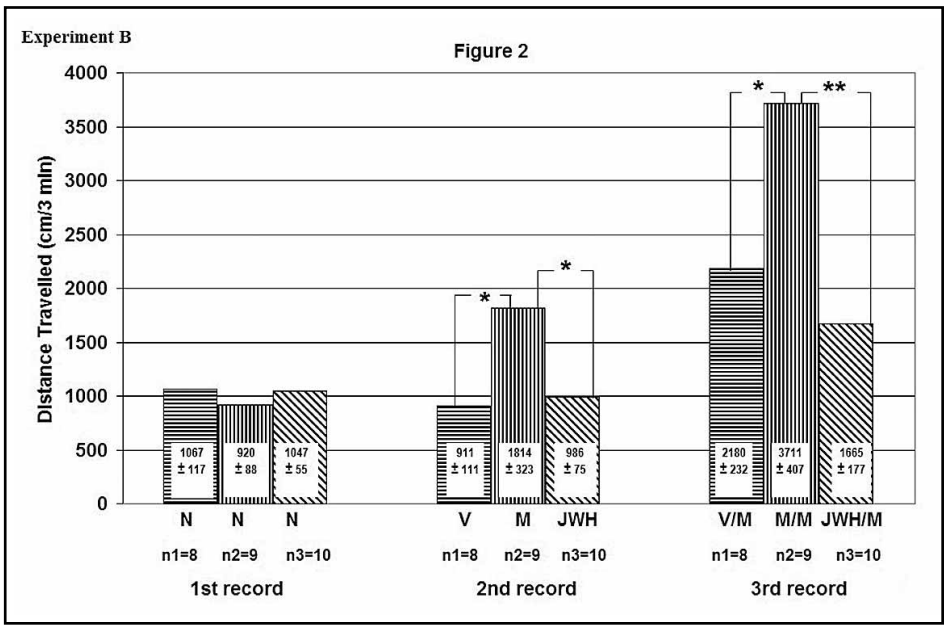

Figure 2. Effects of drug treatments in Experiment B on Distance Travelled (cm/3 min) in the mouse open field test shown as mean ± SEM:
N = naive mice, V = mice after the 1st dose of vehicle, M = mice after the 1st dose of methamphetamine (2.5 mg/kg), JWH = mice after the 1st dose of JWH 015 (5.0 mg/kg), V/M = mice sensitized with vehicle after the challenge dose of methamphetamine (2.5 mg/kg), M/M = mice sensitized with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg), JWH/M = mice sensitized with JWH 015 after the challenge dose of methamphetamine (2.5 mg/kg) * = $p < 0.05$, ** = $p < 0.01$ - the nonparametric Mann-Whitney U test, two tailed

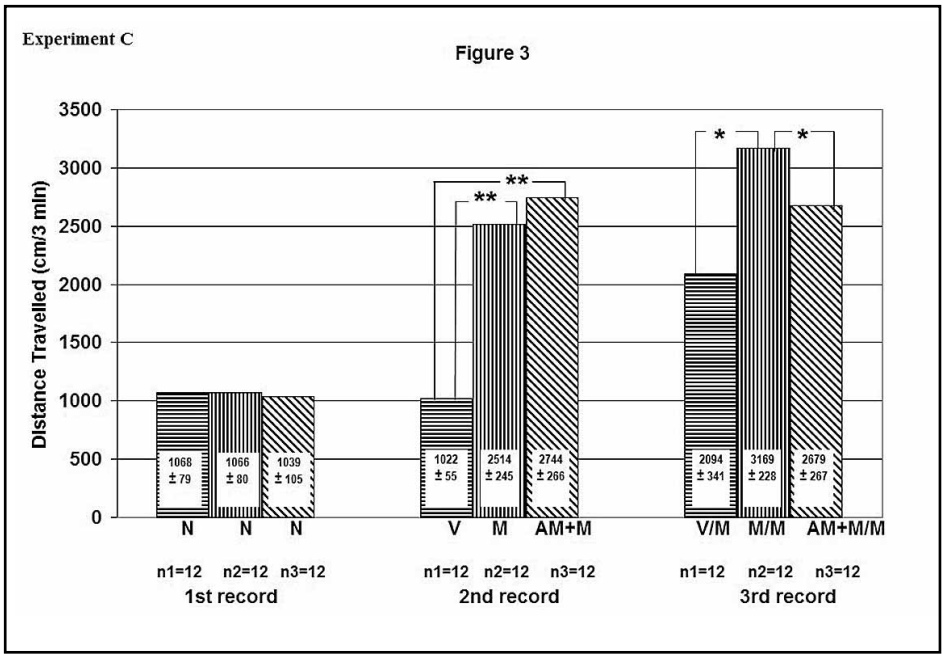

Figure 3. Effects of drug treatments in Experiment C on Distance Travelled (cm/3 min) in the mouse open field test shown as mean ± SEM:
N = naive mice, V = mice after the 1st dose of vehicle, M = mice after the 1st dose of methamphetamine (2.5 mg/kg), AM + M = mice after the 1st dose of the combination of AM 251 + methamphetamine (5.0 mg/kg + 2.5 mg/kg), V/M = mice sensitized with vehicle after the challenge dose of methamphetamine (2.5 mg/kg), M/M = mice sensitized with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg), AM/M = mice sensitized with AM 251 + methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg) * = $p < 0.05$, ** = $p < 0.01$ - the nonparametric Mann-Whitney U test, two tailed

(see Figure 1, columns M/M and V/M). The challenge dose of M administered to animals pre-treated repeatedly with CAN elicited also a significant increase in the same parameter compared to V pre-treated animals which were given M for the first time (see Figure 1, columns CAN/M and V/M).

In Experiment B (Figure 2), a typical significant ($p<0.05$) stimulatory influence in the open field following acute M administration was measured while there was no significant difference between V and JWH 015 (JWH) treated animals. After the challenge dose of M in mice pre-treated with M a significant difference ($p<0.05$, $p<0.01$) was seen for the longer Distance Travelled when comparing to the group pre-treated with both V and JWH (see Figure 2, columns M/M and V/M and JWH/M) which were not different.

In the Experiment C (Figure 3), the combined treatment with M+AM 251 (AM) did not elicit a significantly differential influence on mouse locomotor behaviour comparing with M treatment, and in both cases the stimulation of locomotion was significantly ($p<0.01$) higher versus the V treatment. After the M challenge dose a statistically significant ($p<0.05$) increase in Distance Travelled in mice pre-treated with M was again measured when compared to V pre-treated animals (see Figure 3, columns M/M and V/M). The stimulatory effect of M however, was not apparent in the group of mice pre-treated with M+AM, the parameter measured

did not significantly differ from the group pre-treated with V (see Figure 3, columns AM+M/M and V/M).

No significant signs of habituation were observed in the open field tests after repeated testing performed one week apart.

Discussion

In all three experiments of the current study the development of behavioural sensitization to methamphetamine (the increase in its stimulatory effects on mouse locomotion in the open-field) was confirmed after its repeated administration. Besides this, the chronic treatment with cannabinoid CB_1 receptor agonist methanandamide enhanced the locomotor response to methaphetamine, too. Surprisingly, this cross-sensitization was obtained with methanandamide pre-treatment with the doses which given alone did not elicit a significant influence on mouse behaviour in the open field. These results resemble to high extent those we received earlier in the model of agonistic behaviour in singly-housed aggressive mice on interactions with the non-aggrresive group-housed partners. Methanandamide pre-treatment sensitized to antiaggressive effect of methamphetamine at the doses which did not produce such effect [24].

According to our knowledge such effects of synthetic cannabinoid methanandamide, selective CB_1 receptor agonist, have not yet been described. Nevertheless, our results are in agreement with conclusions of other authors who described behavioural cross-sensitization to another drug of the same class amphetamine with mixed $CB_{1,2}$ cannabinoid receptor agonist tetrahydrocannabinol [25, 9]. According to current literature, various possible mechanisms may contribute.

The mechanism of sensitization to methamphetamine and also of cross-sensitization with cannabinoid CB_1 receptor agonist methanandamide may involve the ability of these drugs to release dopamine in the nucleus accumbens [26], a property common to many drugs that induce sensitization. A reciprocal crosstalk is reported between the cannabinoid CB_1 and dopamine receptors, which are highly co-localized on brain neurones [27, 28]. Several mechanisms have been described dealing with functional interactions between central cannabinoid CB_1 and dopamine receptors which both reduce after stimulation cAMP levels and transmitter release through an inhibitory G protein [29, 30, 31]. Dopamine D_2 and cannabinoid CB_1 receptors are co-localised especially on GABA terminals [32] and their stimulation suppress GABA inhibitory transmission [33]. This inhibition of GABA-transmission may lead to disinhibition at excitatory synapses and some other cellular mechanisms involved in drug addiction [34]. These short-term mechanisms of synaptic plasticity demonstrate a retrograde signalling function for the endocannabinoid system which may also influence longer-lasting modes of synaptic plasticity [34].

The increase in dopaminergic output induced by psychostimulants (e. g. amphetamines) was reported to be counteracted by the activation of cannabinoid CB_1 receptors [25]. However, this process can be reversed in the case of chronic stimulation of the cannabinoid CB_1 receptor by an agonist, leading to desensitization of this system and therefore resulting in increased response to psychostimulants, i. e. in behavioural sensitization to amphetamines.

It also appears that changes in the arachidonic acid cascade induced by exogenously administered cannabinoids (the mobilization of arachidonic acid [35], and activation of phospholipase [36, 37]) may adapt the endocannabinoid system and consequently impact on process of sensitization to psychostimulants [38]. Co-administration of the phospholipase A_2 inhibitor, quinacrine [39], or cyclooxygenase inhibitor, indomethacin [40], during the developmental phase suppressed sensitization to amphetamine in animal studies.

Despite statements that cannabinoid CB_2 receptors are localized mainly outside the CNS [41, 42], in our recent study [43] JWH 015, the CB_2 receptor agonist, elicited psychotropic antiaggressive effects in the model of agonistic behaviour in singly-housed male mice on paired interactions with non-aggressive group-housed partners. However, the data obtained from the present Experiment B show that the pre-treatment with JWH 015 prior to the challenge dose of methamphetamine did not lead to cross-sensitization to its behavioural stimulatory effects. This correlates rather with a more widely accepted belief that cannabinoid CB_2 receptor activity might not be involved in brain processes, in this case, in neurobehavioural plasticity underlying sensitization phenomenon to methamphetamine.

The results of the Experiment C demonstrated that a co-administration of CB_1 receptor antagonist AM 251 with repeated doses of methamphetamine decreased signs of behavioural sensitization measured after the methamphetamine challenge dose. This opposite effect of CB_1 receptor antagonist on methamphetamine sensitization when compared to the influence of methanandamide acting as the CB_1 receptor agonist (showed in the Experiment A and published as an abstract elsewhere [44]) correlates well. Another CB_1 receptor antagonist SR141716 (rimonabant) did not affect cocaine reinforcement and sensitization to its locomotor stimulant effect [45]. However, the same drug reduced the cocaine-seeking and nicotine-seeking behaviour in rats what have been recently validated in humans [46] and is the subject of Clinical Trial (No. NCT00075205) dealing with its impact on reduce of alcohol drinking sponsored by the U.S. National Institute on Alcohol Abuse and Alcoholism.

As endocannabinoids, through binding at CB_1 receptors, act as retrograde synaptic messengers [41] at axon terminals including the midbrain dopamine neurons their effects after CB_1 receptor blockade are prevented. Thus, the reward dopamine mechanism (general pharmacological principle of drugs with abuse potential)

in the ventral mesencephalon with high density of CB_1 receptors can be influenced [47].

In summary, the outcomes of the present behavioural study are threefold. First, they confirmed a well-known fact that repeated administration of methamphetamine produces behavioural sensitization to its stimulatory effects on mouse locomotor activity in the open field test. Secondly, cannabinoid CB_1 receptor stimulation by agonist methanandamide cross-sensitized to metamphetamine, while blocking of the CB_1 receptor with antagonist AM 251 during the sensitizing phase with methamphetamine suppressed this phenomenon in the same animal model. Finally, cannabinoid CB_2 receptor stimulation by agonist JWH 015 did not cause cross-sensitization to methamphetamine in the present study. Concerning that behavioural sensitization accounts for compulsive patterns of drug-seeking and drug-taking behaviour [48] the present results contribute to hypothesis that repeated use of Cannabis derivates may facilitate progression to the consumption of other illicit drugs in vulnerable individuals [9]. Furthermore they support also the findings from both clinical and preclinical studies [46] suggesting that ligands blocking CB_1 receptors offer a novel approach for patients suffering from drug dependence.

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7.2 Impact of cannabinoid receptor ligands on behavioural sensitization to antiaggressive methamphetamine effects in the model of mouse agonistic behaviour

The presented study was aimed at the possible influence of pre-treatments with methanandamide (a cannabinoid CB_1 receptor agonist), JWH 015 (a cannabinoid CB_2 receptor agonist) and AM 251 (a cannabinoid CB_1 receptor antagonist/inverse agonist) on sensitization to methamphetamine antiaggressive effects in the model of mouse agonistic behaviour.

At the time of writing, there was only limited evidence on behavioural sensitization to the inhibitory effects of substances, e.g. sensitization to catalepsy in rats (Schmidt 2004) or sensitization to suppression of defensive-escape behaviour in mice (Votava and Krsiak 2003).

It has been shown in the present study that repeated administration of methamphetamine produced in mice behavioural sensitization to the stimulatory effects of the drug on locomotory behaviour and inhibitory antiaggressive effects in the model of agonistic behaviour.

Furthermore, chronic pre-treatment with the cannabinoid CB_1 receptor agonist methanandamide elicited cross-sensitization to methamphetamine, and, conversely, a blockade of these receptors with the antagonist/inverse agonist AM 251 inhibited this process in the aforementioned model. It was to a large extent in accordance with our previous paper on behavioural sensitization using the mouse open field test (Landa et al. 2006a). Repeated repeated pre-treatment with the cannabinoid CB_2 receptor agonist JWH 015 did not provoke cross-sensitization to methamphetamine antiaggressive effects in the present study.

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Impact of cannabinoid receptor ligands on behavioural sensitization to antiaggressive methamphetamine effects in the model of mouse agonistic behaviour

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Abstract

OBJECTIVES: Psychostimulants and cannabinoids can elicit so called behavioural sensitization after repeated administration, a gradually increased behavioural response to a drug. This phenomenon if conditioned by previous pre-treatment with different drug is termed cross-sensitization. The present study was focused on a possible sensitisation to antiaggressive effect of methamphetamine and cross-sensitization to this effect after repeated pre-treatment with cannabinoid CB_1 and CB_2 receptor ligands with different intrinsic activity (CB_1 agonist methanandamide, CB_2 agonist JWH 015, and CB_1 antagonist AM 251).

METHODS: Behavioural interactions of singly-housed mice with non-aggressive group-housed partners were video-taped and behavioural elements of agonistic behaviour of isolates were recorded in four categories: sociable, timid, aggressive and locomotor.

RESULTS: Repeated administration of methamphetamine elicited a significant sensitization to its antiaggressive effects. Methanandamide pre-treatment provoked cross-sensitization to this methamphetamine effect, whereas pre-treatment with JWH 015 did not. Combined pre-treatment with methamphetamine+AM 251 suppressed the sensitization to antiaggressive effects of methamphetamine.

CONCLUSIONS: Our findings have shown that it is possible to provoke sensitization not only to the stimulatory effects as stated widespread in the literature but also to inhibitory antiaggressive effects of methamphetamine. Furthermore, we confirmed our working hypothesis that it is possible to elicit either cross-sensitization to inhibitory effects of methamphetamine conditioned by repeated pre-treatment with cannabinoid CB_1 receptor agonist methanandamide, or suppression of methamphetamine sensitizing influence by co-administration of CB_1 receptor antagonist.

ORIGINAL ARTICLE

Abbreviations:
AM 251 – CB_1 receptor antagonist,
Al – alert posture,
At – attack,
Cl – climbing over the partner,
De – defensive posture (upright),
Es – escape,
Fo – following the partner,
JWH 015 – CB_2 receptor agonist,
Re – rearing,
Ss – social sniffing,
Tr – tail rattling,
Ur – aggressive unrest (threat),
Wa – walking

Introduction

Repeated administration of various substances can elicit a long-lasting increase in behavioural response, which is well known phenomenon termed behavioural sensitization, described consistently for the first time by Robinson and Berridge [1]. Since that time, behavioural sensitization has been described for instance to cannabinoids [2], opioids [3] or psychostimulants [4, 5].

In addition it has been shown that this increased response to a certain drug can be also achieved by previous repeated administration of another drug, a phenomenon called cross-sensitization. It was documented among others after repeated exposure with THC to opioids [2, 6] or with caffeine and amphetamine to nicotine [7].

The most frequently observed features of behavioural sensitization are stimulatory effects of drugs. In laboratory rodents an increase in locomotor/exploratory activities is considered as the most common symptom of behavioural sensitization. Besides this augmented stimulation, sensitization can occur to some other types of behaviour – like defensive-escape activities [8] and there are also reports on sensitization to inhibitory drug actions such as catalepsy [9].

Results of previous study run in our laboratory suggested an interaction between endocannabinoid system and methamphetamine brain mechanisms in the I.V. drug self-administration model in rats [10]. This was further confirmed by other experiments realised using the mouse open field test where we unambiguously found that pre-treatment with CB_1 receptor agonist methanandamide elicited cross-sensitization to methamphetamine effect and on the contrary, combined pre-treatment with methamphetamine+AM 251 suppressed sensitization to methamphetamine [11]. All these findings speak in favour of the suggested interaction between endocannabinoid system activity and methamphetamine CNS mechanisms and moreover they support further views of other authors that ligands blocking CB_1 receptors offer a novel approach for treatment of addiction [12].

In our earlier experiments acute methamphetamine administration elicited an inhibition of aggressivity in the model of mouse agonistic interactions [13]. Thus, we decided to test in the present study if the repeated administration of methamphetamine would more pronounce this effect, i.e. elicit behavioural sensitization to its antiaggressive effects. Furthermore, the present study was designed to investigate the effects of pre-treatments with cannabinoid CB_1 receptor agonist methanandamide and CB_1 receptor antagonist AM 251 on sensitization to methamphetamine antiaggressive effects. Finally, as the presence of CB_2 receptors was also confirmed in some areas of the brain [14, 15, 16] and we are experienced with behavioural effect of CB_2 receptor agonist JWH 015 in mice [17], we decided to test a possible effect of pre-treatment with CB_2 receptor agonist JWH 015 on sensitization to methamphetamine antiaggressive effects. All these experiments were performed using the model of mouse agonistic behaviour.

Material and methods

Animals

In all experiments mice males (strain ICR, TOP-VELAZ s. r. o., Prague, Czech Republic) with an initial weight of 18–21 g were used. Animals were housed with free access to water and food in a room with controlled humidity and temperature, that was maintained under a 12-h phase lighting cycle. Experimental sessions were always performed in the same light period (8:00 – 11:00 a.m.) in order to minimise possible variability due to circadian rhythms.

The experimental protocols of all experiments comply with the European Community guidelines for the use of experimental animals and were approved by the Animal Care Committee of the Masaryk University Brno, Faculty of Medicine, Czech Republic.

Model of agonistic behaviour

The model of agonistic behaviour used in this study was based on intraspecies social conflict in adult male mice [18, 19] and it consists of observation of behaviour in individually-housed mice on dyadic interactions with group-housed partners in neutral environment of the observational plastic box (base 30 x 20 cm, height 20 cm). After 30 min adaptation of singly-housed mice in the neutral cages their four minute dyadic behavioural interactions of singly-housed mice with non-aggressive group-housed partners were video-taped. After each interaction the neutral cage sawdust bedding was replaced. The behavioural element recording was performed later by an experimenter who was unaware of treatment of the mouse groups using the keyboard of the computer-compatible system OBSERVER 3.1 (Noldus Information Technology b.v., Holland).

Whereas the group-housed partner does not display aggressiveness, individually-housed mice can be according to their behaviour in control interaction (vehicle treatment) divided into 3 categories: a) aggressive mice (showing at least one attack towards the opponents in the control interactions); b) timid mice (showing majority of defensive-escape behaviour even in absence of partner's attacks and no attack); c) sociable mice (animals without aggressive or defensive-escape behaviour, showing however high frequency of approaches to partner and its sniffing or climbing over the partner – acts considered

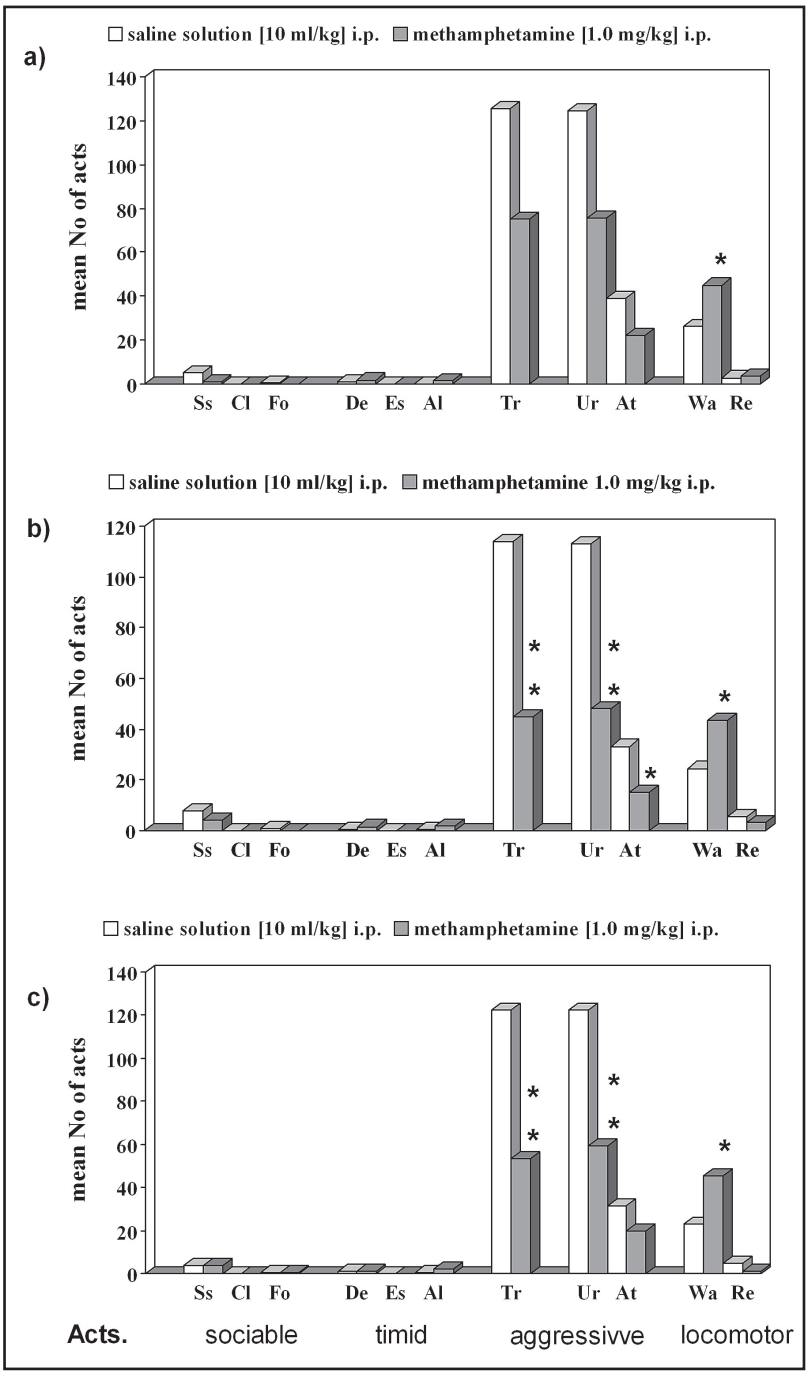

Figure 1: The effect of methamphetamine "challenge dose" in singly-housed aggressive mice on agonistic interactions with non-aggressive group-housed partners: **a)** repeatedly pre-treated with saline solution (n_1=11), **b)** repeatedly pre-treated with methamphetamine (n_2=18), **c)** repeatedly pre-treated with methanadamide (n_3=19). Behavioural acts: Sociable – Ss (social sniffing), Cl (climbing over the partner), Fo (following the partner); Timid: De (defensive posture), Es (escape), Al (alert posture); Aggressive: Tr (tail rattling), Ur (aggressive unrest), At (attack); Locomotor: Wa (walking), Re (rearing). i.p. – intraperitoneally, * = $p < 0.05$, * * = $p < 0.01$, the nonparametric Wilcoxon matched pairs test.

to be sociable. Behavioural elements of four subtypes were recorded: sociable – social sniffing [Ss], following the partner [Fo], climbing over the partner [Cl]; timid – defensive posture (upright) [De], escape [Es], alert posture [Al]; aggressive – attack [At], aggressive unrest (threat) [Ur], tail rattling [Tr]; locomotor – walking [Wa], rearing [Re]. Just aggressive singly-housed mice were chosen as subjects in the present study.

Substances

(+)Methamphetamine, (d-N,α-Dimethylphenyleth ylamine;d-Desoxyephedrine), (Sigma Chemical Co.) dissolved in saline.

(R)-(+)-Methanandamide, (R)-N-(2-hydroxy-1-methylethyl)-5Z,8Z,11Z-eicosotetraenamide) supplied pre-dissolved in anhydrous ethanol 5 mg/ml (Tocris Cookson Ltd., UK) was diluted in saline to the concentration giving the chosen dose to be administered to ani-

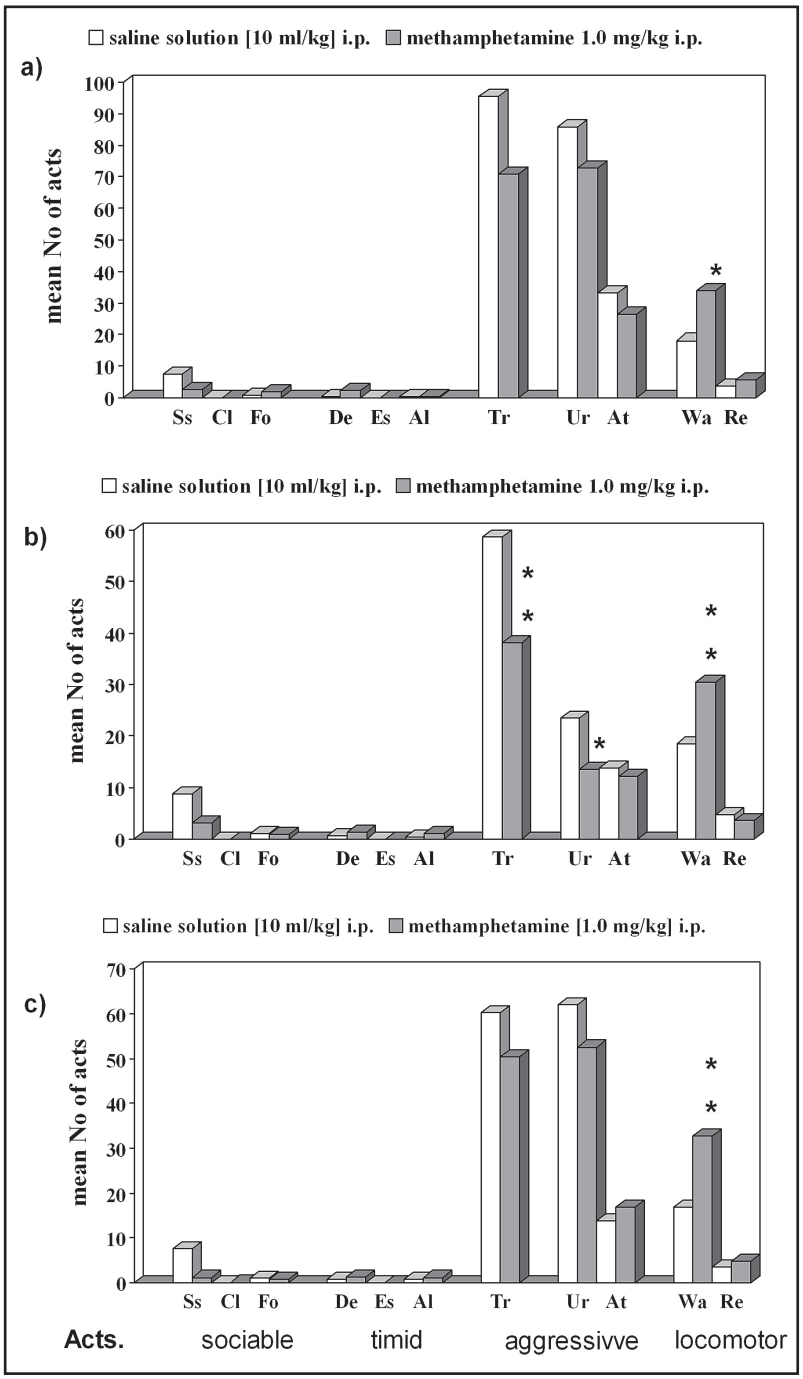

Figure 2: The effect of methamphetamine "challenge dose" in singly-housed aggressive mice on agonistic interactions with non-aggressive group-housed partners: **a)** repeatedly pre-treated with saline solution (n_1=8), **b)** repeatedly pre-treated with methamphetamine (n_2=9), **c)** repeatedly pre-treated with JWH 015 (n_3=11).
Behavioural acts: Sociable – Ss (social sniffing), Cl (climbing over the partner), Fo (following the partner); Timid: De (defensive posture), Es (escape), Al (alert posture); Aggressive: Tr (tail rattling), Ur (aggressive unrest), At (attack); Locomotor: Wa (walking), Re (rearing). i.p. – intraperitoneally, * = $p < 0.05$, * * = $p < 0.01$, the nonparametric Wilcoxon matched pairs test.

mals in a volume of 10 ml/kg; vehicle therefore contained an adequate part of ethanol (a final concentration in the injection below 1%) to make effects of placebo and the drug comparable.

JWH 015, (1 propyl-2-methyl-3-(1-naphthoyl)indole), (Tocris Cookson Ltd., UK), dissolved in ethanol+saline – 1:19; vehicle treatment as a control in this case contained an adequate part of ethanol to make effects of placebo and the drug comparable.

AM 251, (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), (Tocris Cookson Ltd., UK), ultrasonically suspended in Tween 80 (1 drop in 10 ml saline); vehicle treatment as a control in this case contained an adequate part of Tween 80.

Vehicle and all drugs were always given in a volume adequate to drug solutions (10 ml/kg).

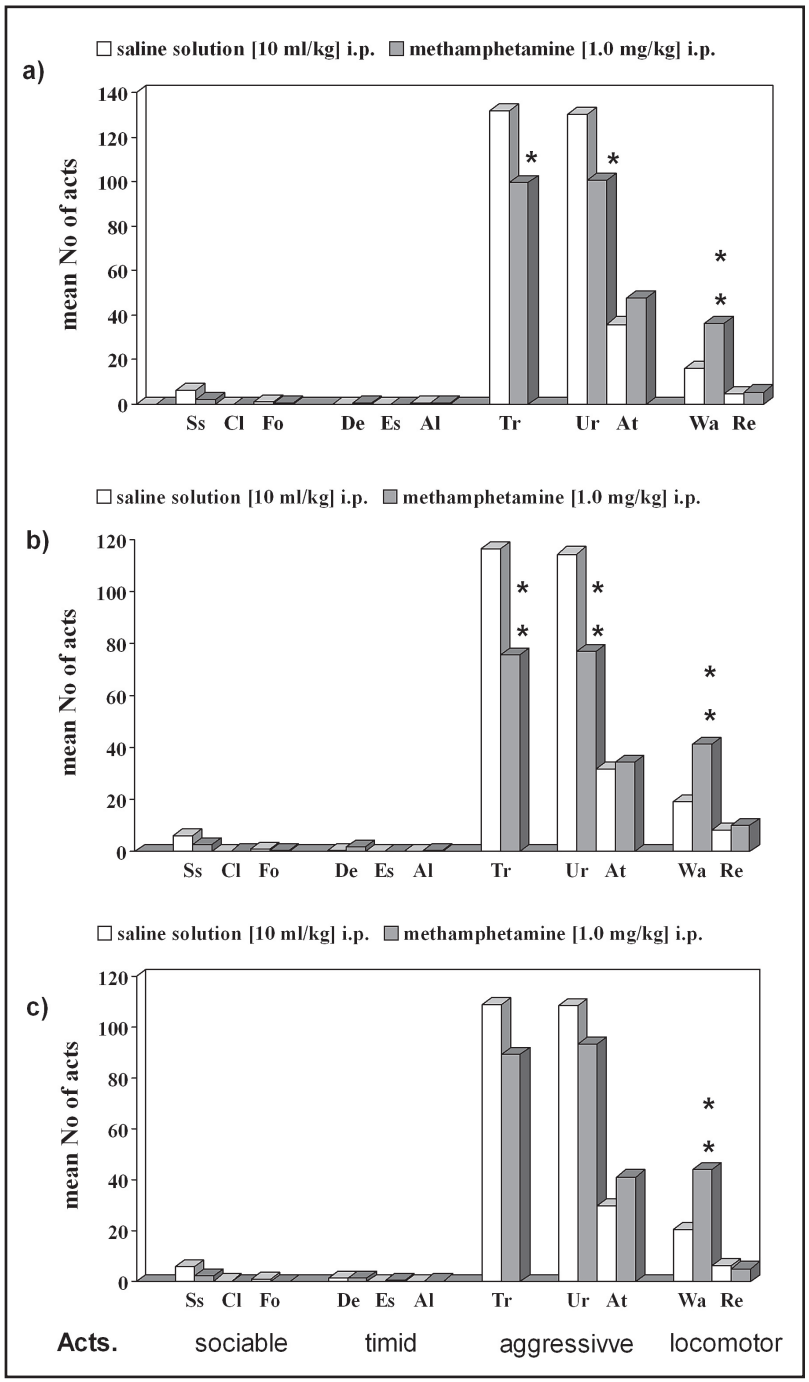

Figure 3: The effect of methamphetamine "challenge dose" in singly-housed aggressive mice on agonistic interactions with non-aggressive group-housed partners: **a)** repeatedly pre-treated with saline solution (n_1=11), **b)** repeatedly pre-treated with methamphetamine (n_2=12), **c)** repeatedly pre-treated with methamphetamine+AM 251 (n_3=14). Behavioural acts: Sociable – Ss (social sniffing), Cl (climbing over the partner), Fo (following the partner); Timid: De (defensive posture), Es (escape), Al (alert posture); Aggressive: Tr (tail rattling), Ur (aggressive unrest), At (attack); Locomotor: Wa (walking), Re (rearing). i.p. – intraperitoneally, * = $p < 0.05$, * * = $p < 0.01$, the nonparametric Wilcoxon matched pairs test.

Procedures

Singly-housed mice were randomly allocated into 3 groups in each of three experiments for the following 5 day drug pre-treatment given intraperitoneally: the Experiment I) n_1=11: saline solution 10 ml/kg/day, n_2=18: methamphetamine 1 mg/kg/day, n_3=19: methanandamide 0.5 mg/kg/day; the Experiment II) n_1=8: saline solution at the dose of 10 ml/kg/day, n_2=9: methamphetamine at the dose of 1 mg/kg/day, n_3=11: JWH 015 at the dose of 10 mg/kg/day; the Experiment III) n_1=11: saline solution at the dose of 10 ml/kg/day, n_2=12: methamphetamine at the dose of 1 mg/kg/day, n_3=14: methamphetamine+AM 251 at the doses of 1 mg/kg/day and 5 mg/kg/day, respectively. There was a wash-out period on the Days 6–10, and on the Day

11 agonistic interactions were performed 15 min after the administration of saline solution to all subjects (10 ml/kg). The "challenge doses" of methamphetamine (1 mg/kg) were given to all subjects 15 min prior to second agonistic interactions on the Day 15 while Days 12–14 present a wash-out.

Statistical data analysis

As the data did not show normal distribution (analysed by Kolmogorov-Smirnov test of normality), the differences between the occurrence of behavioural acts in control and experimental interactions were evaluated by the non-parametric Wilcoxon test, two tailed.

Results

In the Experiment I, administration of the methamphetamine "challenge dose" elicited:

a) non-significant changes in sociable and timid behavioural acts in mice pre-treated with saline solution (group n_1); changes in aggressive acts were also non-significant ($p>0.05$), however there was an apparent trend of decrease in tail rattling, aggressive unrest and attack; there was a significant ($p<0.05$) increase in walking, which represents one of two locomotor behavioural elements (see Figure 1a).
b) non-significant changes in sociable and timid behavioural acts in mice pre-treated with methamphetamine (group n_2), highly significant ($p<0.01$) decrease in tail rattling and aggressive unrest, significant ($p<0.05$) decrease in attack, significant ($p<0.05$) increase in walking – (see Figure 1b).
c) non-significant changes in sociable and timid behavioural acts in mice pre-treated with methanandamide (group n_3), highly significant ($p<0.01$) decrease in tail rattling and aggressive unrest, significant ($p<0.05$) increase in walking (see Figure 1c).

In the Experiment II, administration of the methamphetamine "challenge dose" elicited:

a) in mice pre-treated with saline solution (group n_1) non-significant changes in sociable and timid behavioural acts, as well as in all aggressive acts (tail rattling, aggressive unrest and attack), these, however, showed an apparent trend of decrease; there was a significant ($p<0.05$) increase in walking (see Figure 2a).
b)) in mice pre-treated with methamphetamine (group n_2) non-significant changes in sociable and timid behavioural acts, highly significant ($p<0.01$) decrease in tail rattling, significant ($p<0.05$) decrease in aggressive unrest, highly significant ($p<0.01$) increase in walking (see Figure 2b).
c) in mice pre-treated with JWH 015 (group n_3) non-significant changes in sociable, aggressive and timid behavioural acts, highly significant ($p<0.01$) increase in walking (see Figure 2c).

In the Experiment III administration of the methamphetamine "challenge dose" elicited:

a) in mice pre-treated with saline solution (group n_1) non-significant ($p>0.05$) changes in sociable and timid behavioural acts, significant ($p>0.05$) decrease in tail rattling, aggressive unrest and a highly significant ($p<0.01$) increase in walking (see Figure 3a).
b) in mice pre-treated with methamphetamine (group n_2) non-significant changes in sociable and timid behavioural acts, highly significant ($p<0.01$) decrease in tail rattling and aggressive unrest, highly significant ($p<0.01$) increase in walking (see Figure 3b).
c) in mice pre-treated with methamphetamine+AM 251 (group n_3) non-significant changes in sociable, aggressive and timid behavioural acts and highly significant ($p<0.01$) increase in walking (see Figure 3c).

Discussion

Presented results confirmed with methamphetamine the well known effects of amphetamine and its derivates disrupting aggressive behaviour in various animal species including male mice on agonistic interactions [20, 21, 22]. The behavioural sensitization developed not only to stimulatory effects on locomotion, but also to the inhibitory antiaggressive effects after repeated methamphetamine administration in the present study. Behavioural sensitization to psychostimulant effects of amphetamines and opioids has been already described [for review see 23, 24], however, according to literature available, there is far less evidence on behavioural sensitization to inhibitory effects of substances. It has been described for instance sensitization to catalepsy in rats [25] and also sensitization to suppression of defensive-escape behaviour in mice [8]. Our present experiments showed the development of behavioural sensitization to methamphetamine inhibitory influences on naturally motivated behaviour – male mouse aggression. The results obtained from our study concerning impact of cannabinoid receptor ligands on sensitization to antiaggressive methamphetamine effects confirmed the working hypothesis that it is possible to elicit cross-sensitization to both stimulatory and inhibitory effects of methamphetamine conditioned by repeated pre-treatment with cannabinoid CB_1 receptor agonist methanandamide. The data obtained from these our experiments confirmed an assumption published elsewhere of existing functional interaction between the activity of cannabinoid CB_1 receptors and amphetamine [6, 26, 27, 28, 29] or methamphetamine [11, 30, 31] mechanisms in the CNS.

Despite of the fact that the CB_2 receptor agonist JWH 015 has been shown earlier to produce at the acute dose of 10 mg/kg significant antiaggressive effect in our model of agonistic behaviour in singly-housed male mice on paired interactions with non-aggressive group-housed partners, the repeated pre-treatment with this compound however did not produce the cross-sensitization to these effects of methamphetamine given as a „challenge dose"

after the withdrawal of repeated treatment in the present study. Interestingly, some sign of cross-sensitization was registered in the case of methamphetamine stimulation of locomotion (walking) which occurred on a higher level of significance in JWH 015 pre-treated mice comparing to controls. The presence of CB_2 receptors has been already reported not only in the immune system, but also in the CNS in mice [14] and rats [15], and using specific polyclonal antibodies they were detected in hippocampus and cortex of Alzheimer's disease patients, too [32]. Thus, our findings suggest, that at least some cross-sensitizing processes during combined administration of CB_2 receptor agonist JWH 015 and methamphetamine can exist due to cross-talks between not only CB_1 but also CB_2 receptors and methamphetamine pathways.

The CB_1 receptor blockade attenuates the behavioural manifestations of methamphetamine sensitization in mice pre-treated repeatedly with methamphetamine+AM 251(cannabinoid CB_1 receptor antagonist) in the present study. Just the significant increase of walking was apparent after methamphetamine „challenge dose". Our findings obtained from the model of agonistic interactions are to a large extent in accordance with some other papers. For instance, we have found [10], that AM 251 decreased methamphetamine self-administration under a FR schedule in rats, and similarly the suppression of behavioural sensitization to morphine in the rodent model of drug-seeking behaviour was shown after pre-treatment with another CB_1 antagonist SR141716A [33]. On the other hand there is also a contradictory report available suggesting that endogenous cannabinoids and CB_1 receptors are not involved in behavioural sensitization to psychostimulants, namely cocaine [34].

The endocannabinoid system is thought to be the primary site of action for the rewarding and pharmacological responses induced by cannabinoids [31, 35]. Despite the statement of above mentioned publication of Lescher et al. [34], there are multiple studies supporting that the common neurobiological mechanisms of most drugs of abuse participated in their addictive properties interact in bidirectional manner with the endocannabinoid system involvement in regulation of drug rewarding effects [31].

The main principle of behavioural sensitization to methamphetamine and also of cross-sensitization with cannabinoid CB_1 receptor agonist methanandamide is probably based on the potency of these substances to release dopamine in the nucleus accumbens [36], which is a property common to many drugs that can elicit sensitization, and dopamine activation of endogenous cannabinoid signalling in the CNS has been confirmed [37]. Although not all neurobiological bases of behavioural sensitization are fully clear yet, there are studies indicating that behavioural sensitization has a neural basis and that the neuronal circuit important for behavioural sensitization consists of various structures in the CNS. It involves not only dopaminergic, but also glutamatergic and GABAergic projections between ventral tegmental area, nucleus accumbens, prefrontal cortex, hippocampus and amygdala. The mesolimbic dopaminergic projection from the ventral tegmental area to the nucleus accumbens seems to be of crucial importance for reward-related effects of drugs of abuse [38]. Furthermore, the mesolimbic and nigrostriatal dopamine systems also participate at the reinforcing and locomotor-stimulating effects of psychostimulant drugs [39].

In conclusion, the present study can be summarized as follows: 1) repeated administration of methamphetamine produces behavioural sensitization to its stimulatory effects on locomotion and antiaggressive effects in the mouse model of agonistic behaviour. 2) pre-treatment with cannabinoid CB_1 receptor agonist methanandamide elicited cross-sensitization to metamphetamine, whereas blocking of these receptors with antagonist AM 251 inhibited this process; 3) pre-treatment with cannabinoid CB_2 receptor agonist JWH 015 did not provoke cross-sensitization to methamphetamine antiaggressive effects in this study.

All presented findings received in the model testing antiaggressive drug effects in mice confirmed in fact the similar suggestion on interaction of methamphetamine mechanisms and endocannabinoid system activity we have published earlier [11, 40] using a differential behavioural model, the open field test as a tool for registration of behavioural sensitization to methamphetamine psychostimulant effects.

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7.3 Impact of cannabinoid receptor ligands on sensitization to methamphetamine effects on rat locomotor behaviour

The aim of this study was to investigate the influence of pre-treatments with different doses of methamphetamine on the development of behavioural sensitization to its stimulatory effects in rats. Another aim of the present experiment was to estimate the influence of pre-treatment with methanandamide (a cannabinoid CB_1 receptor agonist) and AM 251 (a cannabinoid CB_1 receptor antagonist/inverse agonist) on behavioural sensitization to methamphetamine effects on rat locomotor activity in the open field test.

Our results showed that, as previously in mice (Landa et al. 2006a, b), a repeated administration of methamphetamine can under certain circumstances provoke behavioural sensitization to its stimulatory effects also in rats, which is in accordance with other reports (e.g. Fukami et al. 2004).

Nevertheless, we were not able to elicit cross-sensitization by repeated application of the cannabinoid CB_1 receptor agonist methanandamide, and similarly, unlike in mice, we did not demonstrate a suppression of the cross-sensitization in rats that were repeatedly pre-treated with combination of the cannabinoid CB_1 receptor antagonist/inverse agonist AM 251 and methamphetamine.

However, in comparison with mice, rats manifested alternative behavioural changes after repeated methamphetamine treatment that are also considered to be signs of behavioural sensitization: the occurrence of stereotypic behaviour (increased frequency of nose rubbing). This is in accordance with findings of other authors (Laviola et al. 1999); unfortunately, it was not possible to quantify precisely these behavioural patterns with our technical equipment.

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Impact of Cannabinoid Receptor Ligands on Sensitisation to Methamphetamine Effects on Rat Locomotor Behaviour

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Abstract

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The repeated administration of various drugs of abuse may lead to a gradually increased behavioural response to these substances, particularly an increase in locomotion and stereotypies may occur. This phenomenon is well known and described as behavioural sensitisation. An increased response to the drug tested, elicited by previous repeated administration of another drug is recognised as cross-sensitisation. Based on our earlier experiences with studies on mice, which confirmed sensitisation to methamphetamine and described cross-sensitisation to methamphetamine after pre-treatment with cannabinoid CB_1 receptor agonist, we focused the present study on the use of another typical laboratory animal - the rat. A biological validity of the sensitisation phenomenon was expected to be enhanced if the results of both mouse and rat studies were conformable. Similar investigation in rats brought very similar results to those described earlier in mice. However, at least some interspecies differences were noted in the rat susceptibility to the development of sensitisation to methamphetamine effects. Comparing to mice, it was more demanding to titrate a dose of methamphetamine producing behavioural sensitisation. Furthermore, we were not able to provoke cross-sensitisation by repeated administration of cannabinoid CB_1 receptor agonist methanandamide and similarly, we did not demonstrate the suppression of cross-sensitisation in rats that were repeatedly given combined pre-treatment with cannabinoid CB_1 receptor antagonist AM 251 and methamphetamine. Finally, unlike mice, an alternative behavioural change was registered after repeated methamphetamine treatment instead: the occurrence of stereotypic behaviour (nose rubbing).

Behavioural sensitisation, cannabinoids, open field test, rats

Repeated administration of various drugs of abuse may lead to an increase in behavioural response to these substances, which has been described as behavioural sensitisation (Robinson and Berridge 1993). Behavioural sensitisation may be observed both in laboratory animals and humans (Tzschentke and Schmidt 1997) and its manifestation may vary in different species (Lanis and Schmidt 2001). It refers to the progressive augmentation of behavioural responses to re-application of the drug and the so-called "challenge dose" of the same drug even after a certain period of its withdrawal. This has been described for several drugs of abuse including psychostimulants (Costa et al. 2001; Elliot 2002), opioids (De Vries et al. 1999) or cannabinoids (Cadoni et al. 2001). It has been also found, that an increased response to a drug may be elicited by previous repeated administrations of a drug different from the challenge dose of the drug tested. This is termed cross-sensitisation and has been manifested for example with tetrahydrocannabinol to opioids (Cadoni et al. 2001; Lamarque et al. 2001). Both behavioural sensitisation and cross-sensitisation are considered to be responsible for reinstating the drug-seeking behaviour (DeVries et al. 2002).

There is increasing evidence indicating that behavioural sensitisation can be parcelled into two temporally defined domains, called development (or initiation) and expression (Kalivas et al. 1993). The term "development" of behavioural sensitisation refers to the

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progressive molecular and cellular alterations that culminate in a change in the processing of environmental and pharmacological stimuli by the CNS. These alterations are transient and may not be detected after a few weeks of withdrawal (Kalivas et al. 1993). The term "expression" of behavioural sensitisation is defined as enduring neural changes that arise from the process of development that directly mediates the sensitised behavioural response (Pierce and Kalivas 1997). Various data indicate that these processes are distinct not only temporally but also anatomically. Development of behavioural sensitisation to psychostimulant drugs occurs in the ventral tegmental area and substantia nigra, which are the loci of the dopamine cells in the ventral midbrain that give rise to the mesocorticolimbic and nigrostriatal pathways. In contrast, the neuronal events associated with expression are distributed among several interconnected limbic nuclei that are centred on the nucleus accumbens (Pierce and Kalivas 1997).

In our previous studies on mice we observed development of behavioural sensitisation to methamphetamine and also cross-sensitisation with cannabinoid CB_1 receptor agonist methanandamide to methamphetamine in the mouse open field test (Landa et al. 2006a) as well as in the mouse model of agonistic behaviour (Landa et al. 2006b). Furthermore, in the same animal models we were able to block this cross-sensitisation using pre-treatment with cannabinoid CB_1 receptor antagonist - substance AM 251 prior to the methamphetamine challenge dose. These data were to a large extent in accordance with earlier findings from our laboratory that supported the hypothesis about interaction between endocannabinoid system and methamphetamine brain mechanisms (Vinklerová et al. 2002). Thus, we decided to extend our research trying to elicit sensitisation to methamphetamine and cross-sensitisation to methanandamide in another laboratory rodent – rat, similarly in the open field test. If the results correlated well, the general biomedical validity of the study would be of greater impact.

Materials and Methods

Animals

Rat males (strain Wistar, BioTest, s.r.o., Konárovice, Czech Republic) with a starting weight of 290 - 310 g were used. Rats were housed with free access to water and food in a room with controlled humidity and temperature maintained under a 12-h phase lighting cycle. Experimental sessions were always performed in the same light period (between 13:00 - 15:00 h) in order to minimise possible variability due to circadian rhythms.

The experimental protocols of all experiments comply with the European Community guidelines for the use of experimental animals and were approved by the Animal Care Committee of the Masaryk University Brno, Faculty of Medicine, Czech Republic.

Open field test

Behavioural activities were measured using an open-field equipped with Actitrack (Panlab, S. L., Spain). This device consists of two square-shaped frames that deliver beams of infrared rays into the space inside the square. A plastic box is placed in this square to act as an open-field arena (base 45 × 45 cm, height 25 cm), in which the animal can move freely. The apparatus software records and evaluates the behavioural activities of the animal by registering the beam interruptions caused by movements of the body. With this device, it is possible to monitor various behavioural indicators. For our purposes, we have chosen Distance Travelled.

Drugs

Vehicle and all drugs were always given in a volume adequate to drug solutions (2 ml/kg):

(+)methamphetamine, (d-N,α-dimethylphenylethylamine;d-desoxyephedrine), (Sigma Chemical Co.) dissolved in saline;

(R)-(+)-methanandamide, (R)-N-(2-hydroxy-1-methylethyl)-5Z,8Z,11Z-eicosotetraenamide), (Tocris Cookson Ltd., UK) in solution (anhydrous ethanol, 5 mg/ml) dissolved in saline;

AM251, (N-(piperidine-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide), (Tocris Cookson Ltd., UK), ultrasonically suspended in Tween 80.

Procedure

In the dose-response Experiments I - III, the effects of different doses of methamphetamine on ambulatory activity in rats were tested. The drug treatments for Days 7 - 13 were provided in the following regimen: Experiment I: 1) vehicle at the dose of 2.0 ml/kg/day ($n_1 = 6$), 2) methamphetamine at the dose of 5.0 mg/kg/day ($n_2 = 6$); Experiment II: 1) vehicle at the dose of 2.0 ml/kg/day ($n_1 = 6$), 2) methamphetamine at the dose of 2.5

mg/kg/day ($n_2 = 6$); Experiment III: 1) vehicle at the dose of 2.0 ml/kg/day ($n_1 = 8$), 2) methamphetamine at the dose of 2.5 mg/kg/day ($n_2 = 8$). On Day 14, all rats in all groups received a "challenge dose" of methamphetamine (in the Experiment I at the dose of 5.0 mg/kg, in the experiment II at a dose of 2.5 mg/kg and in the Experiment III at the dose of 1.0 mg/kg). Locomotor activity in the open field was recorded in naive animals on Day 1 and 15 minutes after each application on Days 7 and 14 of the Experiments using the Actitrack apparatus.

In the Experiment IV rats were randomly divided into three groups, all were administered vehicle intraperitoneally (2.0 ml/kg) and their ambulatory activity in the open field was recorded 15 min after application using the Actitrack apparatus (the 1st record) on Day 1. No observations or drug applications were carried out from Day 2 to Day 6. On Day 7, rats were given a dose of the drug treatment or vehicle (i.p.), followed, after 15 min, by the open field test (the 2nd record). Between Day 8 and Day 14, the animals in all groups were given once a day the same drugs at the same doses. On Day 14, ambulatory activity was recorded in the Actitrack apparatus (3rd record), 15 min after application. There was a pause without applications from Day 15 to Day 20. On Day 21 all animals in all groups received a "challenge dose" of methamphetamine (0.5 mg/kg) and 15 min after application their ambulation was measured in the Actitrack Apparatus (4th record). The drug treatments for Days 7 - 14 were provided in the following design: 1) methamphetamine at the dose of 0.5 mg/kg/day ($n_1 = 6$), 2) methanandamide at the dose of 1.0 mg/kg/day ($n_2 = 7$), 3) combined treatment with methamphetamine + AM 251 at the dose of 0.5 mg/kg/day and 2.0 mg/kg/day, respectively ($n_3 = 6$).

Statistical analysis

Animals in these experiments served as their own controls and because the data were not normally distributed (according to preliminary evaluation in the Kolmogorov-Smirnov test of normality), non-parametric statistics was used: Wilcoxon test, two tailed.

Results

The results obtained from Experiment I showed a significant ($p < 0.05$) increase in Distance Travelled after the acute administration of methamphetamine (5.0 mg/kg) compared to naive animals (the 1st record versus the 2nd record), whereas the "challenge dose" of methamphetamine led to a significant ($p < 0.05$) decrease in Distance Travelled (the 2nd record versus the 3rd record) - see Fig. 1. In this experiment we noticed quite frequent occurrence of stereotypic acts, namely nose rubbing, after the "challenge dose" of methamphetamine, however, an objective quantification was not available.

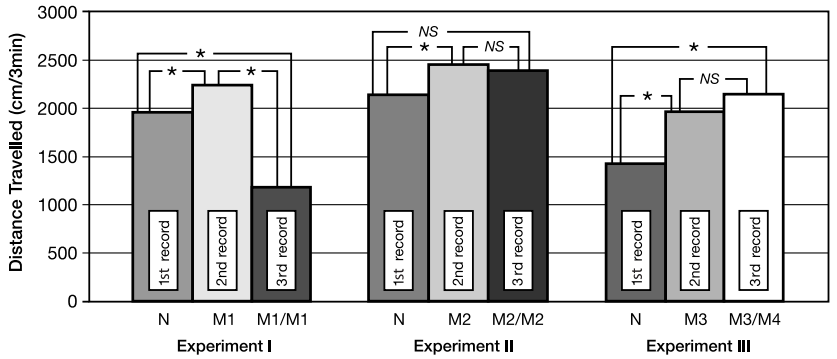
In Experiment II, the acute administration of methamphetamine (2.5 mg/kg) resulted in a significant ($p < 0.05$) increase in Distance Travelled (the 1st record versus the 2nd record), decrease in the same behavioural indicator was non-significant ($p > 0.05$) after the "challenge dose" (the 2nd record versus the 3rd record) (Fig. 1). Also in this experiment we observed an increased frequency of stereotypic nose rubbing after the "challenge dose" of methamphetamine. Unfortunately, similarly to the previous experiment, we were not able to quantify exactly this indicator of behaviour using our technical equipment.

In Experiment III, the acute administration of methamphetamine (2.5 mg/kg) elicited a significant ($p < 0.05$) increase in Distance Travelled (the 1st record versus the 2nd record) and the "challenge dose" of methamphetamine (1.0 mg/kg) following repeated methamphetamine pre-treatment with higher doses led to further increase in Distance Travelled (the 2nd record versus the 3rd record), nevertheless, this change was not significant ($p > 0.05$) (Fig. 1).

In Experiment IV, the acute administration of methamphetamine led to a significant increase ($p < 0.05$) in Distance Travelled (group n_1, the 2nd record) compared to the animals that were given vehicle (group n_1, the 1st record). Repeated administration of methamphetamine resulted in the same group in significant ($p < 0.05$) development of sensitisation (group n_1, the 3rd record versus the 2nd record) and this variable remained increased also after methamphetamine "challenge dose" on Day 21, following a pause lasting for six days without any applications (group n_1, the 4th record versus the 3rd record). Distance Travelled in the 4th record was also significantly increased ($p < 0.05$) comparing to data obtained in this variable during the 1st record (Fig. 2).

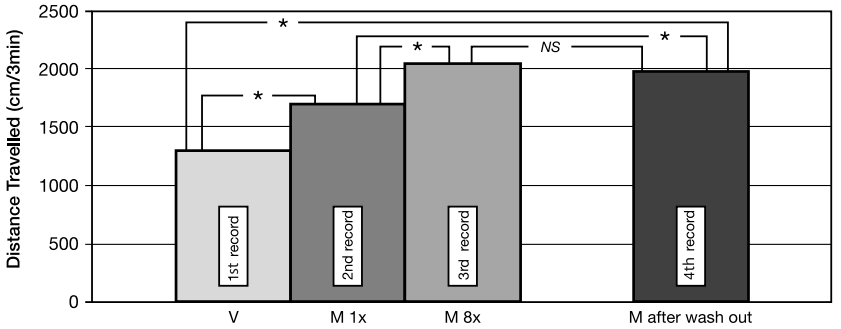
The acute administration of methanandamide resulted in a significant ($p < 0.01$) decrease in Distance Travelled (group n_2, the 2nd record) compared to the animals that were

Fig. 1. Effects of drug treatments in Experiments I, II and III on Distance Travelled (cm/3 min) in the rat open field test shown as median values.



N = naive rats, M1 = rats after the 1st dose of methamphetamine (5.0 mg/kg), M1/M1 rats pre-treated with methamphetamine (5.0 mg/kg) after the methamphetamine "challenge dose" (5.0 mg/kg), M2 = rats after the 1st dose of methamphetamine (2.5 mg/kg), M2/M2 rats pre-treated with methamphetamine (2.5 mg/kg) after the methamphetamine "challenge dose" (2.5 mg/kg), M3 = rats after the 1st dose of methamphetamine (2.5 mg/kg), M3/M4 rats pre-treated with methamphetamine (2.5 mg/kg) after the methamphetamine "challenge dose" (1.0 mg/kg)
* = $p < 0.05$, * * = $p < 0.01$, NS = non-significant - non-parametric Wilcoxon matched pairs test.

Fig. 2. Effects of drug treatments in Experiment IV (subgroup n_1) on Distance Travelled (cm/3 min) in the rat open field test shown as median values.

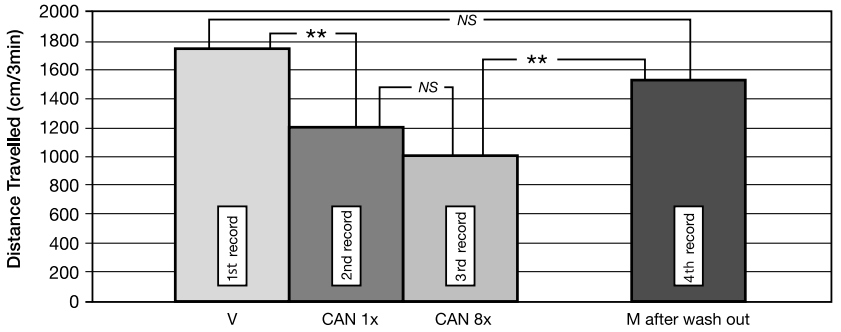


V = rats after the 1st dose of vehicle (2.0 ml/kg), M 1x = rats after the 1st dose of methamphetamine (0.5 mg/kg), M 8x = rats pre-treated with methamphetamine after the 8th methamphetamine dose (0.5 mg/kg), M after wash-out = rats pre-treated with methamphetamine after methamphetamine „challenge dose" (0.5 mg/kg) following six days lasting wash-out period
* = $p < 0.05$, * * = $p < 0.01$, NS = non-significant - the non-parametric Wilcoxon matched pairs test.

administered vehicle (group n_2, the1st record). Repeated administration of methanandamide elicited in the same group a more pronounced decrease in Distance Travelled (group n_2, the 3rd record versus the 2nd record); however, it did not reach statistical significance ($p > 0.05$). After the six day wash-out and application of methamphetamine "challenge dose" on Day 21 Distance Travelled in rats pre-treated with methanandamide was significantly increased (group n_2, the 4th record versus the 3rd record), nevertheless, there was no significant difference in this indicator between the 1st and the 4th record ($p > 0.05$) (Fig. 3).

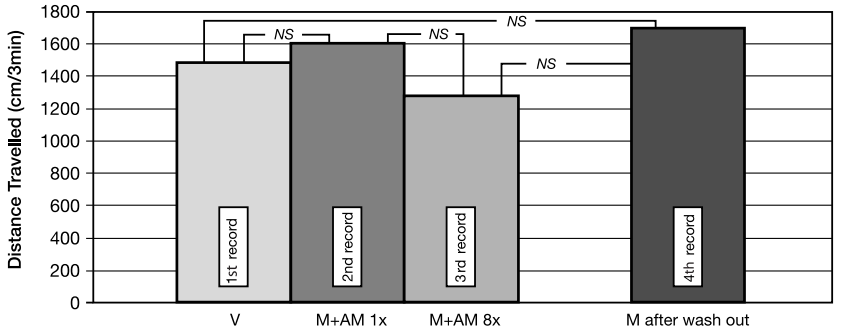
The acute use of a combined treatment with methamphetamine + AM 251 provoked non-significant ($p > 0.05$) changes in Distance Travelled (group n_3, the 2nd record)

Fig. 3. Effects of drug treatments in Experiment IV (subgroup n_2) on Distance Travelled (cm/3 min) in the rat open field test shown as median values.



V = rats after the 1st dose of vehicle (2.0 ml/kg), CAN 1x = rats after the 1st dose of methanandamide (1.0 mg/kg), CAN 8x = rats pre-treated with methanandamide after the 8th methanandamide dose (1.0 mg/kg), M after wash-out = rats pre-treated with methanandamide after methamphetamine "challenge dose" (0.5 mg/kg) following six days lasting wash-out period
* = $p < 0.05$, * * = $p < 0.01$, NS = non-significant – the non-parametric Wilcoxon matched pairs test.

Fig. 4. Effects of drug treatments in Experiment IV (subgroup n_3) on Distance Travelled (cm/3 min) in the rat open field test shown as median values.



V = rats after the 1st dose of vehicle (2.0 ml/kg), M + AM 1x = rats after the 1st dose of combined treatment methamphetamine (0.5 mg/kg) + AM 251 (2.0 mg/kg), M + AM 8x = rats pre-treated with the combined treatment after the 8th dose of this combination (methamphetamine [0.5 mg/kg] + AM 251 [2.0 mg/kg]), M after wash-out = rats pre-treated with the combination of methamphetamine + AM 251 after methamphetamine "challenge dose" (0.5 mg/kg) following six days lasting wash-out period
* = $p < 0.05$, * * = $p < 0.01$, NS = non-significant - the non-parametric Wilcoxon matched pairs test.

compared to the animals that were administered vehicle (group n_3, the 1st record). Repeated administration of the combined treatment led to a non-significant decrease ($p > 0.05$) in Distance Travelled (group n_3, the 3rd record versus the 2nd record). The "challenge dose" of methamphetamine given after the wash-out on Day 21 did not result in significant changes in Distance Travelled (group n_3, the 4th record versus the 3rd record) and the difference between the 4th record and the 1st record also did not reach statistical significance ($p > 0.05$) (Fig. 4).

Discussion

The results of the study of the impact of cannabinoid receptor ligands on sensitisation to methamphetamine effects on locomotor behaviour in rats were not consistent in subsequent

experiments. When compared to acute methamphetamine effects, decreased behavioural responses (development of tolerance?) were manifested after repeated administration of methamphetamine (significant after the dose of 5.0 mg/kg/day; just a trend without statistical significance after the lower dose of 2.5 mg/kg/day, which stimulated locomotion when given acutely) in the same experimental design used earlier in mice in which development of sensitisation (increased behavioural response) to stimulatory effect on locomotion was unambiguously confirmed instead. Nevertheless, in these experiments we observed an increased number of stereotypic acts after the "challenge dose" of methamphetamine, namely increased frequency of nose rubbing after both doses used. The occurrence of this is in accordance with findings of other authors (Laviola et al. 1999) and is suggested to express behavioural sensitisation, too. Unfortunately, we were not able to quantify exactly this indicator of behaviour using our technical equipment.

Concerning these in fact unexpected results of the first two rat experiments we wished to discern whether the decreased locomotor activity after repeated methamphetamine treatment with doses of 2.5 and 5.0 mg/kg was not a result of a too high dosage regimen. Therefore, in the next rat experiment animals were repeatedly pre-treated with the dose of 2.5 mg/kg but the "challenge dose" was only 1 mg/kg. In this experiment we demonstrated a clear trend of increased locomotor activity measured after the "challenge dose" as a sign of potential behavioural sensitisation, although the changes still did not reach statistical significance.

In the further rat experiment we decided to check if the sensitising potential of methamphetamine can be more clearly manifested as an "expression of behavioural sensitisation" when testing of the "challenge dose" (0.5 mg/kg) effects is done after six days of wash-out from repeated drug treatment (0.5 mg/kg/day). Finally, in this last rat experiment both expression and development of behavioural sensitisation to methamphetamine effects on locomotor rat behaviour in the open field occurred.

Thus, these results show that, as previously in mice (Landa et al. 2006a), a repeated administration of methamphetamine can under certain circumstances elicit behavioural sensitisation to its stimulatory effects also in rats, which is in accordance with another report (Fukami et al. 2004). However, we were not able to provoke cross-sensitisation by repeated application of cannabinoid CB_1 receptor agonist methanandamide, and similarly, we did not demonstrate suppression of the cross-sensitisation in rats that were repeatedly given combined pre-treatment of cannabinoid CB_1 receptor antagonist AM 251 and methamphetamine as it was demonstrated in research carried out in mice. Possibly, the suggested interaction between the endocannabinoid system and processing of psychostimulant action of methamphetamine requires a cannabinoid dose that itself does not produce inhibitory effects on locomotion which was found in the present rat study, but not previously in mice.

Some authors discuss the role of habituation in rodents and data available from literature and concerning a possible influence of habituation on the behavioural sensitisation are not completely uniform. Ohmori et al. (2000) mention in their review that animals given a stimulant repeatedly in a test cage but not in other environments may show enhanced drug-induced behaviour in the test cage. Crombag et al. (2001) report that doses of amphetamine or cocaine that fail to induce psychomotor sensitization when given to a rat in its home cage can produce robust sensitisation if given immediately following placement into a relatively novel, distinct environment. They found that the acute psychomotor response produced by an i.v. injection of 0.5 mg/kg amphetamine and the psychomotor sensitisation produced by repeated injections were greater when the drug treatments were given immediately after animals were placed into a distinct and relatively novel test environment, compared to when treatments were performed in a physically identical environment, but in which the animals lived (i.e., at home). Furthermore, habituation to the test environment for

6 - 8 h immediately prior to the drug administration completely abolished the effect of environmental novelty on the acute psychomotor response to amphetamine. This is not to a certain extent in accordance with our findings as our experimental design excluded habituation and despite this we were not able to provoke behavioural sensitisation in the first three rat experiments.

In conclusion, our investigation in these rat experiments showed that there are some interspecies differences in respect of neuronal plasticity changes induced by methamphetamine and underlying behavioural sensitisation to its effects. Those deserve to be analysed further in a wider dose range and with a particular interest paid to the rat stereotypic behaviour observed in our study. Some other authors become increasingly aware of not only species differences but also of strain differences in sensitisation to locomotor stimulation - e.g. after administration of morphine (Shuster 1984), which indicates that this phenomenon could also contribute to the differences between species and their susceptibility to drugs of abuse and in this way to possible elicitation of behavioural sensitisation. For instance, stimulatory effects of cocaine and amphetamine are larger in the Lewis rats than in the Fischer rats and furthermore the Lewis strain is more susceptible to the development of behavioural sensitisation than the animals of the Fischer strain (Kosten et al. 1994). These line differences in behavioural responses to the psychostimulants may be due to the larger amphetamine- and cocaine-induced increase of accumbal dopamine release in animals of the Lewis strain than in those of the Fischer strain (Camp et al. 1994). However, there is some evidence that these dissimilarities are at least partially due to differences in the bioavailability of these drugs (Camp et al. 1994).

Other authors make an attempt to evaluate the age-related differences in amphetamine and methamphetamine sensitization (Fujiwara et al. 1987; Kolta et al. 1990), noting that adult rats pre-treated with amphetamines display an augmentation of locomotor response when subsequently given an amphetamine "challenge dose". It has been shown that this sensitisation response does not occur until 3 - 4 weeks of age. The authors suggested that the appearance of mature presynaptic dopamine autoreceptors may be necessary for sensitisation (Fujiwara et al. 1987) or that maturation of dopamine reuptake sites is the limiting factor in the development of sensitisation (Fujiwara et al. 1987). Nevertheless, these findings related to the age of experimental animals are not in contradiction to our studies, as the age of the rats used for our purposes was about seven weeks at the beginning of each experiment.

Vliv ligandů kanabinoidních receptorů na sensitizaci k účinkům metamfetaminu - ovlivnění lokomoční aktivity u potkanů

Opakovaná aplikace různých zneužívaných látek může vést k postupně se zvyšující behaviorální odpovědi na tyto látky, zejména ke zvýšení lokomoce a možnému výskytu stereotypií. Tento fenomén je dobře znám a popsán jako behaviorální sensitizace. Zvýšená odpověď na testovanou látku vyvolaná předchozí opakovanou aplikací látky odlišné je popisována jako zkřížená sensitizace. Na základě našich předchozích experimentů uskutečněných na myších, ve kterých byla potvrzena sensitizace k metamfetaminu a popsána zkřížená sensitizace k metamfetaminu po předchozí aplikaci agonisty kanabinoidních CB_1 receptorů metanandamidu, jsme se v této studii zaměřili na užití jiného typického laboratorního zvířete - potkana. Pokud by výsledky studií u myší a potkanů byly podobné, zvýšila by se biologická validita sensitizačního fenoménu. Podobný výzkum u potkanů přinesl velmi podobné výsledky popsané dříve u myší. Nicméně, zaregistrovali jsme alespoň některé mezidruhové rozdíly ve vnímavosti potkanů k rozvoji sensitizace k metamfetaminovým účinkům. Ve srovnání s myším modelem bylo náročnější vytitrovat dávku metamfetaminu, která by behaviorální sensitizaci vyvolala. Dále jsme nebyli schopni vyvolat

zkříženou sensitizaci pomocí opakované aplikace agonisty CB_1 kanabinoidních receptorů metanandamidu a podobně se nám nepodařilo demonstrovat potlačení zkřížené sensitizace u potkanů, kterým byla opakovaně podávána kombinace antagonisty kanabinoidních CB_1 receptorů látky AM 251 a metamfetaminu. Konečně, na rozdíl od myší, jsme namísto toho po opakované aplikaci metamfetaminu zaznamenali alternativní behaviorální změnu - výskyt stereotypního chování (otírání nosu).

Acknowledgement

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7.4 Altered cannabinoid CB_1 receptor mRNA expression in mesencephalon from mice exposed to repeated methamphetamine and methanandamide treatments

It is known that the structures involved in the processes of sensitization to psychostimulants are found in the midbrain (particularly in the ventral tegmental area), and moreover that this region contains cannabinoid CB_1 receptors (Maldonado et al. 2006). Therefore, this study was aimed at possible changes in the relative expression of CB_1 receptor mRNA in the mouse mesencephalon during sensitization to methamphetamine and cross-sensitization to methamphetamine induced by repeated pretreatment with methanandamide (a cannabinoid CB_1 receptor agonist) using a quantitative polymerase chain reaction (PCR).

The behavioural part of this experiment confirmed both the development of sensitization to methamphetamine stimulatory effects on mouse locomotor behaviour and cross-sensitization to such effects caused by pre-treatment with the cannabinoid CB_1 receptor agonist methanandamide prior to the methamphetamine challenge dose. Both these findings are consistent with our previous studies (Landa et al. 2006a; b), as well with suggestions by other authors (e.g.: Chiang and Chen 2007; Wiskerke et al. 2008; Panlilio et al. 2010).

Real-time PCR analyses brought rather controversial results. Neither single nor repeated methamphetamine administration caused a significant increase in the relative expression of CB_1 receptor mRNA in the mouse mesencephalon. We did however found an increase in CB_1 receptor mRNA expression after the first dose of methanandamide, which was followed by a decrease after the methamphetamine challenge dose.

Landa, L., Jurajda, M., Sulcova, A. Altered cannabinoid CB_1 receptor mRNA expression in mesencephalon from mice exposed to repeated methamphetamine and methanandamide treatments. Neuroendocrinology Letters, 2011, 32 (6), 841-846.

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Altered cannabinoid CB_1 receptor mRNA expression in mesencephalon from mice exposed to repeated methamphetamine and methanandamide treatments

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Abstract

OBJECTIVES: Since among others also our previous studies suggested an interaction between the endocannabinoid system and methamphetamine brain mechanisms we focused on possible changes in relative expression of cannabinoid CB_1 receptor mRNA in mesencephalon from mice sensitized by repeated treatments to methamphetamine stimulatory effects and cross-sensitized by cannabinoid CB_1 receptor agonist methanandamide pre-treatment.

METHODS: The Open Field Test was used to measure changes in terms of behavioural sensitization or cross-sensitization to drug effects on locomotion in male mice treated repeatedly with either methamphetamine or methamphetamine after pre-treatment with methanandamide. After each measurement one third of animals were sacrificed and the brain was stored. RNA was isolated from the midbrain and used for reverse transcription and subsequent real-time PCR.

RESULTS AND CONCLUSION: The evaluation of behavioural drug effects showed both development of sensitization to methamphetamine stimulatory effects after repeated treatment and cross-sensitization to them by pre-treatment with cannabinoid receptor CB_1 agonist methanandamide. Real-time PCR analyses revealed an increase in CB_1 receptor mRNA expression after the first dose of methanandamide followed by decrease after the combined treatment with methamphetamine challenge dose. Our findings suggest that particularly repeated pre-treatment with CB_1 agonist methanandamide can elicit increase in the mRNA expression level at least in the mouse mesencephalon neurons associated with cross-sensitization to methamphetamine stimulatory effects.

ORIGINAL ARTICLE

Abbreviations:

Bmax	- maximal binding capacity
CAN	- mice after the 1st dose of methanandamide
CAN/M	- mice sensitized with methanandamide after the challenge dose of methamphetamine
DA	- dopamine
GAPDH	- glyceraldehyde-3-phosphate dehydrogenase
M	- mice after the 1st dose of methamphetamine
M/M	- mice sensitized with methamphetamine after the challenge dose of methamphetamine
V	- mice after the dose of vehicle
VTA	- ventral tegmental area

INTRODUCTION

Repeated administration of various psychotropic drugs can elicit behavioural sensitization – a phenomenon characterised by gradually increasing response to the drug (Robinson & Berridge 1993). This phenomenon has been well described for majority of addictive substances including amphetamines (Kameda *et al.* 2011) and cannabinoids (Rubino *et al.* 2003). An increased response to the tested drug may be also elicited by previous repeated administration of a drug different from the drug tested, which is termed as cross-sensitization. Cross-sensitization was observed, for example, after repeated treatment with tetrahydrocannabinol to heroin (Singh *et al.* 2005).

It has been identified, that the crucial neuronal circuits essential for the development of sensitization involve namely dopaminergic, glutamatergic, GABAergic and serotonergic projections between VTA, nucleus accumbens, prefrontal cortex, hippocampus and amygdala (Ago *et al.* 2008). Particularly, the mesolimbic dopaminergic projection from the VTA to nucleus accumbens is considered as the most important for effects associated with reward properties of abused drugs (Kalivas *et al.* 1993). Stimulation of cannabinoid CB_1 receptors present on GABAergic and glutamatergic nerve terminals negatively regulates the release of GABA and glutamate and that way influence the mesolimbic DA functions (Chiang & Chen 2007). The endocannabinoid system consists of cannabinoid receptors (CB_1, CB_2), their endogenous ligands (endocannabinoids), and enzymes for their biosynthesis and degradation. It is known, that CB_1 receptors located in VTA on presynaptic glutamatergic and GABAergic neurons act as retrograde inhibiting modulators and influence their input to VTA dopaminergic neurons which is believed to activate the reward pathway of addictive substances (Maldonado *et al.* 2006).

The first results from our laboratory suggesting an interaction between the endocannabinoid system and methamphetamine brain mechanisms were obtained in the rat I.V. drug self-administration model (Vinklerova *et al.* 2002). Later we have created an original experimental paradigm showing development of behavioural sensitization to psychostimulant methamphetamine effects and also cross-sensitization elicited by cannabinoid CB_1 receptor agonist methanandamide pretreatment (Landa *et al.* 2006a;b) confirming that there exists some relationship between the endocannabinoid system and methamphetamine effect processing.

The present study was designed with respect to results obtained in our previous behavioural studies as well as in the preliminary pilot studies focusing on CB_1 receptor expression (Landa & Jurajda 2007a;b) and density (Sulcova *et al.* 2007) in rodent mesencephalon, and to data confirming that structures responsible for the development of behavioural sensitization to psychostimulants (including methamphetamine) are parts of mesencephalon (namely VTA) with high CB_1 receptor density (Ago *et al.* 2008). The attention was focused on possible changes revealed by quantitative polymerase chain reaction (qPCR) in relative expression of CB_1 receptor mRNA in mouse mesencephalon during a) sensitization to methamphetamine and b) cross-sensitization to methamphetamine induced by repeated pretreatment with CB_1 receptor agonist methanandamide.

MATERIAL AND METHODS

Animals

Male mice (strain ICR, TOP-VELAZ s. r. o., Prague, Czech Republic) with an initial weight of 18–21 g were used. They were randomly allocated into two treatment groups. Experimental sessions in the behavioural part of the experiment were always performed in the same light period between 1:00 p.m. and 3:00 p.m. in order to minimise possible variability due to circadian rhythms.

Apparatus

Locomotor activity was measured using an open-field equipped with Actitrack (Panlab, S.L., Spain). This device consists of two square-shaped frames that deliver beams of infrared rays into the space inside the square. A plastic box is placed in this square to act as an open-field arena (base 30 × 30 cm, height 20 cm), in which the animal can move freely. The apparatus software records locomotor activity of the animal by registering the beam interruptions caused by movements of the body. Using this equipment we have determined the Distance Travelled (trajectory in cm per 3 minutes).

Drugs

Vehicle and all drugs were always given in a volume adequate to drug solutions (10 ml/kg).

(+)-Methamphetamine, (d-N,α-Dimethylphenylethylamine; d-Desoxyephedrine), (Sigma Chemical Co.) dissolved in saline.

(R)-(+)-Methanandamide, (R)-N-(2-hydroxy-1-methylethyl)-5Z, 8Z, 11Z-eicosotetraenamide) supplied pre-dissolved in anhydrous ethanol 5 mg/ml (Tocris Cookson Ltd., UK) was diluted in saline to the concentration giving the chosen dose to be administered to animals in a volume of 10 ml/kg; vehicle therefore contained an adequate part of ethanol (a final concen-

tration in the injection below 1%) to make effects of placebo and the drug comparable.

Procedure

Mice were randomly divided into 2 groups (n_1=24, n_2=24) and all were given vehicle on Day 1 (10 ml/kg). There were no applications from Days 2 to 6. For the next seven days animals were daily treated intraperitoneally as follows: a) n_1: methamphetamine at the dose of 2.5 mg/kg/day, b) n_2: methanandamide at the dose of 0.5 mg/kg. On Day 14 all animals were given intraperitoneally methamphetamine at the dose of 2.5 mg/kg (challenge dose).

Changes in locomotion were measured for the period of 3 minutes in the open field on Days 1 (1st record), 7 (2nd record) and 14 (3rd record) 15 minutes after drug application to assess sensitizing phenomenon. After each measurement one third of both groups was decapitated (75 minutes after drug administration) and the brain was stored in RNAlater (Ambion). For RNA isolation we used excised mesencephalon only. The total RNA was isolated by means of RNAEasy Mini Kit (Qiagene) and the subsequent reverse transcription was performed with Omniscript RT Kit (Qiagene) and RNAse OUT Ribonuclease Inhibitor (Invitrogen). Relative expression of CB_1 receptor (assay Mn00432621_s, Life Technologies) was compared to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (assay Mn99999915_1g, Life Technologies) using real time cycler ABI SDS 7000 (AppliedBiosystems). All real time PCR reactions were performed using TaqMan Gene Expression Master Mix (Life Technologies).

Data analysis

As the data was not normally distributed (according to the Kolmogorov-Smirnov test of normality), non-parametric statistics were used: Mann-Whitney U test, two-tailed (statistical analysis package STATISTICA – StatSoft, Inc., Tulsa, USA).

RESULTS

In the behavioural part of the study (Figure 1), the treatments in the group n_1 caused significant increase (p<0.01) in locomotion after the 1st application of methamphetamine (M) compared to the application of vehicle (V1) (see Figure 1; V1 versus M). The challenge dose of M produced a significant increase in Distance Travelled (p<0.05) in animals pre-treated repeatedly with M when compared to the animals after the 1st application of M (see Figure 1; M versus M/M).

The 1st applications of methanandamide (CAN) compared to the application of vehicle (V2) evoked in the group n_2 significant decrease (p<0.01) in locomotion (see Figure 1, V2 versus CAN). The challenge dose of M produced a significant increase in Distance Travelled (p<0.01) in animals pre-treated repeatedly with

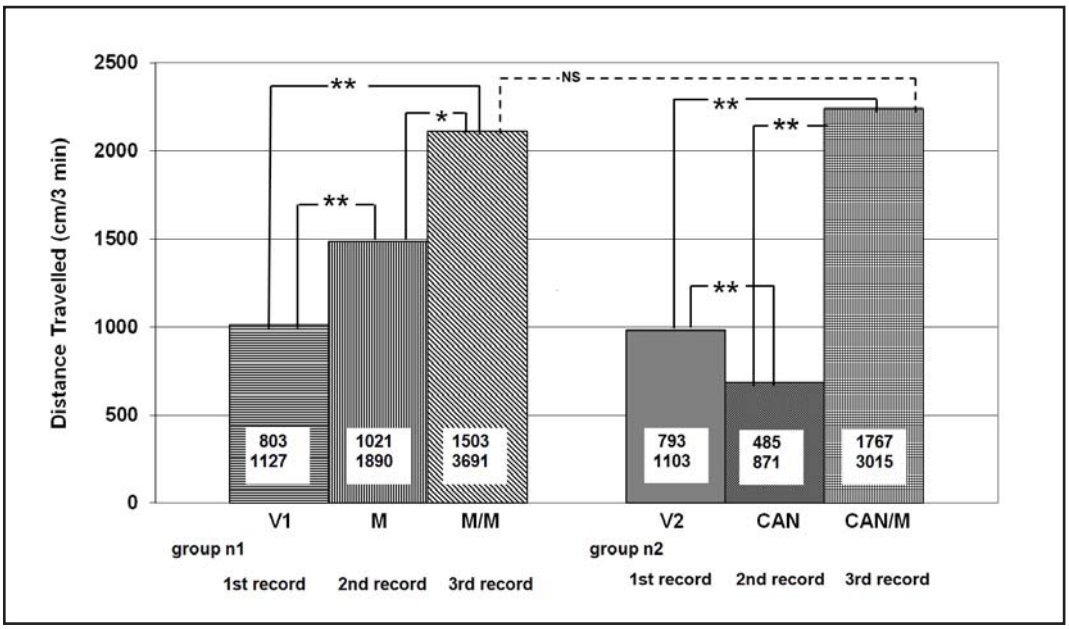

Fig. 1. Effects of drug treatments on Distance Travelled (cm/3 min) in the mouse open field test shown as median (interquartile range Q1 to Q3):
V1 = mice after the dose of vehicle in the group n_1, V2 = mice after the dose of vehicle in the group n_2, M = mice after the 1st dose of methamphetamine (2.5 mg/kg), M/M = mice sensitized with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg), CAN = mice after the 1st dose of methanandamide (0.5 mg/kg), CAN/M = mice sensitized with methanandamide after the challenge dose of methamphetamine (2.5 mg/kg)
*p<0.05, **p<0.01, NS = non-significant, the nonparametric Mann-Whitney U test, two tailed.

CAN when compared to the animals after the 1st application of CAN (see Figure 1; CAN versus CAN/M).

Real-time PCR results showed no significant changes after various treatments in the group n_1 (see Figure 2; V1 and M versus M/M). The treatments in the group n_2 caused significant increase ($p<0.01$) in relative expression of CB_1 receptor mRNA after the 1st application of CAN compared to the application of vehicle (V2) (see Figure 2; V2 versus CAN). The challenge dose of M produced a significant decrease in relative expression of CB_1 receptor mRNA ($p<0.05$) in animals pre-treated repeatedly with CAN when compared to the animals after the 1st application of CAN (see Figure 2; CAN versus CAN/M).

There was no significant change in relative expression of CB_1 receptor mRNA between animals after the MET challenge dose (those were pre-treated with MET) and animals after the MET challenge dose (those were pre-treated with CAN) – see Figure 2; M/M versus CAN/M.

DISCUSSION

The behavioural part of this study confirmed both development of sensitization to methamphetamine stimulatory effects on mouse locomotor behaviour during its repeated administration and cross-sensitization to such effects caused by pre-treatment with cannabinoid CB_1 receptor agonist methanandamide prior to methamphetamine challenge dose administration. Both these findings are in accordance with our previous experimental experiences (Landa *et al.* 2006a;b) as well as suggestions of some others (e.g.: Cadoni et. al. 2001; Wolf *et al.* 2002; Tanda & Goldberg 2003; Chiang & Chen 2007; Wiskerke *et al.* 2008; Panlilio *et al.* 2010).

Neurobiological mechanisms underlying phenomenon of behavioural cross-sensitization are believed to increase vulnerability for use of other drugs of abuse (Steketee & Kalivas 2011). In the case of psychostimulants (including methamphetamine) and cannabinoids it is believed that they induce increase in dopamine activation in the mesolimbic reward pathway. The stimulation of specific cannabinoid CB_1 receptor relieves suppression upon dopaminergic neurons, leading to dopamine release and thus facilitates responses to administration of psychostimulants. However, all outcomes of studies oriented towards involvement of CB_1 receptor in effects of amphetamines have not been consistent (e.g.: Ellgren *et al.* 2004; Solinas *et al.* 2007; Thiemann *et al.* 2008; Panlilio *et al.* 2010).

The part of the present study dealing with relationship between cannabinoid CB_1 receptor agonist methanandamide and methamphetamine effects on the level of CB_1 receptor mRNA expression brought rather controversial results, too. Neither single nor repeated methamphetamine dose of 2.5 mg/kg caused significant increase in relative expression of CB_1 receptor mRNA in the mouse mesencephalon (just a trend to stimulation

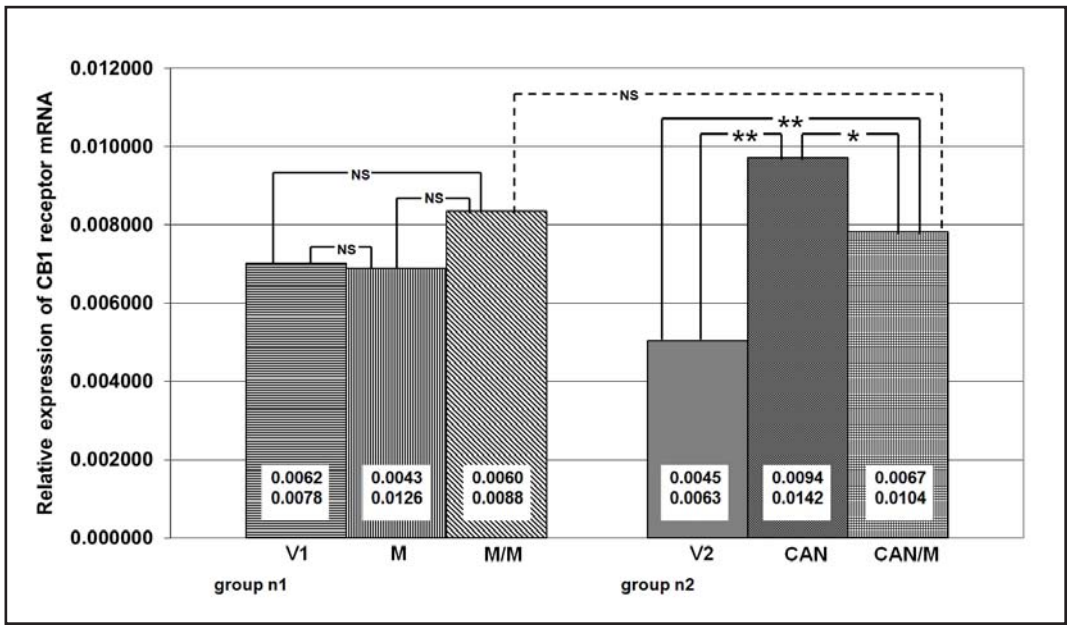

Fig. 2. Effects of drug treatments on relative expression of CB_1 receptor mRNA when compared to GAPDH mRNA shown as median (interquartile range Q1 to Q3):
V1 = mice after the dose of vehicle in the group n_1, V2 = mice after the dose of vehicle in the group n_2, M = mice after the 1st dose of methamphetamine (2.5 mg/kg), M/M = mice sensitized with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg), CAN = mice after the 1st dose of methanandamide (0.5 mg/kg), CAN/M = mice sensitized with methanandamide after the challenge dose of methamphetamine (2.5 mg/kg).
*$p<0.05$, **$p<0.01$, NS = non-significant, the nonparametric Mann-Whitney U test, two tailed.

of expression was registered after the repeated treatment). Increased CB_1 receptor expression across rat brain regions including medial prefrontal cortex, striatum, amygdaloid complex and hippocampal formation was reported after the exposure to methamphetamine treatment, however, with the dosing regimen (4 mg/kg, subcutaneously × 4 injections, 2 h apart), inducing neurotoxic effects (Bortolato *et al.* 2010).

On the contrary, there was measured a decrease in numbers of CB_1 receptor (both Bmax and mRNA) in the mouse nucleus accumbens after the repeated chronic methamphetamine administration (4 mg/kg/day) developing behavioural sensitization while microinjection of CB_1 antagonist into the nucleus accumbens suppressed the behavioural sensitization to methamphetamine (Chiang & Chen 2007). The activation of the CB_1 receptor was evaluated as a cause facilitating adaptive responses to psychostimulants, such as reduction of dopamine and serotonin turnovers resulting in sensitization (Thiemann *et al.* 2008). However, the density of cannabinoid CB_1 receptor mRNA-positive neurons was significantly lower in Cannabis sativa users (Villares 2007).

Thus the mechanisms that regulate CB_1 receptor modifications are far from being completely understood. Moreover adaptations vary by brain region (Sim-Selley 2003) and the results of studies dealing with CB_1 receptor density are dependent also on the method used (e.g. receptor binding, mRNA expression, immunofluorescence). There is also evidence that internalization of CB_1 receptors following agonist treatment can occur (Coutts *et al.* 2001). This could be a reason for discrepant results we have obtained in the present study using PCR evaluation of the relative expression of CB_1 mRNA comparing to another one with immunofluorescent detection of receptors the intensity of which was assayed by image analysis (Sulcova *et al.* 2007). The latter one showed on the surface of VTA neuronal membranes in rats sensitized to methamphetamine I.V. self-administration decreased density of cannabinoid CB_1 receptors while in the present study a trend to the increase in expression of CB_1 receptors in methamphetamine sensitized mice was found.

In spite that the increased expression of CB_1 receptor was associated in the present study with methanadamide cross-sensitization to methamphetamine effects on mouse locomotion, there was measured after the drug challenge dose significantly lower expression of CB_1 receptor but still significantly higher than under the influence of vehicle treatment and with no difference from the level in mice pretreated repeatedly with methamphetamine. Nevertheless, increased expression of CB_1 receptor in mesencephalon was associated with higher sensitivity to methamphetamine psychostimulatory effects.

This is in agreement with findings that CB_1 knockout mice as well as wild type mice pre-treated with CB_1 receptor inverse agonist AM 251 were less sensitive to the psychomotor stimulant as well as locomotor sensitizing effects of amphetamine (Thiemann 2008), and to some extent are also consistent with our earlier study (Vinklerova *et al.* 2002) in which self-administration of methamphetamine was reduced by AM 251, and increased by methanandamide.

In conclusion, the results of the present study brought further evidence that modulation of CB_1 receptor expression may play an important role in behavioural responses to methamphetamine. Pharmacological support of CB_1 receptor activity may increase expression of CB_1 receptor mRNA associated with sensitization to methamphetamine stimulatory effects what supports the hypothesis on increased vulnerability to methamphetamine abuse after neuroplastic changes induced by cannabinoid CB_1 receptor agonists including delta-9-tetrahydrocannabinol from marijuana.

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7.5 Altered dopamine D_1 and D_2 receptor mRNA expression in mesencephalon from mice exposed to repeated treatments with methamphetamine and cannabinoid CB_1 agonist methanandamide

A reciprocal cross-talk was reported among the cannabinoid CB_1 and dopamine D_1 and D_2 receptors, which are highly co-localised on brain neurones (Hermann et al. 2002; Terzian et al. 2011). This experiment was therefore focused on possible changes in the expression of dopamine D_1 and D_2 receptor mRNA in the mouse mesencephalon during sensitization to methamphetamine and cross-sensitization to methamphetamine induced by repeated pre-treatment with methanandamide (a cannabinoid CB_1 receptor agonist) using PCR.

The behavioural part of the present experiment focusing on changes in mouse locomotor behaviour fully confirmed our earlier outcomes published elsewhere (Landa et al. 2011). These results included the development of behavioural sensitization to methamphetamine stimulatory effects after repeated treatment with methamphetamine and the development of cross-sensitization to these effects after repeated pre-treatment with the cannabinoid CB_1 receptor methanadamide prior to the methamphetamine challenge dose.

Real-time PCR analyses revealed an increase in D_1 receptor mRNA expression after the first dose of methamphetamine (which persisted also after the last dose of methamphetamine) and after the first dose of methanandamide (which also persisted after the methamphetamine challenge dose). On the other hand, a significant decrease in D_2 receptor mRNA expression both after the first dose of methamphetamine and methanandamide was found (which persisted also after the methamphetamine challenge doses).

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ORIGINAL ARTICLE

Altered dopamine D_1 and D_2 receptor mRNA expression in mesencephalon from mice exposed to repeated treatments with methamphetamine and cannabinoid CB_1 agonist methanandamide

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Abstract

OBJECTIVES: In our previous studies we found that both acute administration of CB_1 receptor agonist methanandamide and repeated methanandamide pre-treatment prior to methamphetamine challenge dose elicited increase in the CB_1 receptor mRNA expression in the mouse mesencephalon. As a reciprocal cross-talk is reported between the cannabinoid CB_1 and dopamine receptors, that are highly co-localized on brain neurones, we targeted possible changes in relative expression of dopamine D_1 and D_2 receptor mRNA in mesencephalon in mice sensitized by repeated treatments to methamphetamine stimulatory effects and cross-sensitized to methamphetamine by cannabinoid CB_1 receptor agonist methanandamide pre-treatment.

METHODS: To confirm development of behavioural sensitization or cross-sensitization, respectively, we observed changes in locomotion using the open field test. Mice were treated repeatedly with either methamphetamine or methamphetamine after repeated pre-treatment with methanandamide. After each measurement of locomotion one third of animals were sacrificed and the brain was stored. RNA was isolated from the midbrain and used for reverse transcription and subsequent real-time PCR.

RESULTS AND CONCLUSION: As in many of our earlier studies with the same dosage regimen we found in the behavioural part both development of sensitization to methamphetamine stimulatory effects after repeated treatment and cross-sensitization to them by pre-treatment with cannabinoid receptor CB_1 agonist methanandamide. Real-time PCR analyses showed an increase in D_1 receptor mRNA expression after the first dose of methamphetamine (that persisted also after the last dose of methamphetamine) and after the first dose of methanandamide (which also persisted after the methamphetamine challenge dose). In opposite a significant decrease in D_2 receptor mRNA expression both after the first dose of methamphetamine and methanandamide (that persisted also after

the methamphetamine challenge doses) was registered. Thus, our results suggest that both methamphetmine and methanandamide treatment can provoke changes in dopamine receptor density in mouse mesenpcephalon, the increase in D_1 and decrease in D_2 receptor subtypes.

Abbreviations:

CAN	- mice after the 1st dose of methanandamide
CAN/M	- mice sensitized with methanandamide after the challenge dose of methamphetamine
GAPDH	- glyceraldehyde-3-phosphate dehydrogenase
ISHH	- *in situ* hybridization histochemistry
M	- mice after the 1st dose of methamphetamine
M/M	- mice sensitized with methamphetamine after the challenge dose of methamphetamine
PET	- positron emission tomography
V	- mice after the dose of vehicle
VTA	- ventral tegmental area

INTRODUCTION

Increased behavioural response to certain drug conditioned by its previous repeated administration is well-known as behavioural sensitization (Robinson & Berridge, 1993). It has been manifested for the whole range of psychotropic drugs such as amphetamines (Wang *et al.* 2010), cannabinoids (Rubino *et al.* 2003) or opiods (Bailey *et al.* 2010; Liang *et al.* 2010). Moreover, an increased response to a drug tested elicited by previous repeated pre-exposure to another drug is recognized as cross-sensitization; e.g. cross-sensitization between metylphenidate and amphetamine was observed (Yang *et al.* 2011), cross-sensitization with cannabinoid agonist WIN 55,2122 to morphine has been described (Manzanedo *et al.* 2004) or animals pre-treated with amphetamine displayed behavioural cross-sensitization to nicotine and vice versa animals pre-treated with nicotine showed sensitized locomotor response to amphetamine (Santos *et al.* 2009).

Both behavioural sensitization and cross-sensitization represent enduring changes in drug response and although not all neuronal processes involved in these phenomena have been fully elucidated yet, it is clear that the crucial neuronal circuit essential for the development of sensitization involves numerous structures in the central nervous system. Neuroadaptive changes occurred namely in a circuit comprising dopaminergic, GABAergic and glutamatergic interconnections between the ventral tegmental area (VTA), nucleus accumbens, prefrontal cortex and amygdala (Nestler, 2001a; b). Kalivas *et al.* (1993) suggest that the mesolimbic dopaminergic projection from the VTA to nucleus accumbens plays the key role for effects associated with reward properties of abused drugs. In addition, it is well known that dopamine plays a crucial role in the development of behavioural sensitization. This was also confirmed when the established behavioural sensitization to methamphetamine was reversed by administration of dopamine D_1 receptor antagonist R-(+)-SKF38393 (Shuto *et al.* 2006) and signs of behavioural sensitization to amphetamine were decreased by the D_1 receptor antagonist SCH23390 (stereotypical behaviour) and the D_2 receptor antagonist eticlopride (all behavioural activities) (Shi & McGinty, 2011).

An earlier study realized at our laboratory suggested interaction between the endocannabinoid system and methamphetamine brain mechanisms in the rat I.V. drug self-administration model (Vinklerova *et al.* 2002). Furthermore, this finding was confirmed by following research when we provoked behavioural sensitization to psychostimulant methamphetamine and also cross-sensitization to this drug elicited by cannabinoid CB_1 receptor agonist methanandamide pre-treatment (Landa *et al.* 2006a; b).

Our recent study concerning behavioural sensitization to methamphetamine was focused on neuroplastic changes on genomic level. We found that repeated pre-treatment with CB_1 receptor agonist methanandamide elicited increase in the CB_1 receptor mRNA expression in the mouse mesencephalon neurons (Landa *et al.* 2011). Since stimulation of cannabinoid CB_1 receptors present on GABAergic and glutamatergic nerve terminals negatively regulated the release of GABA and glutamate and in this manner affected the mesolimbic dopamine functions (Kelley & Berridge, 2002; Chiang & Chen, 2007) and since a reciprocal cross-talk was reported among the cannabinoid CB_1 and dopamine D_1 and D_2 receptors, which are highly co-localized on brain neurones (Glass and Felder, 1997; de Fonseca *et al.* 1998; Beltramo at al. 2000; Hermann *et al.* 2002; Kearn *et al.* 2005; Martín *et al.* 2008; Dalton & Zavitsanou, 2010; Dowie *et al.* 2010; Terzian *et al.* 2011), we decided to extend the above mentioned research project to these dopamine receptors, too.

With reference to results obtained in our pilot studies focusing on relative expression of D_1 and D_2 receptors (Landa & Jurajda, 2008a; b; c) we designed the present study to reveal possible changes in expression of D_1 and D_2 receptor mRNA in mouse mesencephalon (that involves VTA) by quantitative polymerase chain reaction (qPCR) during: a) sensitization to methamphetamine and b) cross-sensitization to methamphetamine induced by repeated pre-treatment with CB_1 receptor agonist methanandamide.

MATERIAL AND METHODS

Animals

Male mice (strain ICR, TOP-VELAZ s. r. o., Prague, Czech Republic) with an initial weight of 18–21 g were used. Animals were randomly allocated into two treatment groups. In order to minimise possible variability due to circadian rhythms the behavioural observations were always performed in the same period between 1:00 p.m. and 3:00 p.m. of the controlled light/dark cycles (light on 6:00 a.m. – 6:00 p.m.).

APPARATUS

Locomotor activity was measured using an open-field equipped with Actitrack (Panlab, S. L., Spain). This device consists of two square-shaped frames that deliver beams of infrared rays into the space inside the square. A plastic box is placed in this square to act as an open-field arena (base 30 x 30 cm, height 20 cm), in which the animal can move freely. The apparatus software records locomotor activity of the animal by registering the beam interruptions caused by movements of the body. Using this equipment we have determined the Distance Travelled (trajectory in cm per 3 minutes).

Drugs

Vehicle and all drugs were always given in a volume adequate to drug solutions (10 ml/kg).

(+)Methamphetamine, (d-N,α-Dimethylphenylethylamine; d-Desoxyephedrine), (Sigma Chemical Co.) dissolved in saline.

(R)-(+)-Methanandamide, (R)-N-(2-hydroxy-1-methylethyl)-5Z,8Z,11Z-eicosotetraenamide) supplied pre-dissolved in anhydrous ethanol 5 mg/ml (Tocris Cookson Ltd., UK) was diluted in saline to the concentration giving the chosen dose to be administered to animals in a volume of 10 ml/kg; vehicle therefore contained an adequate part of ethanol (a final concentration in the injection below 1%) to make effects of placebo and the drug comparable.

Procedure

Mice were randomly divided into 2 groups (n_1=24, n_2=24) and all were given vehicle on Day 1 (10 ml/kg). There were no applications from Days 2 to 6. For the next seven days animals were daily treated intraperitoneally as follows: a) n_1: methamphetamine at the dose of 2.5 mg/kg/day, b) n_2: methanandamide at the dose of 0.5 mg/kg/day. On Day 14 all animals were given intraperitoneally methamphetamine at the dose of 2.5 mg/kg (challenge dose).

Changes in locomotion were measured for the period of 3 minutes in the open field on Days 1, 7 and 14, fifteen minutes after drug application to assess sensitizing phenomenon. After each measurement one third of both groups was decapitated (75 minutes after drug administration) and the brain was stored in RNAlater (Ambion). For RNA isolation we used excised mesencephalon only. The total RNA was isolated by means of RNAEasy Mini Kit (Qiagene) and the subsequent reverse transcription was performed with Omniscript RT Kit (Qiagene) and RNAse OUT Ribonuclease Inhibitor (Invitrogen). Relative expression of D_1 and D_2 receptors, respectively (assays Mm02620146_s1 and Mm00438541_m1, Life Technologies) was compared to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (assay Mn99999915_1g, Life Technologies) using real time cycler ABI SDS 7000 (AppliedBiosystems). All real time PCR reactions were performed using TaqMan Gene Expression Master Mix (Life Technologies).

Data analysis

As the data was not normally distributed (according to the Kolmogorov-Smirnov test of normality), non-parametric statistics were used: Mann-Whitney U test, two-tailed (statistical analysis package STATISTICA - StatSoft, Inc., Tulsa, USA).

RESULTS

The behavioural part of the present study focused on the changes in mouse locomotion fully confirmed our earlier outcomes published elsewhere (Landa *et al.* 2011): a) development of behavioural sensitization to methamphetamine (M) stimulatory effects after repeated M treatment; b) development of cross-sensitization to these effects after repeated pre-treatment with methanadamide (CAN) prior to the M challenge dose.

Real-time PCR results focused on relative expression of D_1 receptor mRNA showed in the group n_1 a significant increase (p<0.05) after the 1st dose of M (see Figure 1; V1 versus M). This increase was even more pronounced (p<0.01) after the application of M challenge dose (see Figure 1; V1 versus M/M). The treatments in the group n_2 caused significant increase (p<0.01) in relative expression of D_1 receptor mRNA after the 1st application of CAN compared to the application of vehicle (V2) (see Figure 1; V2 versus CAN). The challenge dose of M produced a non-significant decrease (p>0.05) in animals pre-treated repeatedly with CAN when compared to the animals after the 1st application of CAN (see Figure 2; CAN versus CAN/M).

There was no significant change in relative expression of D_1 receptor mRNA between animals after the MET challenge dose (those were pre-treated with MET) and animals after the MET challenge dose (those were pre-treated with CAN) – see Figure 1; M/M versus CAN/M.

Real-time PCR results focused on relative expression of D_2 receptor mRNA showed in the group n_1 a significant decrease (p<0.01) after the 1st dose of M (see Figure 2; V1 versus M). There was no significant difference after the application of M challenge dose (see Figure 1; M versus M/M). The treatments in the group n_2 caused significant decrease (p<0.05) in relative expression of D_2 receptor mRNA after the 1st application of CAN compared to the application of vehicle (V2) (see Figure 2; V2 versus CAN). The challenge dose of M produced a non-significant increase (p>0.05) in animals pre-treated repeatedly with CAN when compared to the animals after the 1st application of CAN (see Figure 2; CAN versus CAN/M).

There was no significant change in relative expression of D_2 receptor mRNA between animals after the MET challenge dose (those were pre-treated with MET) and animals after the MET challenge dose (those were pre-treated with CAN) – see Figure 2; M/M versus CAN/M.

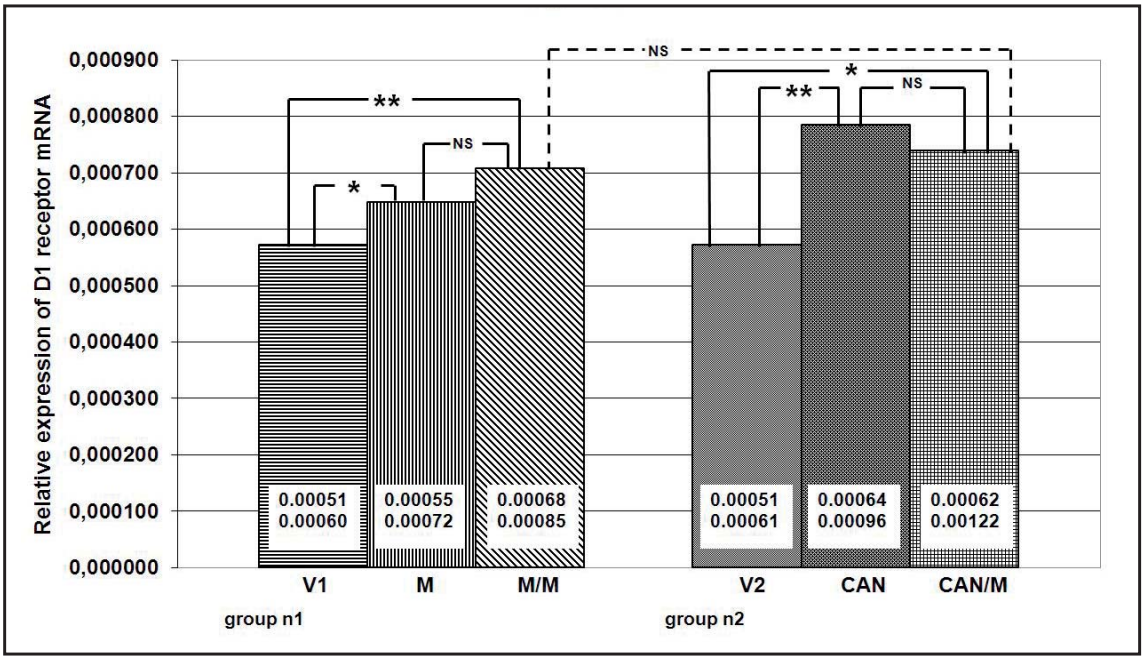

Fig. 1. Effects of drug treatments on relative expression of D_1 receptor mRNA when compared to GAPDH mRNA shown as median (interquartile range Q1 to Q3):
V1 = mice after the dose of vehicle in the group n_1, V2 = mice after the dose of vehicle in the group n_2, M = mice after the 1st dose of methamphetamine (2.5 mg/kg), M/M = mice sensitized with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg), CAN = mice after the 1st dose of methanandamide (0.5 mg/kg), CAN/M = mice sensitized with methanandamide after the challenge dose of methamphetamine (2.5 mg/kg).
*p<0.05, **p<0.01, NS = non-significant, the nonparametric Mann-Whitney U test, two tailed.

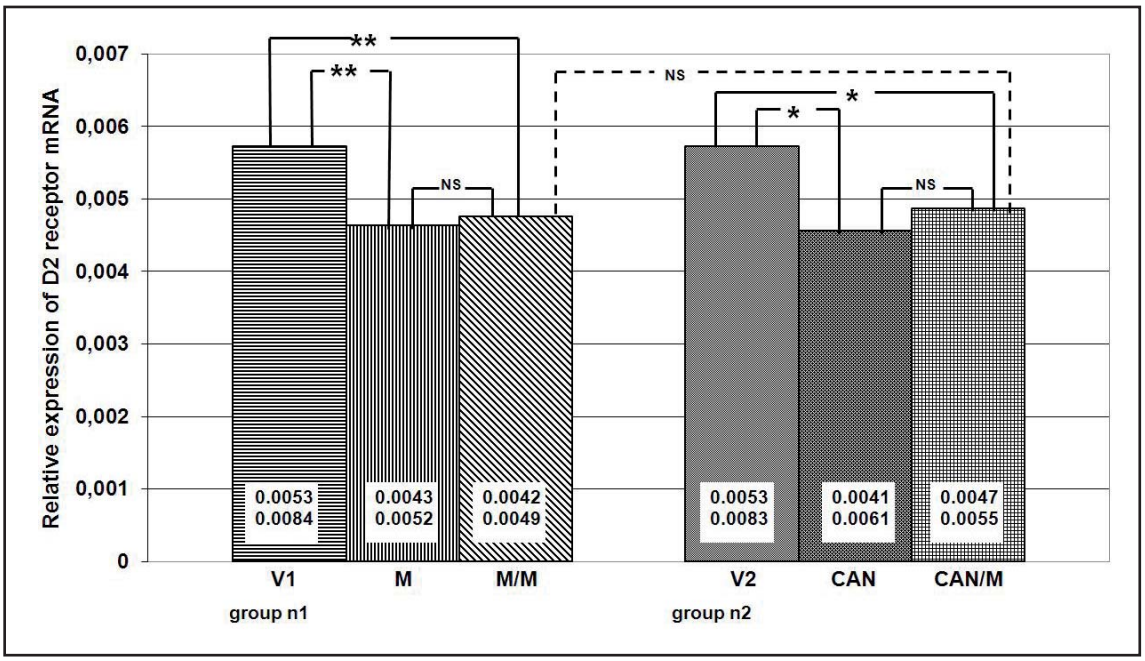

Fig. 2. Effects of drug treatments on relative expression of D_2 receptor mRNA when compared to GAPDH mRNA shown as median (interquartile range Q1 to Q3):
V1 = mice after the dose of vehicle in the group n_1, V2 = mice after the dose of vehicle in the group n_2, M = mice after the 1st dose of methamphetamine (2.5 mg/kg), M/M = mice sensitized with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg), CAN = mice after the 1st dose of methanandamide (0.5 mg/kg), CAN/M = mice sensitized with methanandamide after the challenge dose of methamphetamine (2.5 mg/kg).
*p<0.05, **p<0.01, NS = non-significant, the nonparametric Mann-Whitney U test, two tailed.

DISCUSSION

The start-up behavioural assessment confirmed presence of both sensitization to methamphetamine stimulatory effects and cross-sensitization to methamphetamine induced by pre-treatment with cannabinoid CB_1 receptor agonist methanandamide, which was completely in accordance with our previous experiments (Landa *et al.* 2006a; 2006b; 2011).

Methamphetamine and methanandamide are believed to elicit increase in dopamine activation in the mesolimbic reward pathway. This pathway primarily connects the VTA and nucleus accumbens and both are central to the brain reward system. Increased dopamine activity in the dopamine reward system is associated with neuroadaptive changes, among others in the density of appropriate receptor systems, especially of D_1 and D_2 receptors cooperating in dopamine reward processes (Ikemoto *et al.* 1997).

It is known, that the reinforcing/rewarding effects are common for both methamphetamine and methanadamide (dela Peña *et al.* 2010; Justinova *et al.* 2011) we have tested. Results of Ikemoto *et al.* (1997) indicated that concurrent activation of dopamine D_1 and D_2 receptor subtypes in the shell of nucleus accumbens had a cooperative effect on dopamine-mediated reward processes, which corresponds with our primary hypothesis that both receptor subtypes are involved in the mechanisms of reward. However, despite that also other data attribute to the important role of D_2 and particularly D_1 receptors in the process of reward (including neuroplastic changes underlying behavioural sensitization) not even all of them are completely consistent (Hasbi *et al.* 2011; Bachtell *et al.* 2005; Dias *et al.* 2004; Maneuf *et al.* 1997; Hamamura et al., 1991).

Our present experiments concerning relationship between methamphetamine and cannabinoid CB_1 agonist methanandamide influences on the relative D_1 and D_2 receptor mRNA expression provided quite controversial findings, too. Real-time PCR analyses showed an increase in D_1 receptor mRNA expression after the acute administration of methamphetamine at the dose of 2.5 mg/kg (that persisted also after the last dose of methamphetamine) and also an increase after the acute dose of methanandamide at the dose of 0.5 mg/kg (persisting after the methamphetamine challenge dose). Interestingly, there was a significant decrease in D_2 receptor mRNA expression both after the acute dose of methamphetamine and methanandamide at the same doses as above (that persisted also after the methamphetamine challenge doses).

Probably simultaneously with our experiments there was run a study (Dalton & Zavitsanou, 2010) examining also influence of single and repeated treatments with cannabinoid receptor agonist on dopamine D_1 and D_2 receptor densities in adult and adolescent rats. In the adult rats, using in vitro autoradiography they found after the repeated treatment with cannabinoid CB_1 receptor agonist HU210 significant increase in D_1 and D_2 receptor densities. In adolescent rats the increase in the number of receptors was measured only in the case of D_1 and not D_2 subtypes in the lateral caudate putamen and olfactory tubercle. The authors concluded that the mechanisms stayed unclear to them as previously they registered down-regulation of D_1 receptor density in the rat nucleus accumbens, caudate putamen, substantia nigra and olfactory tubercle (Dalton *et al.* 2009). Shishido *et al.* (1997) received similar outcomes to our behavioural results when measuring by in situ hybridization histochemistry (ISHH) dopamine D_1 receptor and D_2 receptor mRNAs following repeated methamphetamine administration in the dorsal striatum and ventral striatum of rats. Moreover, they revealed, using ISHH, that D_1 receptor mRNA levels in the dorsal striatum were significantly increased and in contrast, repeated methamphetamine treatment did not significantly affect the expression of D_1 receptor mRNA in ventral striatum or D_2 receptor mRNA. Although rather inconsistent, these ISHH-related findings are to certain extent similar to our PCR-results which showed an increase in D_1 receptor mRNA expression in methamphetamine sensitized and methanadamide cross-sensitized mice, respectively, and in opposite a decrease in D_2 receptor mRNA expression. This latter finding is consistent with results of Nader *et al.* (2006) who found using PET, that D_2 receptor availability is decreasing in the brain of rhesus-monkeys by 15–20% within 1 week of initiating cocaine self-administration and remained reduced by similar to 20% during 1 year of exposure.

Vezina (1996) suggested that dopamine D_1 receptors in the VTA played a critical role in the development of sensitization to amphetamine effects, whereas activation of D_2 receptors is not necessary for the induction of sensitization to amphetamine. Although this is in conflict with suggestions of Ikemoto *et al.* (1997) and also with our working hypothesis, it however corresponds very well with our final results, because the relative expression of dopamine D_2 receptor mRNA was decreased in sensitized animals, whereas a significant increase in dopamine D_1 receptor mRNA expression occurred after development of sensitization.

It has been described, that both D_1 and D_2 receptors exist in high- and low-affinity states. High-affinity states of dopamine D_1 (D_1^{High}) and D_2 (D_2^{High}) receptors have much higher affinity for dopamine than D_1 and D_2 receptors in low-affinity states. Dopamine D_1^{High} and D_2^{High} receptors are considered to be the functional state of dopamine receptors and Seeman *et al.* (2002) suggested that the proportion of D_2^{High} receptors was increased in the striatum of amphetamine-sensitized rats, despite of no changes in the density of D_2 receptors (for more details see Shuto *et al.* 2008). From this point of view behavioural sensitization to methamphetamine can be explained by the increased proportion of D_2^{High} receptors in the striatum, which results in substantially higher sensitivity to psychostimulants or dopaminergic

drugs (Shuto *et al.* 2008). Despite Seeman *et al.* (2002) reported that in the animals sensitized to amphetamine the entire density of D_2 receptors was not altered, the question remains whether PCR method is capable to detect mRNA expression of D_2 receptors in both states (high- and low-affinity states), which could explain decrease in the relative D_2 receptor expression of sensitized mice in our experiment.

Our present findings showing the decrease in D_2 receptor mRNA expression after the acute dose of both methamphetamine and methanadamide support hypotheses of those who suggest that drug dependence is associated with a decrease in D_2 receptor availability (Volkow *et al.* 1997; Martinez *et al.* 2004). On the other hand the increase in D_1 receptor density in the mesencephalon associated with development of behavioural cross-sensitization to methamphetamine effects after repeated treatment with cannabinoid receptor agonist methanadamide corresponds with conclusion of Worsley *et al.* (2000) that dependence to methamphetamine might be related to reinforced dopamine D_1 receptor functioning and can support the cannabinoid gateway hypothesis (e.g. Fergusson *et al.* 2006) increasing risk of use of other drugs of abuse.

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7.6 The effect of felbamate on behavioural sensitization to methamphetamine in mice

Many reports have described the important role of the glutamatergic system and NMDA receptors in the process of behavioural sensitization (Wolf 1998; Tzschentke and Schmidt 2003; Lee et al. 2011). This study was therefore aimed at the influence of the antiepileptic drug felbamate (an NMDA receptor antagonist) on behavioural sensitization to the effects of methamphetamine on mouse locomotor activity in the open field test.

Similarly as in our previous studies (Landa et al. 2006a; Landa et al. 2011), repeated administration of methamphetamine produced a robust behavioural sensitization to its stimulatory effects in the mouse open field.

A significant decrease in locomotion in mice sensitized with methamphetamine within one of the experimental groups where mice received a methamphetamine challenge dose along with felbamate is in agreement with a majority of similar studies, which have reported the inhibitory effects of NMDA receptor antagonists on the development of sensitization to amphetamines (Wolf 1998).

The acute dose of felbamate had no behavioural effect, however inhibition of locomotion after repeated administration of the drug was seen.

Despite the fact that felbamate is referred to as an activating antiepileptic drug, its acute administration along with methamphetamine inhibited the stimulatory effects of methamphetamine, and combined repeated pre-treatment with methamphetamine and felbamate facilitated the development of sensitization to metamphetamine stimulatory effects, which are rather contradictory findings compared with other some authors (Wolf et al. 1995).

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The effect of felbamate on behavioural sensitisation to methamphetamine in mice

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ABSTRACT: It has been shown that methamphetamine (Met) similary to other psychostimulants induces a progressive augmentation of behavioural responses after repeated administration, so called behavioural sensitisation. Numerous studies refer to an important role for *N*-methyl-D-aspartate (NMDA) receptors in the development of behavioural sensitisation. Activating antiepileptic drugs of the newer second generation, such as felbamate (Fel), also invoke psychotropic effects. They may possess attention-enhancing and antidepressant activity, causing anxiety, insomnia, and agitation. Although not all pharmacological effects of felbamate are fully elucidated yet, many of its clinical effects may be related to the inhibition of NMDA currents. Thus, the present study was focused on investigating the influence of felbamate on sensitisation to the effects of methamphetamine on mouse locomotor behaviour in the Open field test. Mice of the albino out-bred strain ICR were randomly allocated into four groups and were administered drugs seven times (from the 7th to 13th day of the experiment) as follows: (a) $n_{1, 2}$: 2.5 mg/kg/day of Met; (b) n_3: 240 mg/kg/day of Fel; (c) n_4: Met + Fel. Locomotion in the Open field test was measured (a) after administration of vehicle on the 1st experimental day, (b) after the first dose of drugs given on the 7th day, and (c) on the 14th day after the "challenge doses" given that way (as follows): n_1: Met; n_2: Met + Fel, n_3: Fel; n_4: Met. The following significant behavioural changes were observed: (1) stimulatory influence of Met and sensitisation after repeated treatment (n_1); (2) inhibition of Met sensitisation in the case of a challenge dose combined with Fel (n_2); (3) augmentation of the sensitising effect of Met when sensitisation was induced by pre-treatment with Met + Fel (n_4); (4) no behavioural effect of the first dose of Fel, but inhibition of locomotion after repeated administration of the drug (n_3). The prevention of the development of Met sensitization in the group n_2 in which mice received the Met challenge dose with Fel mirrors the results of a majority of similar studies. Most findings are consistent with inhibitory effects of antagonists of the NMDA receptors on the development of sensitisation to amphetamines; nevertheless, also new findings are reported. In the presented paper, combined pre-treatment with Met + Fel in the group n_4 facilitated the development of sensitisation to Met stimulatory effects.

Keywords: behavioural sensitisation; methamphetamine; felbamate; NMDA receptor antagonist; mice

List of abbreviations

AMPA = α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, **Fel** = febamate, **GABA** = gamma-aminobutyric acid **MDMA** = 3,4-methylenedioxymethamphetamine, **Met** = methamphetamine, **MK-801** = (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine, **NBQX** = 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f] quinoxaline-2,3-dione, **NMDA** = N-methyl-D-aspartate, **THC** = Δ9-tetrahydrocannabinol, **V** = vehicle

Many drugs induce a progressive augmentation of behavioural responses, so called behavioural sensitisation following their repeated administration. This phenomenon was consistently described by Robinson and Berridge (1993) and it occurs in both animals and man (Tzschentke and Schmidt 1997). Behavioural sensitisation was described, for example, to ethanol (Bahi and Dreyer 2012), morphine

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(Farahmandfar et al. 2011), nicotine (Bhatti et al. 2009), THC (Δ9-tetrahydrocannabinol) (Cadoni et al. 2008), or MDMA (3,4-methylenedioxymethamphetamine) (Ball et al. 2011). In our laboratory, we developed an original dosage regimen that produced a reliable and robust behavioural sensitisation to stimulatory effects of methamphetamine (Met) in mice (Landa et al. 2006a,b, 2011).

The phenomenon of behavioural sensitisation is believed to be a consequence of drug-induced neuroadaptive changes in a circuit involving dopaminergic, glutamatergic and GABAergic interconnections between the ventral tegmental area, nucleus accumbens, prefrontal cortex and amygdala (Vanderschuren and Kalivas 2000; Nestler 2001). Numerous studies refer to the important involvement of glutamate *N*-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in the process of behavioural sensitisation (Stewart and Druhan 1993; Ohmori et al. 1994; Subramaniam et al. 1995; Li et al. 1997; Wolf 1998; Tzschentke and Schmidt 2003; Lee et al. 2011).

However, not all studies have reported results that are completely consistent. For example, Mead and Stephens (1998) found that administration of the AMPA receptor antagonist NBQX attenuated amphetamine-induced sensitisation in mice. Boudreau and Wolf (2005) suggested that drug-seeking responses were more effectively triggered in cocaine-sensitised rats due to increased cell surface expression of AMPA receptors in the nucleus accumbens. In contrast, Nelson et al. (2009) concluded that behavioural sensitisation to amphetamine was not accompanied by changes in glutamate receptor surface expression in the rat nucleus accumbens. Xia et al. (2011) showed that the effect of glutamate receptors was not associated solely with sensitisation to psychostimulants, because morphine treatment elicited changes in synaptic AMPA receptor expression in the mice hippocampus, a structure with an important role in learning and memory. Suto et al. (2004) described that in rats with amphetamine-induced sensitisation, a lower AMPA concentration could provoke re-instatement of cocaine seeking.

Felbamate (Fel) is an activating antiepileptic drug of the newer second generation (Vohora et al. 2010), and is therapeutically used in both humans and animals (Ruehlmann et al. 2001). Fel is characterised as an NMDA receptor antagonist (Germano et al. 2007), that blocks NMDA receptor-mediated currents (Kuo et al. 2004). Generally, antiepileptic drugs from this generation invoke psychotropic effects. They may exert attention-enhancing and antidepressant effects, and cause anxiety, insomnia, and agitation (Nadkarni and Devinsky 2005; Sharma et al. 2008). Felbamate was also reported to significantly inhibit the nociception induced by glutamate (Beirith et al. 2002). It has been shown that felbamate reduced the locomotor hypoactivity induced by repeated stress in mice (Pistovcakova et al. 2005).

Most findings are consistent with the hypothesis that antagonists of the NMDA receptors have inhibitory effects on behavioural sensitisation to amphetamines (Wolf 1998); however, there are also reports that co-administration of NMDA-receptor antagonists, e.g., dizocilpine enhances the effect of the sensitising drug (Tzschentke and Schmidt 1998). Thus, this issue remains quite controversial. According to our knowledge, none of the experiments which support the notion of inhibitory effects and summarised in the review of Wolf (1998) tested felbamate and methamphetamine together. Thus, the present study was designed to investigate the influence of felbamate on sensitisation to the effects of methamphetamine on mouse locomotor behaviour in the open field test; we particularly focused on possible changes in the development of methamphetamine sensitisation.

MATERIAL AND METHODS

Animals

Male mice (strain ICR, TOP-VELAZ s.r.o., Prague, Czech Republic) with an initial weight of 18–21 g were used. Animals were randomly allocated into four treatment groups. In order to minimise possible variability due to circadian rhythms the behavioural observations were always performed in the same period between 1:00 p.m. and 3:00 p.m. of controlled light/dark cycles (light on 6:00 a.m.–6:00 p.m.).

Apparatus

Locomotor activity was measured using an open-field equipped with Actitrack (Panlab, S.L., Spain). This device consists of two square-shaped frames that deliver beams of infrared rays into the space inside the square. A plastic box is placed in this square to act as an open-field arena (base 30 × 30 cm,

height 20 cm), in which the animal can move freely. The apparatus software records locomotor activity of the animal by registering the beam interruptions caused by movements of the body. Using this equipment we have determined the Distance Travelled (trajectory in cm per 3 min).

Drugs

Vehicle and all drugs were always given in a volume adequate for drug solutions (10 ml/kg).

(+)Methamphetamine, (D-*N*,α-Dimethylphenylethylamine; D-Desoxyephedrine) (Sigma Chemical Co.) dissolved in saline.

Felbamate (Taloxa® 600 mg, Schering-Plough) dissolved in distilled water.

Procedure

For the purposes of this study we used our in-house dosage regimen. Mice were randomly divided into four groups ($n_1 = 10$, $n_2 = 10$, $n_3 = 10$, $n_4 = 10$) and all were given vehicle on Day 1 (10 ml/kg). There were no applications from Days 2 to 6. For the next seven days animals were daily treated as follows: (a) $n_{1, 2}$ 2.5 mg/kg/day of Met, (b) n_3 240.0 mg/kg/day of Fel; (c) n_4 combination of Met + Fel at doses of 2.5 mg/kg/day and 240 mg/kg/day, respectively. On Day 14 all animals were given challenge doses in the following way: n_1: Met at the dose of 2.5 mg/kg, n_2: Met + Fel at the doses of 2.5 mg/kg and 240 mg/kg, respectively, n_3: Fel at the dose of 240 mg/kg, n_4: Met at the dose of 2.5 mg/kg. All doses of Met were administered intraperitoneally and all doses of Fel were administered orally. Changes in locomotion were measured for a period of 3 min in the open field on Days 1, 7 and 14 to assess the sensitising phenomenon.

The experimental protocol complies with the European Community guidelines for the use of experimental animals and was approved by the Animal Care Committee of the Masaryk University Brno, Czech Republic.

Data analysis

As the data were not normally distributed (according to the Kolmogorov-Smirnov test of normality), non-parametric statistics were used: Wilcoxon matched-pairs signed-ranks test, two tailed (statistical analysis package Statistica-StatSoft, Inc., Tulsa, USA).

RESULTS

The treatments in the group n_1 caused a significant increase ($P < 0.05$) in locomotion after the 1st application of methamphetamine (Met) compared to the application of vehicle (V) (see Figure 1; V versus MET). The challenge dose of Met produced a significant increase in Distance Travelled ($P < 0.05$) in animals pre-treated repeatedly with Met when compared to the animals after the 1st Met dose (see Figure 1; Met versus Met/Met).

Similarly, in the group n_2 the first administration of Met caused a significant increase ($P < 0.05$) in Distance Travelled compared to the application of V (see Figure 2; V versus Met). In contrast, the challenge dose of Met + Fel evoked a significant decrease ($P < 0.05$) in locomotion in animals pre-treated repeatedly with Met when compared to the animals after the 1st application of Met (see Figure 2; Met versus Met/Met + Fel).

In the group n_3 the 1st application of Fel did not affect locomotor activity in mice significantly ($P > 0.05$) (see Figure 3; V versus Fel), whereas the challenge dose of Fel induced a significant decrease ($P < 0.05$) in locomotion in animals pre-treated repeatedly with Fel when compared to the animals after the 1st dose of Fel (see Figure 3; Fel versus Fel/Fel).

Finally, in the group n_4 the first application of the Met + Fel combination did not affect Distance Travelled significantly ($P > 0.05$) (see Figure 4; V versus Met + Fel) and the challenge dose of Met evoked a significant increase ($P < 0.05$) in locomotion in animals pre-treated repeatedly with the combination Met + Fel when compared to the animals after the 1st dose of Met + Fel (see Figure 4; Met + Fel/Met versus Met + Fel).

DISCUSSION

The results obtained in the group n_1 are completely in accordance with the results from our previous studies that confirmed the development of sensitisation to methamphetamine stimulatory effects in an original dosage regimen applied in mice (Landa et al. 2006 a,b, 2011). A significant decrease in locomotion in mice sensitised with Met in the group n_2 in which mice received the Met challenge

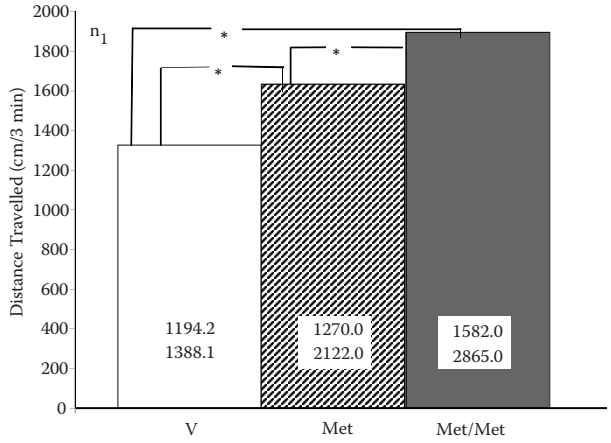

Figure 1. Effects of drug treatments in the group n_1 on Distance Travelled (cm/3 min) in the mouse open field test shown as median (interquartile range Q1 to Q3)

V = mice after the 1st dose of vehicle, Met = mice after the 1st dose of methamphetamine (2.5 mg/kg), Met/Met = mice sensitised with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg)

*$P < 0.05$, NS = non-significant; the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

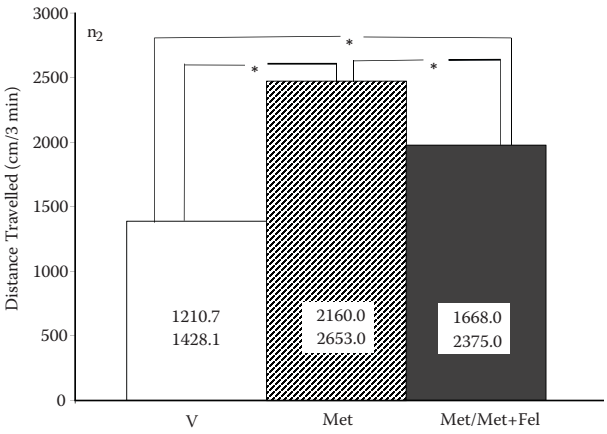

Figure 2. Effects of drug treatments in the group n_2 on Distance Travelled (cm/3 min) in the mouse open field test shown as median (interquartile range Q1 to Q3)

V = mice after the 1st dose of vehicle, Met = mice after the 1st dose of methamphetamine (2.5 mg/kg), Met/Met + Fel = mice sensitised with methamphetamine after the challenge dose of methamphetamine + felbamate (2.5 mg/kg + 240.0 mg/kg)

*$P < 0.05$, NS = non-significant; the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

dose with Fel is in agreement with a majority of similar studies which described inhibitory effects of NMDA receptor antagonists on the development of sensitisation to amphetamines (Wolf 1998).

Despite the fact that felbamate is referred to as an activating antiepileptic drug its acute administration along with methamphetamine also inhibited the stimulatory effects of methamphetamine in the group n_4. Wolf et al. (1995) found that co-administration of N-methyl-D-aspartate antagonists MK-801 (dizocilpine maleate) with amphetamine prevented the development of behavioural sensitisation in rats. In their study animals were given either water + amphetamine or MK-801 + ampheta-

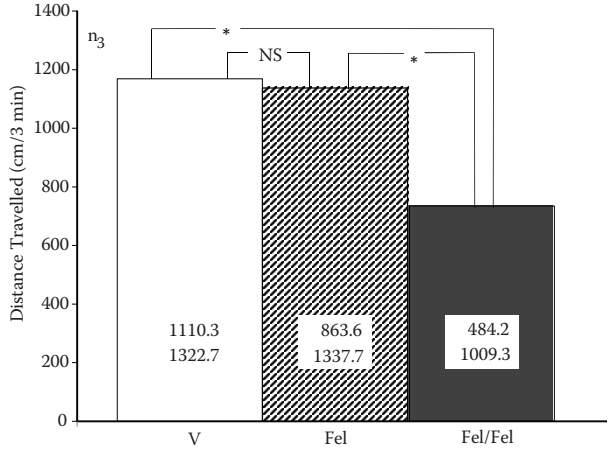

Figure 3. Effects of drug treatments in the group n_3 on Distance Travelled (cm/3 min) in the mouse open field test shown as median (interquartile range Q1 to Q3)

V = mice after the 1st dose of vehicle, Fel = mice after the 1st dose of felbamate (240.0 mg/kg), Fel/Fel = mice sensitised with felbamate after the challenge dose of felbamate (240.0 mg/kg)

*$P < 0.05$, NS = non-significant; the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

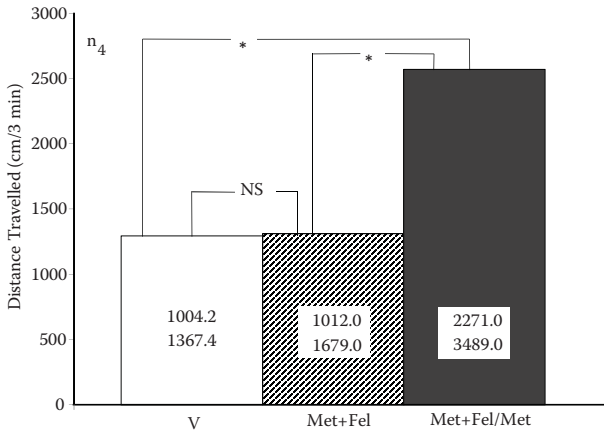

Figure 4. Effects of drug treatments in the group n_4 on Distance Travelled (cm/3 min) in the mouse open field test shown as median (interquartile range Q1 to Q3)

V = mice after the 1st dose of vehicle, Met + Fel = mice after the 1st dose of combination methamphetamine + felbamate (2.5 mg/kg + 240.0 mg/kg), Met + Fel/Met = mice sensitised with the combination methamphetamine + felbamate after the challenge dose of methamphetamine (2.5 mg/kg)

*$P < 0.05$, NS = non-significant; the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

mine for six consecutive days. The challenge dose of amphetamine alone was administered on Day 8. Co-administration of MK-801 increased the locomotor response to acute amphetamine administration and repeated pre-treatment with the MK-801 + amphetamine combination prevented the development of sensitisation to a subsequent challenge dose of amphetamine. Similarly Wolf et al. (1995) found that co-administration of NMDA antagonist CGS 19755 augmented the locomotor response to acute amphetamine application and prevented the development of sensitisation after amphetamine challenge dose. Both these results are run counter to our findings because co-administration of felbamate and methamphetamine did not increase locomotory behaviour at all and repeated pre-treatment with the methamphetamine + felbamate combination elicited, after methamphetamine challenge, a significant increase in locomotion, i.e., development of behavioural sensitisation.

Similar findings to Wolf et al. (1995) and contradictory to our results were published by Shim et al. (2002). They also tested the effect of the NMDA receptor antagonist MK-801 on the development of sensitisation to nicotine in rats. The authors described that application of MK-801 plus nicotine evoked a marked increase in locomotor activity for the first four testing days; nevertheless, pre-treatment with MK-801 during the developmental phase inhibited nicotine-induced sensitisation in response to the nicotine challenge dose.

Abekawa et al. (2007) prenatally treated rats with MK-801; however, it was shown that prenatal exposure to MK-801 neither enhanced the acute effects of methamphetamine on postnatal day 35 nor the development of behavioural sensitisation to methamphetamine.

Carey et al. (1995) found that an NMDA receptor antagonist enhanced behavioural responses evoked by drug stimuli (cocaine) and in this way promoted behavioural sensitisation in rats, which is consistent with our results obtained in the group n_4 where repeated co-administration of methamphetamine + felbamate resulted, after the methamphetamine challenge dose, in the development of behavioural sensitisation to the stimulatory effects of methamphetamine.

Other reports suggest that the involvement of NMDA receptors in the processes of behavioural sensitisation could be substance-dependent. For example, Meyer and Phillips (2007) concluded that ethanol-induced behavioural sensitisation was not associated with increased behavioural sensitivity to NMDA receptor antagonists or altered sensitivity to NMDA receptor agonists. They concluded that their results were inconsistent with the hypothesis that ethanol-induced sensitization is associated with alterations in NMDA receptor-mediated processes.

On the other hand, Shim et al. (2002) found that the non-competitive NMDA receptor antagonist MK-801 prevented behavioural sensitisation to nicotine. Hong et al. (2006) focused on the effect of MK-801on nicotine sensitisation of nucleus accumbens dopamine release and found that MK-801 blocked this sensitisation, which speaks to a role for NMDA receptors in the development of behavioural sensitisation to nicotine.

Yang et al. (2008) studied the effects of ifenprodil, a selective antagonist of the NR2B subunit of NMDA receptors on morphine-induced reward and drug-seeking behaviour and behavioural sensitisation. They found that morphine-induced reward and drug-seeking behaviour were abolished when the NR2B subunits of NMDA receptors at the nucleus accumbens were blocked by ifenprodil. On the other hand, morphine-induced reward and drug-seeking behaviour and behavioural sensitisation were not affected when ifenprodil was injected at the ventral tegmental area. Only when ifenprodil was co-administered with morphine did it partially inhibit morphine-induced behavioural sensitisation. These results suggest that the role of the NMDA receptor in the development of sensitization could be dependent not only on the particular substance but also on the particular brain region that is affected.

Some authors have examined possible changes in the brain at the level of receptors. Nelson et al. (2009) tested whether behavioural sensitisation to amphetamine was associated with redistribution of glutamate receptors in the rat nucleus accumbens or dorsolateral striatum but revealed no significant changes in AMPA or NMDA receptor surface expression in both brain structures after withdrawal from the sensitising regimens of amphetamine. They compared these results with previous experiments suggesting increased surface and synaptic levels of AMPA receptors in the nucleus accumbens in rats with cocaine-induced sensitisation (Boudreau and Wolf 2005; Boudreau et al. 2007).

Taken together, behavioural sensitisation is a very complex phenomenon that evokes diverse neurophysiological and behavioural effects via various brain areas and neurochemical pathways. The involvement of glutamatergic receptors in the processes of behavioural sensitisation represents "only" one component. It can be concluded that the

role of NMDA receptors in the processes of sensitisation is of large importance, despite the rather conflicting results obtained from different studies that have dealt with various substances. Since the processes of behavioural sensitisation are believed to reflect neuroadaptive changes involved in psychotic disorders, particularly in addiction and since glutamatergic modulators show promise as a treatment for addiction in pre-clinical models (Bowers et al. 2010), it would be therefore worthwhile to perform further research aimed at elucidating the role of glutametergic component in behavioural sensitisation.

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7.7 The effect of memantine on behavioural sensitization to methamphetamine in mice

With regard to the results obtained in the previous study with felabamate, this experiment investigated the influence of another NMDA receptor antagonist, memantine, on behavioural sensitization to the effects of methamphetamine on mouse locomotor activity in the open field test.

The results from the group repeatedly administered methamphethamine, were identical to the findings in our previous studies and confirmed the development of sensitization to methamphetamine stimulatory effects (e.g. Landa et al. 2006a, b; 2011; 2012a). In this study we moreover focused on the expression of behavioural sensitization to methamphetamine, and although there was a clear trend towards an increase in locomotion after the second methamphetamine challenge dose, it did not reach statistical significance.

Neither development nor expression of behavioural sensitization occurred in mice sensitized with methamphetamine in which animals were given methamphetamine challenge doses in combination with memantine. This finding is in accordance with the majority of similar studies reporting the inhibitory effects of NMDA receptor antagonists on the development of sensitization to amphetamines (Wolf 1998). The results of the experiment are also to a certain extent in accordance with our previous study wherein we tested the possible effect of another NMDA receptor antagonist, felbamate, on behavioural sensitization to methamphetamine (Landa et al. 2012a).

Repeated pre-treatment with the combination methamphetamine + memantine did not produce sensitization after methamphetamine challenge doses.

Memantine alone did not change the measured behavioural parameters after the acute dose but it significantly decreased locomotion after its repeated administration.

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The effect of memantine on behavioural sensitisation to methamphetamine in mice

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ABSTRACT: After repeated administration the psychostimulant methamphetamine (Met) produces a substantial increase in behavioural responses, which is termed behavioural sensitisation. Many studies have reported that *N*-methyl-D-aspartate (NMDA) receptors play an important role in the development and expression of behavioural sensitisation. Memantine (Mem) is used particularly for the treatment of Alzheimer's disease and acts as a non-competitive NMDA glutamate receptor antagonist, possessing a variety of psychotropic effects. For example, there are studies indicating that memantine prevents the expression of withdrawal symptoms in mice and causes reversal of opioid dependence. Although not all pharmacological mechanisms of memantine have been clarified yet, it is known that memantine inhibits NMDA receptor inward currents. Thus, the present study was designed to assess whether memantine would influence behavioural sensitisation to the stimulatory effects of methamphetamine on mouse locomotion. Mice were randomly allocated into four groups. They were given vehicle on Day 1of the experiment and after five days without application they were administered seven drug daily doses (*i.p.*) from Day 7 to Day 13 of the study, as follows: (a) $n_{1, 2}$: 2.5 mg/kg/day of Met; (b) n_3: combination Met + Mem at the doses of 2.5 mg/kg/day and 5 mg/kg/day, respectively; (c) n_4: Mem at the dose of 5 mg/kg/day. On Day 14 mice were given the first "challenge treatment" (a) n_1: Met, (b) n_2: Met + Mem, (c) n_3: Met, (d) n_4: Mem. The second "challenge treatment" was given after a six day wash-out period on Day 21: (a) n_1: Met, (b) n_2: Met + Mem, (c) n_3: Met, (d) n_4: Mem. Changes in locomotion were measured for a period of 3 min in the Open field on Days 1, 7, 14 and 21 to assess the sensitising phenomenon. Met pre-treatment significantly sensitised to the effects of the challenge doses (n_1). Mem given alone did not change the measured behavioural parameters after the acute dose but it significantly decreased locomotion after its repeated administration (n_4). Repeated pre-treatment with the Met + Mem combination (n_3) did not produce sensitisation after Met challenge doses and similarly, repeated pre-treatment with Met did not induce sensitisation after the challenge dose of Met + Mem (n_2). Thus, our results suggest that the role of the NMDA receptor antagonist memantine in the development and expression of behavioural sensitisation to Met seems to be an inhibitory one.

Keywords: behavioural sensitisation; methamphetamine; memantine; NMDA receptor antagonist; mice

List of abbreviations

GABA = gamma-aminobutyric acid, **i.p.** = intraperitoneally, **Mem** = memantine, **Met** = methamphetamine, **NAc** = nucleus accumbens, **NMDA** = *N*-methyl-D-aspartate, **V** = vehicle, **VTA** = ventral tegmental area

Robinson and Berridge (1993) first consistently describe a phenomenon that was termed behavioural sensitisation. This phenomenon occurs after repeated administration of a whole range of abused drugs and its typical features involve progressively increasing behavioural responses to the effects of the particular substances. It has been described in both laboratory animals and man (Tzschentke and Schmidt 1997; Steketee and Kalivas 2011). Behavioural sensitisation was, for example, re-

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ported for cocaine (Schroeder et al. 2012; Ramos et al. 2012), methylphenidate (Freese et al. 2012), morphine (Hofford et al. 2012), ethanol (Pastor et al. 2012) and methamphetamine (Horio et al. 2012; Landa et al. 2011, 2012).

It has been shown that behavioural sensitisation is a consequence of drug-induced neuroadaptive changes in a circuit involving particularly dopaminergic, glutamatergic and GABAergic interconnections between the ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex and amygdala (Vanderschuren and Kalivas 2000; Nestler 2001). It has also been demonstrated that the phenomenon of sensitisation can be subdivided into two temporally defined domains, that are termed development (or initiation) and expression (Kalivas et al. 1993). The development of behavioural sensitisation is connected with progressive molecular and cellular alterations that culminate in a change in the processing of environmental and pharmacological stimuli by the CNS. Expression has been described as the enduring neural changes, which arise from the process of the development that directly mediate the sensitised behavioural response (Pierce and Kalivas 1997). There are data indicating that these processes differ not only temporally but also anatomically. Development of behavioural sensitisation to psychostimulant drugs is associated with the VTA and substantia nigra, whereas expression is particularly related to the neurotransmission in the NAc (Kalivas and Duffy 1993).

Various articles have described that interference with glutamatergic neurotransmission at *N*-methyl-D-aspartate (NMDA) receptors can disrupt both the development and the expression of sensitisation (Wolf 1998; Tzschentke and Schmidt 2003). It has been accepted that in particular NMDA-receptor antagonists block or interfere with behavioural plasticity. Nevertheless, there are also reports that co-administration of NMDA-receptor antagonists enhanced the effect of the sensitising drug (Tzschentke and Schmidt 1998).

In our previous study we tested the effect of the activating antiepileptic drug felbamate (that acts as an NMDA receptor antagonist) on behavioural sensitisation to methamphetamine (Landa et al. 2012). Another substance that also blocks NMDA glutamate receptors is memantine. Memantine is widely used in human medicine as a medication for Alzheimer's disease (Cummings et al. 2006). However, the full potential of memantine use has likely not been revealed so far. For example, it has been shown on the experimental level that memantine was able to attenuate chronic morphine-induced place-preference in rats (Chen et al. 2012). And moreover, there is also a recent report on the use of memantine in veterinary medicine for the treatment of canine compulsive disorders (Schneider et al. 2009).

Thus, since the role of glutamatergic transmission in the processes of behavioural sensitisation remains quite controversial and with regard to our previous results concerning the involvement of felbamate in sensitisation, we designed the present study to investigate a possible influence of memantine on sensitisation to methamphetamine in mice. In comparison with our previous study involving felbamate, in the present experimental design we focused on possible changes not only during the phase of development but also during the phase of expression.

MATERIAL AND METHODS

Animals

Mice (males, strain ICR, TOP-VELAZ s.r.o., Prague, Czech Republic) with an initial weight of 18–21 g were used. They were randomly allocated into four treatment groups. Animals were housed with free access to water and food in a room with controlled humidity and temperature, that was maintained under a 12-h phase lighting cycle. In order to minimise possible variability due to circadian rhythms behavioural measurements were always performed in the same time period between 1:00 p.m. and 3:00 p.m.

Apparatus

Locomotor activity was tested using an open-field equipped with Actitrack (Panlab, S.L., Spain). This device consists of two square-shaped frames that deliver beams of infrared rays into the space inside the square. A plastic box is placed in this square to act as an open-field arena (base 30 × 30 cm, height 20 cm), in which the animal can move freely. The apparatus software records the locomotor activity of the animal (such as Distance Travelled, fast movements, resting time, etc.) by registering the beam interruptions caused by movements of the body. Using this equipment we measured the Distance Travelled (trajectory in cm per 3 min).

Drugs

Vehicle and all drugs were always given in a volume adequate for the drug solutions (10 ml/kg).

(+)Methamphetamine, (*d-N*,α-Dimethylphenylethylamine;*d*-Desoxyephedrine), (Sigma Chemical Co.) and memantine hydrochloride, (3,5-Dimethyl-1-adamantanamine hydrochloride), (H. Lundbeck A/S) were dissolved in saline.

Procedure

For the purposes of this study we devised an original dosage regimen. Mice were randomly divided into four groups (n_1 = 10, n_2 = 10, n_3 = 10, n_4 = 10). All animals were given vehicle on Day 1 of the experiment and after five days without application were administered drug doses on seven occasions – intraperitoneally, once daily from Day 7 to Day 13 of the study – as follows: (a) n_1, n_2: 2.5 mg/kg/day of Met; (b) n_3: combination Met + Mem at the doses of 2.5 mg/kg/day and 5.0 mg/kg/day, respectively; (c) n_4: Mem at the dose of 5.0 mg/kg/day. On Day 14 mice were given the first "challenge doses" (a) n_1: Met at the dose of 2.5 mg/kg, (b) n_2: Met + Mem at the doses of 2.5 mg/kg and 5.0 mg/kg, respectively, (c) n_3: Met at the dose of 2.5 mg/kg, (d) n_4: Mem at the dose of 5.0 mg/kg). The second "challenge doses" were given after a six day wash-out period on Day 21 (a) n_1: Met at the dose of 2.5 mg/kg, (b) n_2: Met + Mem at the doses of 2.5 mg/kg and 5.0 mg/kg/day, respectively, (c) n_3: Met at the dose of 2.5 mg/kg, (d) n_4: Mem at the dose of 5.0 mg/kg. Changes in locomotion were measured for a period of 3 minutes in the open field on Days 1, 7, 14 and 21 to assess the development and expression of behavioural sensitisation.

The experimental protocol of the experiment complied with the European Community guidelines for the use of experimental animals and was approved by the Animal Care Committee of Masaryk University Brno, Czech Republic.

Data analysis

As the data were not normally distributed (according to the Kolmogorov-Smirnov test of normality), non-parametric statistics were used: Wilcoxon matched-pairs signed-ranks test, two tailed (statistical analysis package Statistica – StatSoft, Inc., Tulsa, USA).

RESULTS

Locomotion significantly increased ($P < 0.01$) after the 1st application of methamphetamine (Met) in the n_1 group compared to the application of vehicle (V) (see Figure 1; V versus Met). The 1st challenge dose of methamphetamine (Met1) produced a significant increase in Distance Travelled ($P < 0.01$) in animals pre-treated repeatedly with Met (see Figure 1; Met versus Met1). The 2nd challenge dose of methamphetamine (Met2) did not elicit any further significant increase ($P > 0.05$), (see Figure 1; Met1 versus Met2), however a highly significant increase ($P < 0.01$) occurred when comparing animals after the 2nd Met challenge dose to the mice after the 1st Met dose (see Figure 1; Met versus Met2).

In the group n_2 the 1st application of Met caused a significant increase ($P < 0.01$) in Distance Travelled

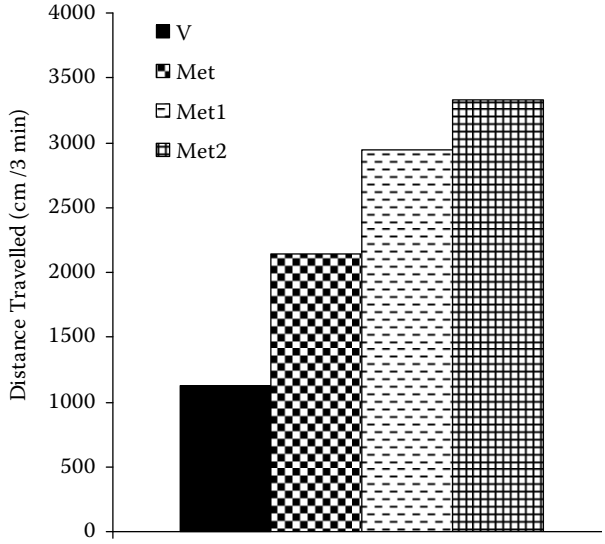

Figure 1. Effects of drug treatments in the group n_1 on Distance Travelled (cm/3 min) in the mouse open field test shown as medians (interquartile ranges Q1 to Q3)

V = mice after the 1st dose of vehicle, (interquartile range Q1 to Q3 = 1097.5–1258.3); Met = mice after the 1st dose of methamphetamine (2.5 mg/kg), (interquartile range Q1 to Q3 = 1632.0–2363.0); Met1 = mice repeatedly pre-treated with methamphetamine (2.5 mg/kg/day) after the 1st challenge dose of methamphetamine (2.5 mg/kg), (interquartile range Q1 to Q3 = 2416.0–3540.0); Met2 = mice repeatedly pre-treated with methamphetamine after the 2nd challenge dose of methamphetamine (2.5 mg/kg) following wash-out period, (interquartile range Q1 to Q3 = 2378.0–4049.0)
Statistical significances are as follows: V : Met ($P < 0.01$), Met : Met1 ($P < 0.01$), Met1 : Met2 (non-significant), Met : Met2 ($P < 0.01$); the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

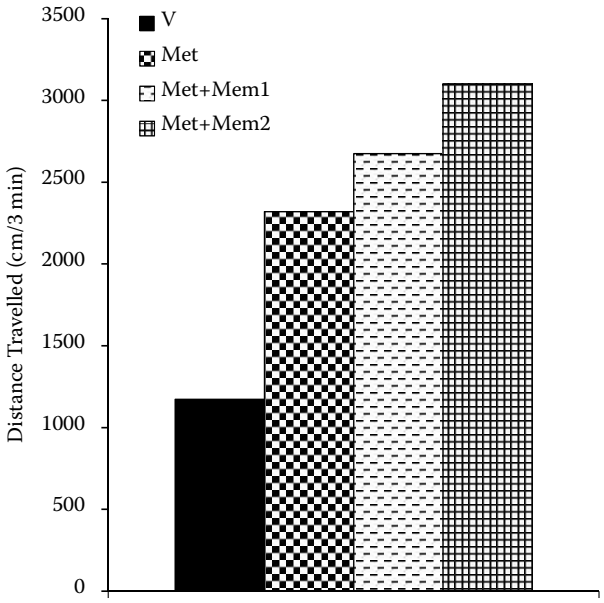

Figure 2. Effects of drug treatments in the group n_2 on Distance Travelled (cm/3 min) in the mouse open field test shown as medians (interquartile ranges Q1 to Q3)

V = mice after the 1st dose of vehicle, (interquartile range Q1 to Q3 = 1059.5–1380.8); Met = mice after the 1st dose of methamphetamine (2.5 mg/kg), (interquartile range Q1 to Q3 = 1914.0–3131.0); Met + Mem1 = mice repeatedly pre-treated with methamphetamine (2.5 mg/kg/day) after the 1st challenge dose of methamphetamine+memantine (2.5 mg/kg + 5.0 mg/kg), (interquartile range Q1 to Q3 = 2390.0–3248.0); Met + Mem2 = mice repeatedly pre-treated with methamphetamine after the 2nd challenge dose of methamphetamine + memantine (2.5 mg/kg + 5.0 mg/kg) following wash-out period, (interquartile range Q1 to Q3 = 2852.0–3326.0)
Statistical significances are as follows: V : Met ($P < 0.01$), Met : Met + Mem1 (non-significant), Met + Mem1 : Met + Mem2 (non-significant), Met : Met + Mem2 (non-significant); the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

compared to the application of V (see Figure 2; V versus Met). The 1st challenge dose of the methamphetamine + memantine combination (Met + Mem1) did not significantly increase locomotion in animals pre-treated repeatedly with Met ($P > 0.05$) (see Figure 2; Met versus Met + Mem1) and there were also no significant change after the 2nd challenge dose of methamphetamine + memantine (Met + Mem2) (see Figure 2; Met + Mem1 versus Met + Mem2). Similarly, no statistically significant change was found between animals after the 1st Met administration and animals that received the 2nd challenge dose of Met + Mem2 (see Figure 2; Met versus Met + Met2).

In group n_3 the 1st application of the methamphetamine+memantine (Met + Mem) combination increased locomotor activity compared to the application of V in a highly significant manner ($P < 0.01$) (see Figure 3; V versus Met + Mem). The 1st challenge dose of methamphetamine (Met1) did not result in any significant change in locomotion when compared to animals after the 1st dose of Met + Mem ($P > 0.05$), (see Figure 3; Met + Mem versus Met1). There were no significant changes in locomotion after the 2nd methamphetamine challenge dose (Met2) compared to animals after the 1st Met challenge dose (see Figure 3; Met1 versus Met2). No statistically significant changes were

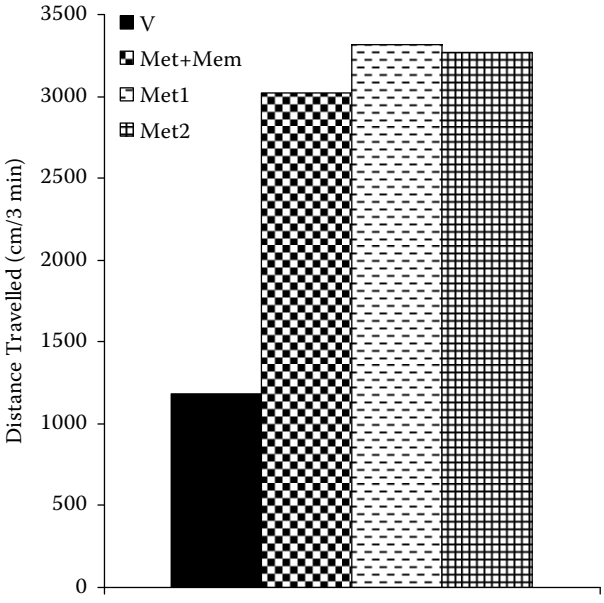

Figure 3. Effects of drug treatments in the group n_3 on Distance Travelled (cm/3 min) in the mouse open field test shown as medians (interquartile ranges Q1 to Q3)

V = mice after the 1st dose of vehicle, (interquartile range Q1 to Q3 = 1015.5–1333.7); Met + Mem = mice after the 1st dose of methamphetamine + memantine (2.5 mg/kg + 5.0 mg/kg), (interquartile range Q1 to Q3 = 1721.0–3519.0); Met1 = mice repeatedly pre-treated with combination Met + Mem (2.5 mg/kg/day + 5.0 mg/kg/day) after the 1st challenge dose of Met (2.5 mg/kg), (interquartile range Q1 to Q3 = 2031.0–4477.0); Met2 = mice repeatedly pre-treated with combination Met + Mem after the 2nd challenge dose of Met (2.5 mg/kg) following wash-out period, (interquartile range Q1 to Q3 = 2902.0–4409.0)
Statistical significances are as follows: V : Met + Mem ($P < 0.01$), Met + Mem : Met1 (non-significant), Met1 : Met2 (non-significant), Met + Mem:Met2 (non-significant); the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

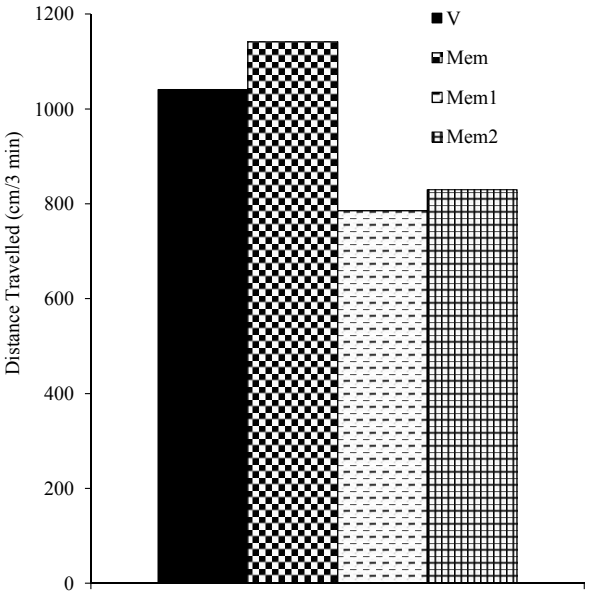

Figure 4. Effects of drug treatments in the group n_4 on Distance Travelled (cm/3 min) in the mouse open field test shown as medians (interquartile ranges Q1 to Q3)

V = mice after the 1st dose of vehicle, (interquartile range Q1 to Q3 = 939.9–1169.0); Mem = mice after the 1st dose memantine (5.0 mg/kg), (interquartile range Q1 to Q3 = 967.5–1373.5); Mem1 = mice repeatedly pre-treated with memantine (5.0 mg/kg/day) after the 1st challenge dose of Mem (5.0 mg/kg), (interquartile range Q1 to Q3 = 731.2–903.8); Mem2 = mice repeatedly pre-treated with Mem after the 2nd challenge dose of Mem (5.0 mg/kg) following wash-out period, (interquartile range Q1 to Q3 = 711.0–973.0) Statistical significances are as follows: V : Mem (non-significant), Mem : Mem1 ($P < 0.01$), Mem1 : Mem2 (non-significant), Mem : Mem2 ($P < 0.05$); the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

found between animals after the 1st Met + Mem administration and animals that received the 2nd Met challenge dose (see Figure 3; Met+Mem versus Mem2).

Finally, in the group n_4 the first application of Mem did not affect Distance Travelled significantly ($p>0.05$) (see Figure 4; V versus Mem). The 1st memantine challenge dose (Mem1) provoked a highly significant decrease ($P < 0.01$) in locomotion in animals pre-treated repeatedly with Mem (see Figure 4; Mem versus Mem1). Mice that received the 2nd memantine challenge dose (Mem2) showed no statistically significant changes when compared with animals after the 1st Mem challenge dose (see Figure 4; Mem1 versus Mem2). There was, however, a significant decrease ($P < 0.05$) in locomotion between animals after the 1st dose of Mem and animals after administration of the 2nd Mem challenge dose (see Figure 4; Mem versus Mem2).

DISCUSSION

The results from the group n_1 were identical to the results from numerous of our previous studies and confirm the development of sensitisation to the stimulatory effects of methamphetamine (e.g. Landa et al. 2006a,b, 2011, 2012). In our experimental design we focused also on the expression of behavioural sensitisation and although there was a clear trend towards an increase in locomotion in mice after the second methamphetamine challenge dose when compared to sensitised animals, it did not reach statistical significance. Nevertheless behavioural sensitisation to the stimulatory effects of methamphetamine unambiguously persisted in this group even after the wash-out period.

Neither development, nor expression of behavioural sensitisation occurred in mice sensitised with methamphetamine (group n_2) in which mice were administered methamphetamine challenge doses in combination with memantine. This result is in accordance with the majority of similar experiments reporting the inhibitory effects of NMDA receptors antagonists on the development of sensitisation to amphetamines (Wolf 1998). The findings obtained in this experiment are also to a certain extent in compliance with our previous study where we tested the possible influence of another NMDA receptor antagonist, felbamate, on behavioural sensitisation to methamphetamine (Landa et al. 2012). This substance also inhibited, even in a more pronounced manner, sensitisation in mice repeatedly pre-treated with methamphetamine that were given a methamphetamine challenge dose together with felbamate. A felbamate challenge dose administered along with methamphetamine after repeated methamphetamine pre-treatment significantly decreased locomotion in the previous experiment, which was, however, not the case in the group of animals in the present study. These animals were repeatedly administered methamphetamine and the challenge dose consisted of a methamphetamine + memantine combination. There was a trend towards an increase in locomotion although this was non-significant. This difference between the effects of felbamate and memantine could support the hypothesis suggesting that NMDA antagonists

affect behavioural sensitisation in a substance-dependent manner. It is, for example, in accordance with the report of Bespalov et al. (2000) indicating that cocaine-conditioned behaviours can be selectively modulated by some, but not all, NMDA receptor antagonists.

Although the involvement of glutamatergic neurotransmission in the processes of behavioural sensitisation is widely reported (Stewart and Druhan 1993; Ohmori et al. 1994; Subramaniam et al. 1995; Li et al. 1997; Wolf 1998; Tzschentke and Schmidt 2003; Lee et al. 2011), there are also reports suggesting that NMDA receptor antagonists affect the action of addictive substances by different means. For example, Glick et al. (2001) reported that the non-competitive NMDA receptor antagonist dextromethorphan significantly decreased methamphetamine self-administration in rats; the authors nevertheless suggested that these findings could have been mediated via non-NMDA mechanisms. Similarly, Chen et al. (2012) reported that the NMDA receptor antagonist memantine significantly attenuated chronic morphine-induced place-preference in rats. These authors hypothesised that the development of opioid addiction could be associated with neuronal inflammation and degeneration and thus the attenuation of morphine-induced addiction behaviour by memantine may be due to its anti-inflammatory and neurotrophic effects rather than through NMDA receptor blockade. Despite these findings, results supporting the role of NMDA receptor in processes associated with drug addiction are reported much more frequently (Wolf et al. 1995; Shim et al. 2002; Hong et al. 2006; Yang et al. 2008).

Popik et al. (2003) in their study tried to compare the effects of memantine in mice on expression of place preferences that were conditioned with morphine administration (10 mg/kg) and furthermore with sexual encounters with females and consumption of regular laboratory food. Memantine in this experiment inhibited the expression of place preference conditioned with morphine and sexual encounter; however, it did not affect food-conditioned animals. Thus, these results suggested that antagonizing the NMDA receptor may not only affect drug-reinforced behaviour (Popik et al. 2003).

Similarly, Aguilar et al. (2009) tested the influence of memantine on sensitisation to the motor and rewarding effects of morphine. They revealed in mice that administration of morphine at the dose of 2 mg/kg was ineffective in animals pre-exposed to saline but induced a clear conditioned place preference in those pre-exposed to morphine. In contrast, mice pre-exposed to morphine + memantine did not acquire conditioned place preference. Only mice pre-exposed to morphine showed an increased motor response to morphine at a dose of 2 mg/kg. These results indicate that NMDA glutamatergic receptors were involved in the development of sensitisation to conditioned rewarding effects and that memantine blocked sensitisation to the rewarding effects of morphine (Aguilar et al. 2009). This is in accordance with our findings where repeated pre-treatment with the methamphetamine+memantine combination blocked the development of behavioural sensitisation to methamphetamine. On the other hand, the results obtained by Aguilar et al. (2009) are in contradiction with our previous results obtained in the study with another NMDA receptor antagonist felbamate (Landa et al. 2012), where pre-treatment with felbamate+methamphetamine resulted, after the methamphetamine challenge dose, in the development of sensitisation to the stimulatory effects of methamphetamine.

The concept of behavioural sensitisation formulated by Robinson and Berridge (1993, 2003) clearly indicates that sensitisation plays a very important role in the processes of craving and the reinstatement of compulsive drug-seeking behaviour. The majority of studies, including this article, suggest that glutamatergic modulators, in particular NMDA receptor antagonists, affect the sensitising phenomenon and that the influence of these substances is largely inhibitory. Our results support this suggestion also. Moreover, this notion has been successfully tested in humans dependent on opioids where memantine attenuated the expression of opioid physical dependence (Bisaga et al. 2001).

Despite somewhat controversial results reported in the literature, the use of NMDA receptor antagonists could in many cases serve as a useful method for blocking behavioural sensitisation, decrease the risk of relapses in ex-addicts and thus represents a promising pharmacological tool for possible treatment of substance dependence (David et al. 2006).

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7.8 The effect of sertindole on behavioural sensitization to methamphetamine in mice

It has been described that the chronic administration of the dopamine D_2 receptor antagonist sertindole in rats deactivated dopaminergic neurons in the ventral tegmental area (Skarsfeldt 1992), which is an important structure for the development of behavioural sensitization (Kalivas and Duffy 1993), and dopaminergic transmission plays a key role in the process of behavioural sensitization. This study was therefore aimed at the influence of the antipsychotic (neuroleptic) drug sertindole on behavioural sensitization to the effects of methamphetamine on mouse locomotor activity in the open field test.

The results from the group of mice treated repeatedly with methamphetamine are completely consistent with the findings from our previous studies (Landa et al. 2006a, b; 2011; 2012a, b) and again confirm the development of sensitization to the stimulatory effects of methamphetamine on locomotion in mice.

A challenge dose of methamphetamine and sertindole combination given to animals repeatedly pre-treated with methamphetamine inhibited locomotion compared to the acute methamphetamine dose, which is similar to results obtained in human subjects dependent on methamphetamine, who were given another D_2 receptor antagonist, risperidone, which produced a decrease in methamphetamine use (Meredith et al. 2007).

There was an increase in locomotion in mice that were repeatedly pre-treated with the methamphetamine + sertindole combination and challenged with a dose of methamphetamine.

The acute dose of sertindole elicited a significant decrease in locomotion that persisted also after the last of the eight daily doses.

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The effect of sertindole on behavioural sensitisation to methamphetamine in mice

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ABSTRACT: Similarly to various other addictive substances, methamphetamine (Met) produces, following repeated application, a strong increase in behavioural responses (particularly locomotor behaviour), a phenomenon termed behavioural sensitisation. In our previous studies we tested the effects of various psychotropic drugs on behavioural sensitisation to Met, particularly the effects of cannabinoid receptor ligands with different intrinsic activities and felbamate and memantine, antagonists of *N*-methyl-D-aspartate (NMDA) receptors. In the present study we investigated the influence of the antipsychotic drug sertindole (Srt) on sensitisation to the effects of Met on mouse locomotor behaviour in the Open field test. Male mice were randomly divided into 4 groups and were administered drugs seven times (from the 7th to 13th day of the experiment) as follows: (a) $n_{1, 2}$: Met at the doses of 2.5 mg/kg/day; (b) n_3: Met + Srt at the doses of 2.5 mg/kg/day + 10.0 mg/kg/day; (c) n_4: Srt at the dose of 10.0 mg/kg/day. Locomotion in the Open field test was measured (a) after administration of vehicle on the 1st day, (b) after the 1st dose of drugs given on the 7th day, and (c) on the 14th day after the "challenge doses" administered in the following way: n_1: Met; n_2: Met+Srt, n_3: Met; n_4: Srt. We found the following significant behavioural changes: (1) a stimulatory influence of Met and development of sensitisation after repeated treatment (n_1); (2) an inhibition of Met sensitisation in the case of a combined challenge dose of Met + Srt (n_2); (3) a stimulatory effect of Met when animals were repeatedly pre-treated with Met + Srt (n_3); (4) a significant inhibition of locomotion after the 1st dose of Srt, that persisted even after the last Srt dose (n_4). Data concerning the involvement of sertindole in reward processes associated with drug addiction are not completely consistent and our results reflect this ambiguity to a certain extent. A combined challenge dose of Met + Srt administered after repeated pre-treatment with Met inhibited the development of behavioural sensitisation; on the other hand a Met challenge dose alone administered after repeated pre-treatment with Met + Srt produced a significant increase in locomotion.

Keywords: behavioural sensitisation; methamphetamine; sertindole; mice

List of abbreviations

AM 251 = *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide, **JWH 015** = 1 propyl-2-methyl-3-(1-naphthoyl)indole, **Met** = methamphetamine, **NMDA** = *N*-methyl-D-aspartate, **Srt** = sertindole, **V** = vehicle

It is well established that repeated administration of the psychostimulant drug methamphetamine results in an increased behavioural response to this substance. This phenomenon is termed behavioural sensitisation and was described for the first time by Robinson and Berridge (1993). Behavioural sensitisation occurs not only for psychostimulants – amphetamine (Enman and Unterwald 2012; Fukushiro et al. 2012) or cocaine (Aracil-Fernandez et al. 2012; Ramos et al. 2012) but also for other psychotropic substances – e.g., morphine (Hofford et al. 2012; Niu et al. 2012), delta(9)-tetrahydrocannabinol (Cadoni et al. 2008), ethanol (Bahi and Dreyer 2012) and nicotine (Lee et al. 2012).

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It has been suggested that behavioural sensitisation is a consequence of drug-induced neuroadaptive changes in a circuit which involves particularly dopaminergic, glutamatergic and GABAergic interconnections between the ventral tegmental area, nucleus accumbens, prefrontal cortex and amygdala (Vanderschuren and Kalivas 2000; Nestler 2001). In our previous studies we investigated the possible effects of various psychotropic drugs on behavioural sensitisation to methamphetamine (particularly cannabinoids and NMDA receptor antagonists). We tested the effects of the CB_1 receptor agonist methanandamide, CB_1 receptor antagonist AM 251 and CB_2 receptor agonist JWH 015 (Landa et al. 2006a,b), and furthermore the effects of the glutametergic NMDA receptor antagonists felbamate (Landa et al. 2012a) and memantine (Landa et al. 2012b).

In the present set of experiments we investigated a possible interference of the antipsychotic drug sertindole with the sensitising phenomenon. Sertindol is a second-generation antipsychotic (neuroleptic) agent used in human medicine that was recently reintroduced into the market for the treatment of schizophrenia (Spina and Zoccali 2008). It acts as an antagonist of dopamine D_2, serotonin 5-HT_{2A}, 5-HT_{2C}, and α_1-adrenergic receptors (Muscatello et al. 2010). According to our knowledge, there are no reports on the use of sertindole in veterinary medicine; however, other drugs from the same group of antipsychotics (e.g., chlorpromazine) have been used for the treatment of aggressive behaviour in dogs (Blackshaw 1991).

It has been shown in experimental pharmacology that chronic administration of sertindole to rats inactivated dopamine neurons in the ventral tegmental area (Skarsfeldt 1992), which is a crucial structure for the development of behavioural sensitisation (Kalivas and Duffy 1993). Dopaminergic transmission also plays a substantial role in the process of sensitisation. Suzuki and Misawa (1995) reported that the dopamine D_2 receptor antagonist sertindole antagonised place preference in rats induced by morphine, cocaine and methamphetamine. Since these experiments with sertindole showed an unambiguous interference with dopaminergic neurotransmission and with methamphetamine brain mechanisms in the model of place preference, we therefore focused on possible effects of this substance on the development of behavioural sensitisation to the stimulatory effects of methamphetamine in mice, which is believed to play an important role in the processes of drug addiction.

MATERIAL AND METHODS

Animals

Male mice (strain ICR, TOP-VELAZ s.r.o., Prague, Czech Republic) with an initial weight of 18–21 g were used. Animals were randomly allocated into four treatment groups. In order to minimise possible variability due to circadian rhythms the behavioural observations were always performed in the same period between 1:00 p.m. and 3:00 p.m. and the animals were maintained under a 12-h light/dark cycle.

Apparatus

Locomotor activity was measured using an open-field equipped with Actitrack (Panlab, S.L., Spain). This device consists of two square-shaped frames that deliver beams of infrared rays into the space inside the square. A plastic box is placed in this square to act as an open-field arena (base 30 × 30 cm, height 20 cm), in which the animal can move freely. The apparatus software records locomotor activity of the animal by registering the beam interruptions caused by movements of the body. Using this equipment we have determined the Distance Travelled (trajectory in cm per 3 min).

Drugs

Vehicle and all drugs were always given in a volume adequate for drug solutions (10 ml/kg).

(+)Methamphetamine, (*d-N*,α-dimethylphenylethylamine;*d*-desoxyephedrine) (Sigma Chemical Co.) was dissolved in saline.

Sertindole, (1-(2-{4-[5-chloro-1-(4-fluorophenyl)-1H-indol-3-yl]-1-piperidinyl}ethyl)-2-imidazolidinone), (H. Lundbeck A/S) was ultrasonically suspended in Tween 80 (one drop in 10 ml saline); vehicle treatment as a control in this case contained the corresponding amount of Tween 80.

Procedure

Mice were randomly allocated into four groups ($n_1 = 9$, $n_2 = 10$, $n_3 = 10$, $n_4 = 10$) and all were given vehicle on Day 1 (10 ml/kg). There were no applications from Days 2 to 6. For the next seven days

animals were treated daily as follows: (a) $n_{1,2}$ 2.5 mg/kg/day of Met; (b) n_3 combination of Met + Srt at the doses of 2.5 mg/kg/day and 10.0 mg/kg/day, respectively; (c) n_4 10.0 mg/kg/day of Srt. On Day 14 all animals were given challenge doses in the following way: n_1: Met at the dose of 2.5 mg/kg, n_2: Met + Srt at the doses of 2.5 mg/kg and 10.0 mg/kg, respectively, n_3: Met at the dose of 2.5 mg/kg, n_4: Srt at the dose of 10.0 mg/kg. All doses of both Met and Srt were administered intraperitoneally. Changes in locomotion were measured for a period of 3 min in the open field on Days 1, 7 and 14 to assess the sensitising phenomenon.

The experimental protocol complies with the European Community guidelines for the use of experimental animals and was approved by the Animal Care Committee of the Masaryk University Brno, Czech Republic.

Data analysis

As the data was not normally distributed (according to the Kolmogorov-Smirnov test of normality), non-parametric statistics were used: Wilcoxon matched-pairs signed-ranks test, two tailed (statistical analysis package Statistica – StatSoft, Inc., Tulsa, USA).

RESULTS

The treatment administered to group n_1 caused a highly significant increase ($P < 0.01$) in locomotion after the 1st application of methamphetamine (Met) compared to the application of vehicle (V) (see Figure 1; V versus Met). The challenge dose of Met produced a further significant increase in

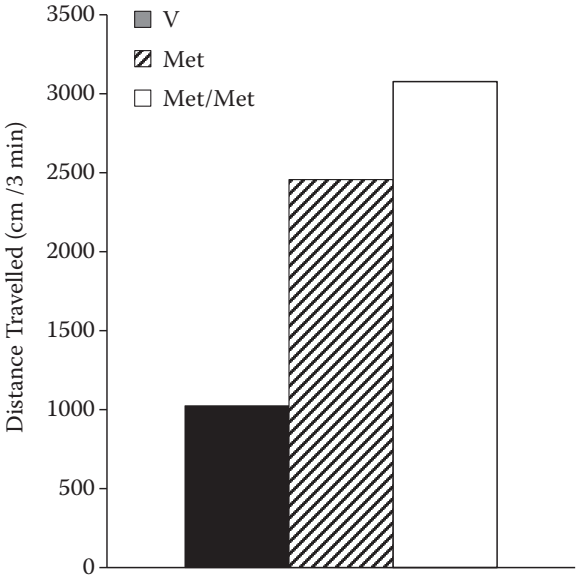

Figure 1. Effects of drug treatments in the group n_1 on Distance Travelled (cm/3 min) in the mouse open field test shown as medians (interquartile ranges Q1 to Q3)

V = mice after the 1st dose of vehicle, (interquartile range Q1 to Q3 = 798.6–1143.7); Met = mice after the 1st dose of methamphetamine (2.5 mg/kg), (interquartile range Q1 to Q3 = 1962.0–1603.0); Met/Met = mice repeatedly pretreated with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg), (interquartile range Q1 to Q3 = 2392.0–3182.0)

Statistical significances are as follows: V : Met ($P < 0.01$), Met : Met/Met ($P < 0.05$), V : Met/Met ($P < 0.01$); the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

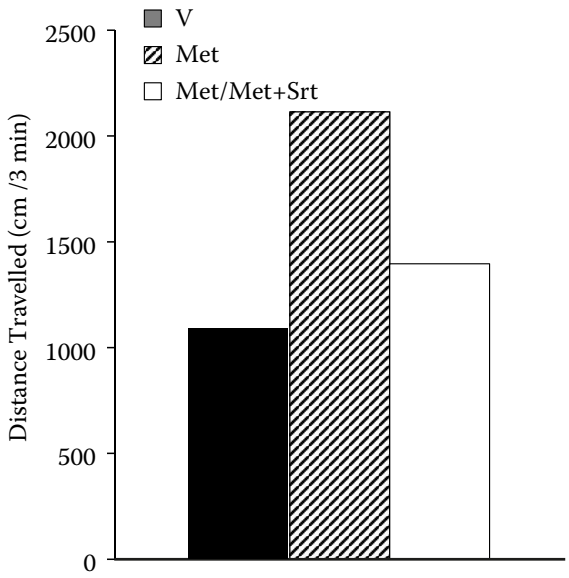

Figure 2. Effects of drug treatments in the group n_2 on Distance Travelled (cm/3 min) in the mouse open field test shown as medians (interquartile ranges Q1 to Q3)

V = mice after the 1st dose of vehicle, (interquartile range Q1 to Q3 = 880.2–1375.5); Met = mice after the 1st dose of methamphetamine (2.5 mg/kg), (interquartile range Q1 to Q3 = 1540.0–2493.0); Met/Met + Srt = mice repeatedly pretreated with methamphetamine after the challenge dose of methamphetamine + sertindole (2.5 mg/kg + 10.0 mg/kg), (interquartile range Q1 to Q3 = 743.0–2092.0)

Statistical significances are as follows: V : Met ($P < 0.01$), Met : Met/Met + Srt ($P < 0.05$), V : Met/Met + Srt (non-significant); the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

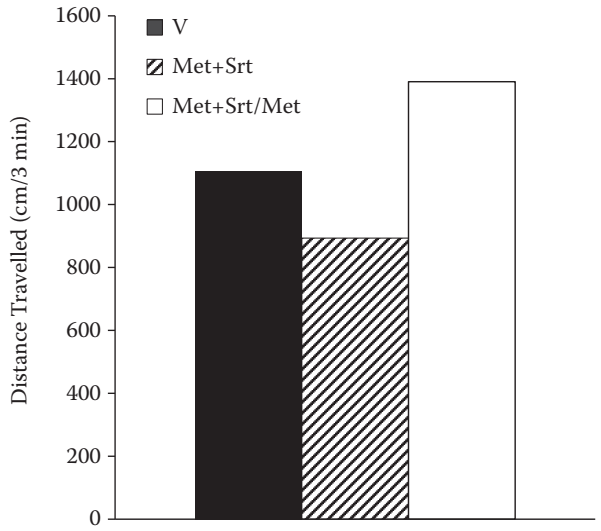

Figure 3. Effects of drug treatments in the group n_3 on Distance Travelled (cm/3 min) in the mouse open field test shown as medians (interquartile ranges Q1 to Q3)

V = mice after the 1st dose of vehicle, (interquartile range Q1 to Q3 = 795.7–1188.0); Met + Srt = mice after the 1st dose of combination methamphetamine + sertindole (2.5 mg/kg + 10.0 mg/kg), (interquartile range Q1 to Q3 = 735.2–1122.3); Met + Srt/Met = mice repeatedly pre-treated with the combination methamphetamine + sertindole after the challenge dose of methamphetamine (2.5 mg/kg), (interquartile range Q1 to Q3 = 1121.0–1869.0)

Statistical significances are as follows: V : Met + Srt (non-significant), Met + Srt : Met + Srt/Met ($P < 0.05$), V : Met + Srt/Met ($P < 0.05$); the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

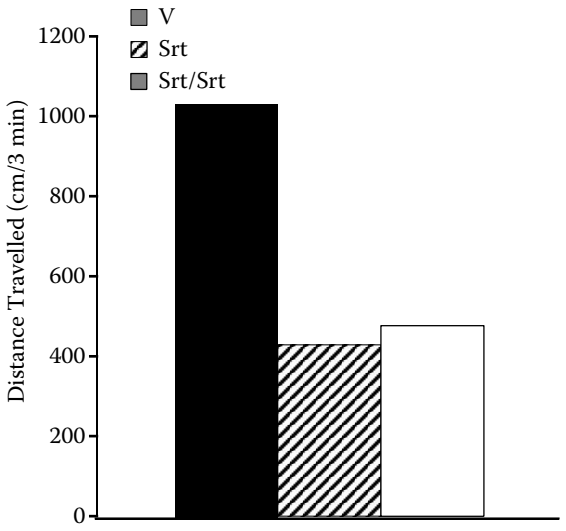

Figure 4. Effects of drug treatments in the group n_4 on Distance Travelled (cm/3 min) in the mouse open field test shown as medians (interquartile ranges Q1 to Q3)

V = mice after the 1st dose of vehicle, (interquartile range Q1 to Q3 = 922.2–1202.2); Srt = mice after the 1st dose of sertindole (10.0 mg/kg), (interquartile range Q1 to Q3 = 309.5–700.0); Srt/Srt = mice repeatedly pre-treated with sertindole after the challenge dose of sertindole (10.0 mg/kg), (interquartile range Q1 to Q3 = 410.3–639.8)

Statistical significances are as follows: V : Srt ($P < 0.01$), Srt : Srt/Srt (non-significant), V : Srt/Srt ($P < 0.01$); the non-parametric Wilcoxon matched-pairs signed-ranks test, two tailed

Distance Travelled ($P < 0.05$) in animals pre-treated repeatedly with Met (see Figure 1; Met versus Met/Met). A highly significant difference in locomotion was also found between mice after the administration of V and animals that received the Met challenge dose (see Figure 1; V versus Met/Met).

In group n_2 the 1st administration of Met caused a highly significant increase ($P < 0.01$) in Distance Travelled compared to the application of V (see Figure 2; V versus Met). In contrast, the challenge dose of Met + Srt provoked a significant decrease ($P < 0.05$) in locomotion in animals pre-treated repeatedly with Met (see Figure 2; Met versus Met/Met + Srt). No statistically significant increases ($P > 0.05$) were found between animals after the application of V compared to animals that were given the Met + Srt combination after repeated Met treatment (see Figure 2; V versus Met/Met + Srt).

In group n_3 the 1st application of the Met + Srt combination did not affect locomotor activity in mice significantly ($P > 0.05$) (see Figure 3; V versus Met + Srt), whereas the challenge dose of Met provoked a significant increase ($P < 0.05$) in locomotion in animals pre-treated repeatedly with Met + Srt (see Figure 3; Met + Srt versus Met + Srt/Met). There was a significant increase ($P < 0.05$) in locomotion in animals pre-treated with the Met + Srt combination after the Met challenge dose when compared with the animals that were administered V (see Figure 3; V versus Met + Srt/Met).

Finally, in group n_4 the 1st application of Srt caused a highly significant decrease in locomotion when compared with animals that received vehicle ($P < 0.01$) (see Figure 4; V versus Srt). The challenge dose of Srt did not affect Distance Travelled significantly ($P > 0.05$) in animals pre-treated repeatedly with Srt when compared with animals after the 1st Srt dose (see Figure 4; Srt versus Srt/Srt). A highly significant decrease ($P < 0.01$) in locomotion was found in mice after the administration of

V compared to animals that were repeatedly pre-treated with Srt and were administered the Srt challenge dose (see Figure 4; V versus Srt/Srt).

DISCUSSION

The results from the group of mice treated repeatedly with methamphetamine are entirely consistent with results from our previous studies (Landa et al. 2006a,b; 2011; 2012a,b) and again confirm the development of sensitisation to the stimulatory effects of methamphetamine on locomotor behaviour in this original dosage regimen used in mice. The 1st dose in the mice under the repeated treatment with sertindole elicited a significant decrease in locomotion that persisted also after the last of the eight daily doses. This finding is in agreement with the results of Suzuki and Misawa (1995) who reported that sertindole given alone produced neither preference nor aversion for the drug-associated place. Therefore, they suggested that sertindole had no potential for abuse. A challenge dose of a methamphetamine + sertindole combination given to animals repeatedly pre-treated with methamphetamine inhibited locomotion compared to the 1st methamphetamine dose, which is similar to observations made in human subjects dependent on methamphetamine, who were given another D_2 receptor antagonist risperidone, which went on to produce a decrease in methamphetamine use (Meredith et al. 2007). On the other hand, the use of a further antipsychotic drug, olanzapine, in humans dependent on cocaine did not support the usefulness of this substance for the treatment of cocaine dependence (Kampman et al. 2003).

Akdag et al. (2011) tested the effects of risperidone (a substance that similarly to sertindole also belongs to the group of atypical antipsychotics with similar multiple mechanisms of action and with a high selectivity for mesolimbic pathways) on nicotine-induced locomotor sensitisation in rats. Risperidone affects serotonin 5-HT_{2A-C} receptors, dopamine D_2 receptors, α_1- and α_2-adrenergic receptors and also histamine H_1 receptors (Akdag et al. 2011). These authors focused on both development and expression of sensitisation and found that repeated administration of nicotine provoked in their experimental design a robust sensitisation. Furthermore, they described that pre-treatment with risperidone inhibited the expression but not the development of nicotine-induced locomotor sensitisation in rats (Akdag et al. 2011). Thus, they concluded that risperidone blocked the continuation of nicotine-type addictive behaviour, whereas it was ineffective against the early adaptations in the development of nicotine addiction. Despite this, the antipsychotic drug risperidone may be of limited beneficial use in nicotine dependence treatment (Akdag et al. 2011). On the other hand, Meng et al. (1998) reported that a typical and an atypical antipsychotic drug, haloperidol and clozapine, respectively, blocked the development of behavioural sensitisation to amphetamine in rats. Our results showed an increase in animals that were repeatedly pre-treated with the methamphetamine + sertindole combination and challenged with a dose of methamphetamine, however this increase cannot be considered as development of sensitisation.

Prinssen et al. (2004) examined whether the ability of the dopamine D_2 receptor antagonists eticlopride and raclopride (substances primarily used in basic pharmacological research) to decrease cocaine-induced locomotion varied between non-sensitised and sensitised mice if they were challenged with cocaine. In this experiment the dopamine D_2 receptor antagonists eticlopride and raclopride were less efficient in inhibiting the locomotor effects of cocaine in sensitised mice compared to the non-sensitised animals. However, when the authors used the lowest doses to maximally increase locomotion in each of the repeated treatment conditions (10 and 40 mg/kg) both dopamine D_2 receptor antagonists inhibited the influence of cocaine on locomotor activity in non-sensitised and sensitised mice to a similar extent (Prinssen et al. 2004). Thus, these results indicate that the possible effects of dopamine receptor agonists are dose-dependent.

Data concerning the involvement of sertindole in reward processes associated with drug addiction are not completely consistent. Suzuki and Misawa (1995) reported that the dopamine D_2 receptor antagonist sertindole antagonised place preference in rats induced by morphine, cocaine and methamphetamine. On the other hand, Arnt (1992) tested the effect of various antipsychotic drugs (sertindole, clozapine, flupentixol, haloperidol) on the discriminative stimulus properties of amphetamine (i.e., dopamine stimulant) and LSD (i.e. 5-HT_2 receptor agonist) and found in rats that sertindole antagonised the effects of LSD, whereas those of *d*-amphetamine were unchanged. In contrast, Jackson et al. (1994) found that sertindole blocked amphetamine and phencyclidine-induced

motor stimulation in rats and similarly, Artn (1995) described that sertindole inhibited hypermotility induced by two dose levels of amphetamine. These data indicate that there is not only variability in doses but also probable differences among substances from the groups of antipsychotics in their ability to interfere with the action of various drugs of abuse. In addition, there are also further factors contributing to diversity in the action of D_2 receptor antagonists. For example, it has been shown that there was a difference between the effects of acute and chronic antipsychotic drug treatment on dopamine neurons. Whereas acute application increased dopamine neuron population activity, chronic administration (21 days) led to inactivation of dopamine neurons in the substantia nigra of rats (Grace et al. 1997).

Despite these controversies, it can be concluded that findings such as those reported by Suzuki and Misawa (1995) and also results from our study suggest that the use of sertindole holds therapeutic promise for the treatment of drug addiction, though further research is certainly required.

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7.9 The effect of the cannabinoid CB_1 receptor agonist arachidonylcyclopropylamide (ACPA) on behavioural sensitization to methamphetamine in mice

In our previous studies, pre-treatment with the CB_1 receptor agonist methanandamide elicited cross-sensitization to methamphetmine (Landa et al. 2006a). In this experiment, we aimed at the possible influence of another selective cannabinoid CB_1 receptor agonist arachidonylcyclopropylamide (ACPA) on behavioural sensitization to the effects of methamphetamine on mouse locomotor activity in the open field test.

Similarly to previous studies (Landa et al. 2006a, b; 2011; 2012a, b, c), there was a robust development of behavioural sensitization to the stimulatory effects of methamphetamine.

The first dose of the CB_1 receptor selective agonist ACPA led to a significant decrease in locomotor behaviour. This, however, runs to some extent counter to the results of our previous experiments using another CB_1 receptor agonist methanandamide, which did not change mouse locomotor behaviour (Landa et al. 2006a).

There was an increase in locomotion in mice pretreated with ACPA after the methamphetamine challenge dose, however the cross-sensitization phenomenon was not fully developed, which is also in contradiction to our previous experiments using methanandamide (Landa et al. 2006a).

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The effect of the cannabinoid CB_1 receptor agonist arachidonylcyclopropylamide (ACPA) on behavioural sensitisation to methamphetamine in mice

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ABSTRACT: The psychostimulant methamphetamine (Met), similarly to other drugs of abuse, is known to produce an increased behavioural response after its repeated application (behavioural sensitisation). It has also been described that an increased response to a drug may be elicited by previous repeated administration of another drug (cross-sensitisation). We have previously shown that the CB_1, CB_2 and TRPV (vanilloid) cannabinoid receptor agonist methanandamide, cross-sensitised to Met stimulatory effects in mice. The present study was focused on ability of the more selective and potent CB_1 receptor activator arachidonylcyclopropylamide (ACPA) to elicit cross-sensitisation to the stimulatory effects of Met on mouse locomotor behaviour in the Open field test. Male mice were randomly divided into three groups and on seven occasions (from the 7th to 13th day of the experiment) were administered drugs as follows:(a) n_1: vehicle at the dose of 10 ml/kg/day; (b) n_2: Met at the dose of 2.5 mg/kg/day; (c) n_3: ACPA at the dose of 1.0 mg/kg/day. Locomotor behaviour in the Open field test was measured (a) after administration of vehicle on the 1st experimental day, (b) after the 1st dose of drugs given on the 7th day, and (c) on the 14th day after the "challenge doses" administered in the following manner: n_1: saline at a dose of 10 ml/kg, $n_{2,3}$: Met at a dose of 2.5 mg/kg. The observed behavioural changes consisted in: (a) gradual development of habituation to the open field conditions in three consecutive tests; (b) development of behavioural sensitisation to the stimulatory effects of Met after repeated treatment; (c) insignificant effect of repeated pre-treatment with ACPA on the stimulatory effects of Met challenge dose. The results of our study give rise to the question which of the cannabinoid receptor mechanisms might be most responsible for the neuroplastic changes inducing sensitisation to the stimulatory effects of Met.

Keywords: behavioural sensitisation; methamphetamine; cannabinoids; ACPA; mice

List of abbreviations

ACPA = N-(cyclopropyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (alternative name: arachidonylcyclopropylamide); **AM 251** = N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; **GPR55** = G protein-coupled receptor 55; **JWH 015** = 1 propyl-2-methyl-3-(1-naphthoyl)indole; **Met** = methamphetamine; **Sal** = saline; **THC** = delta 9-tetrahydrocannabinol; **TRPV1** = transient receptor potential cation channel subfamily V member 1; **V** = vehicle

It has been consistently described that repeated administration of dependence-producing substances leads to an increased behavioural response, defined as behavioural sensitisation (Robinson and Berridge 1993). This phenomenon was observed after repeated administration of both legal and illegal drugs and has been described for ethanol (Broadbent 2013; Kim and Souza-Formigoni 2013; Linsenbardt and Boehm 2013), nicotine (Hamilton et al. 2012; Lenoir et al. 2013; Perna and Brown 2013), caffeine (Zancheta et al. 2012), cannabinoids (Rubino et al. 2003; Cadoni et al. 2008), psycho-

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stimulants (Landa et al. 2006a,b; 2011; 2012a,b; Wang et al. 2010; Ball et al. 2011; Kameda et al. 2011) or opioids (Bailey et al. 2010; Liang et al. 2010; Farahmandfar et al. 2011; Hofford et al. 2012; Rezayof et al. 2013).

When an increased response to a tested substance is elicited by previous repeated administration of a different drug, such a phenomenon is termed as cross-sensitisation. Cross-sensitisation was described (among others) after repeated treatment of nicotine to amphetamine (Adams et al. 2013) with tetrahydrocannabinol to heroin (Singh et al. 2005) or with methamphetamine (Met) to modafinil (Merhautova et al. 2012).

Vinklerova et al. (2002) reported that in animals trained to self-administer Met (rat *i.v.* drug self-administration model) the cannabinoid CB_1 receptor antagonist/inverse agonist AM 251 decreased Met intake. This finding obtained in our laboratory suggested an interaction between the endocannabionoid system and Met brain mechanisms. Thus, we then focused on interactions of cannabinoid receptor ligands with different intrinsic activities and Met. Using our original experimental design in the mouse Open Field Test and the model of agonistic behaviour we found that repeated pre-treatment with the CB_1 receptor agonist methanandamide elicited cross-sensitisation to the stimulatory effects of Met, whereas pre-treatment with the CB_2 receptor agonist JWH 015 did not (Landa et al. 2006a,b). Furthermore, combined pre-treatment with methamphetamine and CB_1 receptor antagonist/inverse agonist AM 251, suppressed sensitisation to Met, which is in accordance with the attenuation of behavioural sensitisation to amphetamine reported after co-administration with AM 251 (Thiemann et al. 2008).

Both Met and herbal cannabinoids, particularly delta 9-tetrahydrocannabinol (THC; the main psychotropic component of marijuana) are well known substances with dependence potential. Nevertheless, there are also reports on the therapeutic potential of pharmacological manipulation of the endocannabinoid system; besides addiction, this system has also been studied with respect to possible treatment of multiple sclerosis, chronic neuropathic pain, nausea and vomiting, loss of appetite, cancer or AIDS patients, psychosis, epilepsy, metabolic disorders, asthma and glaucoma (Fisar 2009; Robson 2014). In veterinary medicine attention has focused mainly on the cases of intoxication with marijuana (Donaldson 2002; Meola et al. 2012). Nevertheless, there is an increasing number of reports (as yet anecdotal) on the therapeutic use of cannabinoids in small animals. However, this issue still need to be investigated thoroughly.

In the present experiment we examined the possible influence of the selective cannabinoid CB_1 receptor agonist arachidonylcyclopropylamide (ACPA) on behavioural sensitisation to Met. Repeated use of cannabinoid CB_1 receptor agonists is believed to facilitate consumption of other dependency producing substances (Lamarque et al. 2001). On the other hand, the use of CB_1 receptor antagonists was described as a possible approach for treatment of drug dependence (LeFoll and Goldberg 2005; Thiemann et al. 2008). We believe that our study may contribute to better understanding of the mutual relationship between cannabinoids and Met interactions in the processes of behavioural sensitisation.

MATERIAL AND METHODS

Animals. Mice (males, strain ICR, TOP-VELAZ s.r.o., Prague, Czech Republic) weighing 18–21 g at the beginning of the experiment were used. Animals were randomly allocated into three equal groups. In order to minimise possible variability due to circadian rhythms the behavioural observations were always performed in the same period between 1:00 p.m. and 3:00 p.m. The animals were maintained under a 12-h light/dark cycle.

Apparatus. Locomotor activity was measured in an open-field arena using the Actitrack instrument (Panlab, S.L., Spain). This device consists of two square-shaped frames that deliver beams of infrared rays into the space inside the square. A plastic box is placed in this square and it acts as an open-field arena (base 30 × 30 cm, height 20 cm), in which the animal can move freely. The apparatus software records locomotor activity of the animal by registering the beam interruptions caused by movements of its body. Using this equipment we determined the Distance Travelled (trajectory in cm per 3 min).

Drugs. Vehicle and all drugs were always given in a volume adequate for drug solutions (10 ml/kg).

(+)Methamphetamine, (d-N,α-dimethylphenylethylamine;d-desoxyephedrine), (Sigma Chemical Co.) was dissolved in saline.

Arachidonylcyclopropylamide, *N*-(cyclopropyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide was supplied

pre-dissolved in anhydrous ethanol 5 mg/ml (Tocris Cookson Ltd., UK) and was further diluted in saline to the appropriate concentration; the vehicle contained an adequate part of ethanol (final concentration in the injection below 1%) to make the effects of the placebo and the drug comparable.

The adjustment of all drug doses was based on both literature data and results obtained in our earlier behavioural experiments.

Procedure. Mice were randomly divided into three treatment groups (n_1 = 10, n_2 = 11, n_3 = 10) and all were given vehicle on Day 1 (10 ml/kg). There were no applications from Days 2 to 6. For the next seven days animals were daily treated as follows: (a) n_1: saline at the dose of 10 ml/kg/day; (b) n_2: Met at the dose of 2.5 mg/kg/day; (c) n_3: ACPA at the dose of 1.0 mg/kg/day. On Day 14 animals were given challenge doses in the following manner: n_1: saline at the dose of 10 ml/kg, $n_{2, 3}$: Met at the dose of 2.5 mg/kg. All substances were administered intraperitoneally. Changes in horizontal locomotion were measured for a period of 3 min in the open field on Days 1, 7 and 14 to evaluate the sensitising phenomenon.

The experimental protocol complies with the European Community guidelines for the use of experimental animals and was approved by the Animal Care Committee of the Masaryk University Brno, Czech Republic.

Data analysis. As the data were normally distributed (according to the Kolmogorov-Smirnov test of normality), parametric statistics were used: paired *t*-test, two tailed for comparison within the individual groups and unpaired *t*-test, two tailed for comparison across the individual groups (statistical analysis package Statistica – StatSoft, Inc., Tulsa, USA).

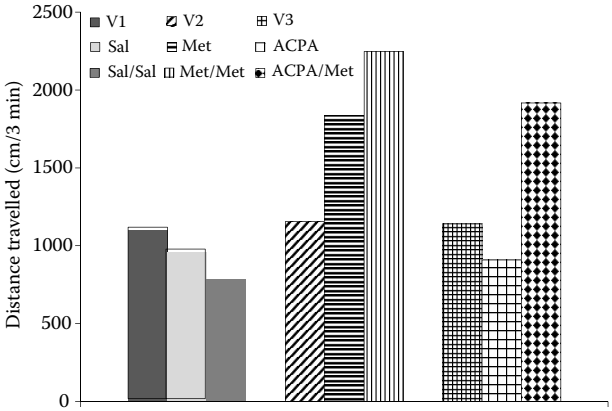

Figure 1. Effects of drug treatments on Distance Travelled (cm/3 min) in the mouse open field test shown as mean values with standard deviation (SD): V1 = mice in the group n_1 after the 1st dose of vehicle, (SD = 145.4); Sal = mice in the group n_1 after the 1st dose of saline, (SD = 379.0); Sal/Sal = mice in the group n_1 after the last dose of saline, (SD = 157.9); V2 = mice in the group n_2 after the 1st dose of vehicle, (SD = 207.2); Met = mice in the group n_2 after the 1st dose of methamphetamine (2.5 mg/kg), (SD = 413.0); Met/Met = mice in the group n_2 repeatedly pre-treated with methamphetamine after the challenge dose of methamphetamine (2.5 mg/kg), (SD = 491.0); V3 = mice in the group n_3 after the 1st dose of vehicle, (SD = 283.9); ACPA = mice in the group n_3 after the 1st dose of arachidonylcyclopropylamide (1.0 mg/kg), (SD = 228.2); ACPA/Met = mice in the group n_3 repeatedly pre-treated with ACPA (1.0 mg/kg) after the challenge dose of methamphetamine (2.5 mg/kg), (SD = 396.0). Statistical significances are as follows: V1 : Sal (non-significant), Sal : Sal/Sal (non-significant), V1 : Sal/Sal ($P < 0.01$), V2 : Met ($P < 0.01$), Met : Met/Met ($P < 0.01$), V2 : Met/Met ($P < 0.01$); V3 : ACPA ($P < 0.05$), ACPA : ACPA/Met ($P < 0.01$), V3 : ACPA/Met ($P < 0.01$); paired *t*-test, two tailed. ACPA/Met : Met/Met (non-significant), ACPA/Met : Met (non-significant); unpaired *t*-test, two tailed

RESULTS

The applications in the group n_1 induced highly significant decreases ($P < 0.01$) in locomotion after the last application of saline (Sal/Sal) compared to the 1st application (V1) (see Figure 1; V1 versus Sal/Sal).

The applications in group n_2 led to highly significant increases ($P < 0.01$) in locomotion after the 1st application of methamphetamine (Met) compared to the application of vehicle (V2) (see Figure 1; V2 versus Met). The challenge dose of Met produced a further highly significant increase in Distance Travelled ($P < 0.01$) in animals that were repeatedly given Met (see Figure 1; Met versus Met/Met). Highly significant increases ($P < 0.01$) in locomotion were also observed between the group of mice after the administration of V2 and the group that received the Met challenge dose (see Figure 1; V2 versus Met/Met).

In group n_3 the 1st administration of ACPA caused a significant decrease ($P < 0.05$) in Distance Travelled compared to the application of V3 (see Figure 1; V3 versus ACPA). In contrast, the challenge of Met caused a highly significant increase ($P < 0.01$) in locomotion in animals pre-treated repeatedly with

ACPA (see Figure 1; ACPA versus ACPA/Met). A highly significant increase in Distance Travelled ($P < 0.01$) was also found between animals after the application of V3 and animals that were given a Met challenge dose following repeated ACPA administration (see Figure 1; V3 versus ACPA/Met).

There was no statistically significant difference between animals pre-treated repeatedly with Met after the Met challenge dose and animals repeatedly pre-treated with ACPA after the Met challenge dose (see Figure 1; Met/Met versus ACPA/Met). No significant difference was found between the group that was given Met for the 1st time and the group repeatedly pre-treated with ACPA after the Met challenge dose (see Figure 1; Met versus ACPA/Met).

DISCUSSION

The robust development of behavioural sensitisation to the stimulatory effects of Met on locomotion observed in the present study is fully in accordance with results obtained earlier (Landa et al. 2006a,b; 2011; 2012a,b,c).

In the current study the first dose of the CB_1 receptor selective agonist ACPA led to a significant decrease in locomotor behaviour. This, to some extent runs counter to the results of our previous experiments using the CB_1 receptor agonist methanandamide, which did not change mouse locomotor behaviour (Landa et al. 2006a).

Cannabinoids delta-9-THC, ACPA, methanandamide, and endocannabinoid anandamide were reported to produce comparable discriminative stimulus effects (McMahon 2009). The modulatory effects of the cannabinoid CB_1 receptor-selective agonist ACPA on brain reward systems were described many times. For example, ACPA influences conditioned place preference and conditioned place aversion (Rezayof et al. 2011, 2012). Rezayof et al. (2011) found that microinjection of ACPA into the central amygdala of rats (0.5, 2.5 and 5 ng/rat) potentiated morphine-induced (2 mg/kg) conditioned place preference in a dose-dependent manner. In addition, the application of ACPA alone (5 ng/rat) led to a significant conditioned place preference. In their more recent experiments Rezayof et al. (2012) observed significant conditioned place preference after bilateral injection of ACPA into basolateral amygdala whereas co-administration of ACPA with ethanol produced conditioned place aversion. Rezayof et al. (2011) also reported that microinjection of the cannabinoid CB_1 antagonist/inverse agonist AM 251 (90 and 120 ng/animal) into central amygdala suppressed morphine-induced place preference. These results are similar to our previously published data obtained with AM 251 and Met (Landa et al. 2006a,b), where AM 251 (5.0 mg/kg) given together with Met inhibited behavioural sensitisation to this psychostimulant drug in the Open Field Test and in the model of agonistic behaviour in mice.

In the present study the locomotor activity of mice treated repeatedly with saline for three consecutive exposures in the Open Field Test decreased significantly which clearly shows the development of habituation to exploration of the open field arena. Despite that, the stimulatory effects of Met were significantly increased in the third Open Field Test in mice repeatedly pre-treated with either Met or ACPA, with no significant difference between them. However, the cross-sensitisation phenomenon was not fully confirmed with ACPA pre-treatment as there was no significant difference between the stimulatory effects of a single Met dose administered after the vehicle and Met challenge dose after repeated ACPA pre-treatment. This finding is also in contradiction with our earlier experiments using the less selective CB_1 receptor agonist methanandamide which also activates other cannabinoid receptor subtypes such as TRPV1 (vanilloid) receptors (Malinowska et al. 2001) and GPR55 receptors (Pertwee 2010).

Both ACPA and methanandamide are CB_1 receptor agonists with very low affinity for the cannabinoid CB_2 receptor subtype, with ACPA exhibiting a potency ratio of CB_2/CB_1 325 (Hillard et al. 1999) whereas the value for methanandamide is 41 (Khanolkar et al. 1996). The development of cross-sensitisation to Met by methanandamide pre-treatment was clearly observed in our previous studies (Landa et al. 2006a,b). On the other hand, methanandamide was reported to produce no changes in locomotor activities and to block amphetamine-induced behavioural sensitisation in rats (Rasmussen 2010). A decrease in locomotion after acute methanandamide treatment was observed in rats (Landa et al. 2008) while in mice the drug did not change locomotor behaviour (Landa 2006a). These results might speak in favour of possible interspecific differences in sensitivity to modulation of cannabinoid CB_1 receptor mechanisms.

Important distinctions which may underlie the different pharmacological actions of these sub-

stances also include susceptibility to hydrolytic enzymes, namely FAAH (fatty amino acid hydrolase). ACPA, similarly to ananadamide, is more susceptible, while methanandamide is more resistant probably because of the presence of a methyl substituent in its molecular stucture (Pertwee 2006). Methanandamide exhibits enhanced biological stability when compared to endocannabinoid anadamide and although the metabolic rate of ACPA has not so far been directly compared with anandamide, it is thought that the rate of metabolism is similar in primates (McMahon 2009). Thus, it is possible to speculate that different behavioural actions can be explained by faster elimination of ACPA compared to methanandamide and also by other differences. Jarbe et al. (1998) suggested that agonists of cannabinoid receptors may have various mechanisms of action. Indeed, it has been shown that both methanandamide and ACPA also possess other activities. Stimulation of CB_1 receptors in the basal ganglia and cerebellum-induced motor deficits and sedative effects of ACPA have been reported (Patel and Hillard 2001).

Our present results with the CB_1 receptor agonist ACPA diverge from those acquired earlier with methanandamide, an analogue of the endocannabinoid anandamide. However, it is clear that the endocannabinoid system is involved in modulating the brain reward pathway induced by Met and thus exploration of functional interactions with CB_1 cannabinoid receptor ligands might be a promising approach to discover potential treatments for addiction to psychostimulants (Oliere et al. 2013).

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8. List of publications in extenso related to the habilitation thesis

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10. Appendices

Appendix 1

Slais K, Machalova A, Landa L, Vrskova D, Sulcova A (2012): Could piracetam potentiate behavioural effects of psychostimulants? Medical Hypotheses, 79 (2), 216-218.

Appendix 2

Landa L, Slais K, Machalova A, Sulcova A (2014): Interaction of CB_1 receptor agonist arachidonylcyclopropylamide with behavioural sensitisation to morphine in mice. Veterinarni Medicina, 59 (6), 307-314.

Appendix 3

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Appendix 4

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Appendix 5

Landa L, Jurica J, Sliva J, Pechackova M, Demlova R (2018): Medical cannabis in the treatment of cancer pain and spastic conditions and options of drug delivery in clinical practice. Biomedical Papers, 162 (1), 18-25.

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Could piracetam potentiate behavioural effects of psychostimulants?

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ABSTRACT

Press and internet reports mention abuse of nootropic drug piracetam (PIR) in combination with psychostimulants methamphetamine (MET) or 4-methylenedioxymethamphetamine (MDMA). These combinations are believed to produce more profound desirable effects, while decreasing hangover. However, there is a lack of valid experimental studies on such drug–drug interactions in the scientific literature available. Our hypothesis proposes that a functional interaction exists between PIR and amphetamine psychostimulants (MET and MDMA) which can potentiate psychostimulant behavioural effects.

Our hypothesis is supported by the results of our pilot experiment testing acute effects of drugs given to mice intraperitoneally (Vehicle, $n = 12$; MET 2.5 mg/kg, $n = 10$; MDMA 2.5 mg/kg, $n = 11$; PIR 300 mg/kg, $n = 12$; PIR + MET, $n = 12$; PIR + MDMA, $n = 11$) in the Open Field Test (Actitrack, Panlab, Spain). PIR given alone caused no significant changes in mouse locomotor/exploratory behaviour, whereas the same dose combined with either MET or MDMA significantly enhanced their stimulatory effects.

Different possible neurobiological mechanism underlying drug–drug interaction of PIR with MET or MDMA are discussed, as modulation of dopaminergic, glutamatergic or cholinergic brain systems. However, the interaction with membrane phospholipids seems as the most plausible mechanism explaining PIR action on activities of neurotransmitter systems.

Despite that our behavioural experiment cannot serve for explanation of the pharmacological mechanisms of these functional interactions, it shows that PIR effects can increase behavioural stimulation of amphetamine drugs. Thus, the reported combining of PIR with MET or MDMA by human abusers is not perhaps a coincidental phenomenon and may be based on existing PIR potential to intensify acute psychostimulant effects of these drugs of abuse.

Introduction

Reports have shown that human abusers tend to combine illicit psychostimulant drug methamphetamine (MET) or 4-methylenedioxymethamphetamine (MDMA; "ecstasy") with the neurodynamic (nootropic) drug piracetam (PIR). Cases of more profound effects of such combinations associated with less adverse effects are discussed in the internet forums. Such references may lead to spiral effect and even wider usage of PIR in abuser community, however without enough relevant evidence concerning its real functioning. In fact, there are very few research data on PIR interaction with psychostimulants. PIR is a nootropic drug chemically related to neurotransmitter γ-aminobutyric acid (GABA). It was the first drug reported to act on cognition without causing sedation [1]. In spite of structural similarity of PIR with GABA and its ability to modify functions of many neurotransmitter systems, its actions are currently considered not to be based on direct interactions with any major postsynaptic neurotransmitter receptor. Its actions in the nervous tissue include indirect modulation of several neurotransmitter systems, neuroprotective and anticonvulsant effects and positive influence on neuronal plasticity. It enhances glucose and oxygen metabolism in hypoxic brain tissue [2]. On the other hand, in both animals and humans, PIR is practically free of toxic effects, what could be considered to some extent as a lack of specific pharmacological activity. Although there is large number of published works concerning PIR actions, many areas of interest remain to be clarified including possible involvement in drug–drug interactions.

Hypothesis

Our hypothesis proposes that a functional interaction exists between piracetam and amphetamine psychostimulants (methamphetamine and MDMA) and such interaction can potentiate psychostimulant behavioural effects.

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Empirical data

According to the available literature only one study [3] was previously focused on changes of MET effects on locomotor activity and shuttle-box avoidance acquisition in mice (C57BL/6 strain) by co-administration of PIR (100 mg/kg) or oxiracetam (50 mg/kg/day). Single dose of these nootropics significantly increased avoidance responses when combined with MET (0.5; 1.0; 2.0 mg/kg) but did not affect locomotor stimulation induced by MET. The authors concluded that nootropic drugs may interact with the effects of MET on processes involved in learning and memory.

The aim of our pilot study was to evaluate modification of MET or MDMA behavioural stimulatory effects by PIR co-administration in effort to assess possible existence of pharmacodynamic interaction between these drugs on locomotor/exploratory behaviour in the mouse Open Field Test (Actitrack, Panlab, Spain). Acute effects of drugs were tested after intraperitoneal drug administration in mice (ICR strain): (a) MET 2.5 mg/kg; (b) MDMA 2.5 mg/kg; (c) PIR 300 mg/kg; (d) PIR + MET or PIR + MDMA. Under the dosing regimen used in this study PIR given alone caused no significant changes in the mouse horizontal locomotor/exploratory behaviour in the novel environment of the Open Field. Both MET and MDMA given alone significantly increased locomotion. Stimulatory effects of both MET and MDMA administered as combined treatment with PIR were significantly higher in comparison with either MET or MDMA administered alone (Fig. 1).

Evaluation of the hypothesis

Potency of PIR to pharmacodynamic drug–drug interactions is generally considered to be very low. Although a facilitation of anticonvulsant effects of carbamazepine and diazepam was reported and suggested to be related to shared GABAergic influences [4–7].

When considering possibility of pharmacologic drug–drug interaction increasing MET and MDMA psychostimulant action, one of the most self-offering theories is involvement in mechanisms facilitating a turnover of monoaminergic neurotransmitters (dopamine, noradrenalin, serotonin), which is shared by the most of psychostimulant drugs including MET [8]. However, mechanisms of pharmacodynamic interactions between PIR and MET or MDMA remain unclear. In previous studies PIR indirectly influenced a number of neurotransmitter systems including dopaminergic one [9]. It was shown that PIR interferes with level of dopamine in the perfused isolated rat brain by increasing the K^+-stimulated dopamine release [10]. Other publications describe changed levels of dopamine or its metabolite homovanillic acid in the brain after administration of high doses of PIR [11,12]. However, studies in which lower doses of PIR were used showed no significant influence on dopaminergic brain system [13]. With respect to studies listed above the dose of PIR used in our study was high enough to influence dopaminergic system. Therefore results of our study could serve for setting up one of possible and plausible explanations supporting the theory that dopamine system is involved in PIR effects.

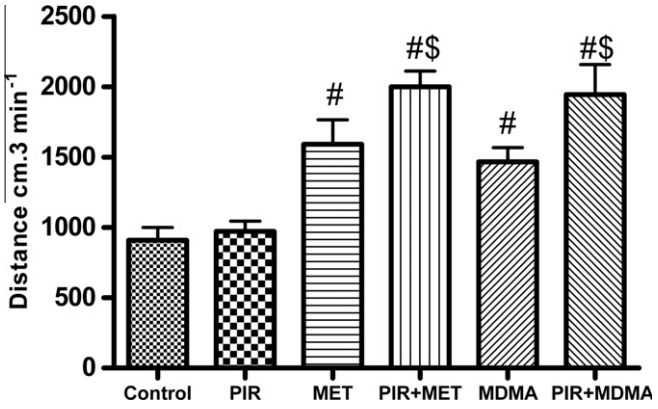

Fig. 1. Acute effects of drugs on mouse horizontal locomotor activity in the Open Field Test. Piracetam (PIR) enhanced both metamphetamine (MET) and MDMA (4-methylenedioxymethamphetamine) stimulatory effect. Control: vehicle 10 ml/kg i.p.; MET: 2.5 mg/kg i.p.; MDMA: 2.5 mg/kg i.p.; PIR: piracetam 300 mg/kg i.p.; # – $p < 0.05$ vs. control; $ – $p < 0.05$ vs. MET or MDMA; data are shown as Mean ± SEM.

Another neurobiological pharmacological system with important impact on addiction developing to psychostimulants is excitatory amino acid transmission [14]. Glutamate is the major excitatory neurotransmitter of the brain and the reward circuit consisting of dopamine releasing neurons receives multiple glutamatergic input [15]. Many studies have already shown that amphetamines as well as other psychostimulants elevate extracellular levels of glutamate [14,16]. PIR is known to interact with glutamatergic system in a yet not fully understood way [9]. As the glutamatergic system impairment was described in cognitive deficits [17,18], it is possible that glutamatergic effects of PIR contribute to its positive impact on cognition. This influence can increase perception of psychostimulant effects of simultaneously acting MET or MDMA. Furthermore, PIR was shown to allosterically modulate glutamatergic AMPA receptors [19], structures involved in positive reinforcement in addiction [20]. Their functionality is also altered by amphetamines [21].

Evidence suggests that the cholinergic system contributes at least to the reinforcing effects of psychostimulants. Since the beginning of PIR usage, indications for prescription include treatment of learning and memory dysfunctions. This leads to premise that one of the most important mechanisms of PIR action is of cholinergic origin [22]. However, closer evaluation of these first studies decreased plausibility of this theory [9,23,24].

Nowadays, PIR is not generally considered to be a significant agonist or inhibitor of neurotransmitter receptors [22]. The interaction with membrane phospholipids is the mostly suggested mechanism of its action on activities of neurotransmitter systems [25–28]. This proposition is supported by reported clinical effects of PIR in conditions with impaired membrane fluidity (e.g. in ageing) and the rheological properties of PIR [22,29,30]. Neurotransmitter signals depend on binding to specific membrane protein structures (receptors, transporters). Changing the membrane fluidity by PIR can have impact on binding sites for neurotransmitters and that way indirectly affect their functioning [29]. Possibility that PIR acts as a potentiator of current activities of neurotransmitters probably due to modulatory influences on differential ion channels was presented elsewhere [9].

Conclusion

Our pilot experiment confirmed that while acute PIR treatment did not elicit psychostimulant-like effects on locomotor/exploratory behaviour in mice, the same dose combined with either MET or MDMA significantly enhanced their stimulatory effects. Despite that our behavioural study cannot serve for explanation of the pharmacological mechanisms of these functional interactions, it shows that PIR effects can increase behavioural stimulation of these drugs of addiction. Thus, the reported combining of PIR and MET or MDMA by human abusers is not perhaps coincidental and may be based on existing PIR potential to intensify acute psychostimulant effects of these drugs of abuse. Such use of PIR may contribute to higher risk of development of dependence on MET or MDMA and increase susceptibility to relapse in abstaining individuals. Certainly, further research on this topic would be worthwile.

Conflict of interest statement

None declared.

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Interaction of CB_1 receptor agonist arachidonylcyclopropylamide with behavioural sensitisation to morphine in mice

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ABSTRACT: Activities of the endocannabinoid system are believed to be substantially involved in psychostimulant and opioid addiction. Nevertheless, interactions between cannabinoid and opioid systems are not yet fully understood. Thus, the aim of the present study was to investigate the interaction between morphine and the cannabinoid CB_1 receptor agonist arachidonylcyclopropylamide (ACPA) in behavioural sensitisation. Sensitisation occurs after repeated exposure to drugs of abuse including morphine and cannabinomimetics and it has been suggested to mediate craving and relapses. Male mice were randomly allocated into three groups and were seven times (from the 7th to 13th day of the experiment) administered drugs as follows: (a) n_1: vehicle at the dose of 10 ml/kg/day; (b) n_2: morphine at the dose of 10.0 mg/kg/day; (c) n_3: ACPA at the dose of 1.0 mg/kg/day. Changes in locomotor behaviour were measured in the Open Field Test: (a) after administration of vehicle on the 1st experimental day, (b) after the 1st dose of drugs given on the 7th day, and (c) on the 14th day after "challenge doses" given in the following way: n_1: saline at the dose of 10 ml/kg, $n_{2, 3}$: morphine at the dose of 10.0 mg/kg. Registered behavioural changes unambiguously showed the development of behavioural sensitisation to the stimulatory effects of morphine on locomotion after its repeated administration ($P < 0.05$). However, surprisingly, taking into account reports on synergistic effects of opioids and cannabinoid receptor stimulation, a significant decrease ($P < 0.05$) in behavioural sensitisation to morphine occurred when the drug challenge dose was given following repeated pre-treatment with the CB_1 receptor agonist ACPA, i.e. suppression of cross-sensitisation to morphine.

Keywords: behavioural sensitisation; morphine; cannabinoids; ACPA; mice

List of abbreviations

ACPA = *N*-(cyclopropyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide (alternative name: arachidonylcyclopropylamide, selective CB_1 receptor agonist), **AM 251** = *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (synthetic CB_1 receptor antagonist/inverse agonist), **CP 55,940** = (–)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (mixed $CB_{1,2}$ receptor agonist), **CPP** = conditioned place preference, **HU 210** = (6aR)-trans-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo[b,d]pyran-9-methanol (synthetic mixed $CB_{1,2}$ receptor agonist), **JWH 015** = 1 propyl-2-methyl-3-(1-naphthoyl)indole (selective CB_2 receptor agonist), **Met** = methamphetamine, **Mo** = morphine, **Sal** = saline, **THC** = delta 9-tetrahydrocannabinol (mixed $CB_{1,2}$ receptor agonist), **V** = vehicle, **WIN 55,212-2** = (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone (synthetic $CB_{1,2}$ receptor agonist)

Repeated administration of various psychotropic substances may result in an increasing behavioural response to their effects, which has been termed as behavioural sensitisation. This phenomenon can for example develop to amphetamines (Landa et al. 2006; Slamberova et al. 2011; Enman and Unterwald

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2012; Herrera et al. 2013; Hutchinson et al. 2014; Jing et al. 2014), cannabinoids (Rubino et al. 2001; Rubino et al. 2003; Cadoni et al. 2008), opioids (Vanderschuren and Kalivas 2000; Farahmandfar et al. 2011a; Hofford et al. 2012), caffeine (Hu et al. 2014), nicotine (Lee et al. 2012) or ethanol (Bahi and Dreyer 2012). It has also been described that an increased response to a drug may be elicited by previous repeated administration of a drug different from the drug tested - so called cross-sensitisation. This was reported for heroin after pre-treatment with THC (Singh et al. 2005) or for morphine after pre-treatment with the cannabinoid agonist WIN 55,212-2 (Manzanedo et al. 2004). Similar results were observed even across generations. Adolescent female rats were exposed to the cannabinoid agonist WIN 55,212-2 and as adults mated with drug-naïve males. Their adult female offspring were tested for behavioural sensitisation to the effects of morphine and showed cross-sensitisation development and a significantly higher density of mu opioid receptors in the nucleus accumbens (Vassoler et al. 2013).

After its development, behavioural sensitisation lasts for a long period of time (Coelhoso et al. 2013). Its neurobiological background consists in drug-induced neuroadaptive changes in a circuit involving dopaminergic and glutamatergic interconnections between the ventral tegmental area, the nucleus accumbens, prefrontal cortex and amygdala (Vanderschuren and Kalivas 2000; Nestler 2001; Landa et al. 2014a). A simultaneous impact of both endogenous opioid and cannabinoid systems on the development of behavioural sensitisation can be the result of a cross-talk between opioid and cannabinoid receptors (Robledo et al. 2008).

Despite increasing evidence for functional synergistic interactions between the endocannabinoid and opioid systems (Braida et al. 2008; Robledo et al. 2008; Zarrindast et al. 2008; Lopez-Moreno et al. 2010; Parolaro et al. 2010), our pilot study using the model of agonistic behaviour in singly housed male mice on paired interactions with non-aggressive group-housed partners showed no cross-sensitisation to the anti-aggressive effects of morphine after repeated pre-treatment with the cannabinoid methanandamide (Sulcova et al. 2004). As behavioural sensitisation and cross-sensitisation are suggested to play a role in relapses in drug abusers (De Vries et al. 2002) the aim of the present study was to further investigate functional interactions between morphine and the selective CB_1 receptor agonist arachidonylcyclopropylamide (ACPA) in a model of behavioural sensitisation using the mouse Open Field Test.

MATERIAL AND METHODS

Animals. Thirty one male mice (strain ICR, TOP-VELAZ s.r.o., Prague, Czech Republic) with an initial weight of 18–21 g were used. The mice were randomly allocated into three experimental groups and were housed with free access to water and food in a room with controlled humidity and temperature, that was maintained under a 12-h phase lighting cycle. Experimental sessions were always performed in the same light period between 1:00 p.m. and 3:00 p.m. in order to minimise possible variability due to circadian rhythms.

Apparatus. Locomotor activity was measured using an open-field equipped with Actitrack (Panlab, S.L., Spain). This device consists of two square-shaped frames that deliver beams of infrared rays into the space inside the square. A plastic box is placed in this square to act as an open-field arena (base 30 × 30 cm, height 20 cm), in which the animal can move freely. The apparatus software records and evaluates the locomotor activity of the animal by registering the beam interruptions caused by movements of the body. Using this equipment we have determined trajectory in cm per 3 min (Distance Travelled).

Drugs. Vehicle and all drugs were always given in a volume adequate for drug solutions (10 ml/kg).

Morphine hydrochloride (Tamda a.s., Czech Republic) was dissolved in saline.

Arachidonylcyclopropylamide, *N*-(cyclopropyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide was supplied pre-dissolved in anhydrous ethanol at a concentration of 5 mg/ml (Tocris Cookson Ltd., UK) and was diluted in saline to a concentration that allowed administration of the drug in a volume of 10 ml/kg; therefore, the vehicle contained an adequate amount of ethanol (a final concentration in the injection of below 1%) to make the effects of the placebo and the drug comparable.

The adjustment of all drug doses was based on both literature data and results obtained in our earlier behavioural experiments.

Procedure. Animals were randomly divided into three groups ($n_1 = 10$, $n_2 = 11$, $n_3 = 10$) and all were given vehicle on Day 1 (10 ml/kg). There were no applications from Days 2 to 6. For the next seven

days animals were daily treated as follows: (a) n_1: saline at the dose of 10 ml/kg/day; (b) n_2: morphine at the dose of 10.0 mg/kg/day; (c) n_3: ACPA at the dose of 1.0 mg/kg/day. On Day 14 all mice received challenge doses in the following way: n_1: saline at the dose of 10 ml/kg, n_2, n_3: morphine at the dose of 10.0 mg/kg. All substances were administered intraperitoneally. Changes in horizontal locomotion were measured for a period of 3 min in the open field on Days 1, 7 and 14 to evaluate the sensitising and cross-sensitising phenomenon, respectively.

The experimental protocol complies with the European Community guidelines for the use of experimental animals and was approved by the Animal Care Committee of the Masaryk University Brno, Czech Republic.

Data analysis. As the data were normally distributed (according to the Kolmogorov-Smirnov test of normality) and the following parametric statistics were used: unpaired *t*-test, two tailed for comparison across the individual groups and paired *t*-test, two tailed for comparison within the individual groups (statistical analysis package Statistica – StatSoft, Inc., Tulsa, USA).

RESULTS

No significant differences were found in Distance Travelled across the groups that were given vehicle for the first time (see Figure 1; vehicle1 versus vehicle2, vehicle2 versus vehicle3, vehicle1 versus vehicle3).

The first doses of saline, morphine and ACPA, respectively, did not elicit any significant behavioural changes among the three experimental groups (see Figure 1; saline versus morphine, morphine versus ACPA, saline versus ACPA.

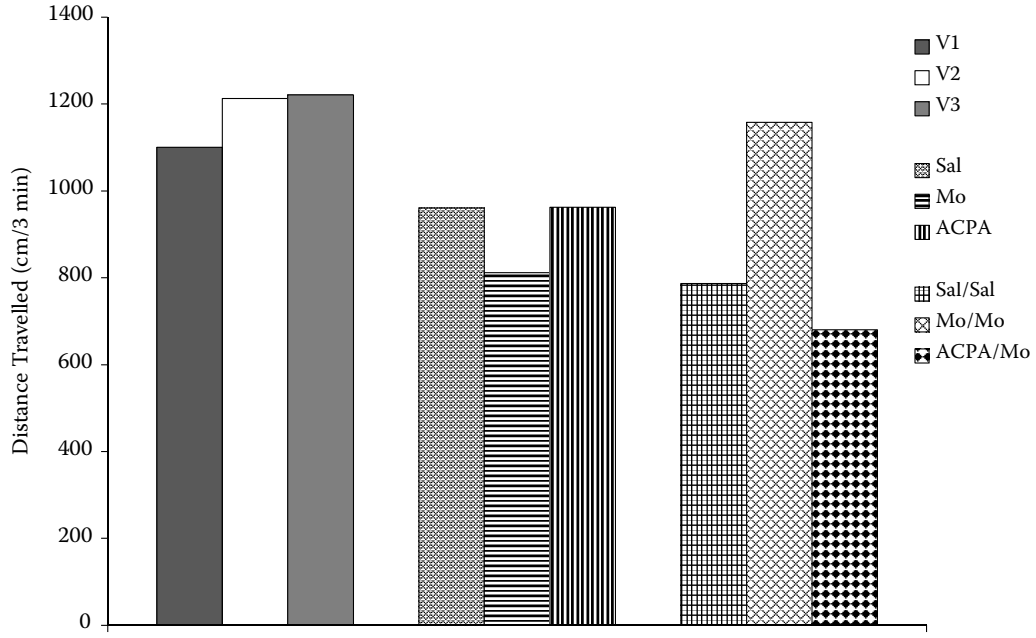

Figure 1. Effects of drug treatments on Distance Travelled (cm/3 min) in the mouse open field test shown as mean values with standard deviation (SD): vehicle1 = mice in the group n_1 after the 1st dose of vehicle, (SD = 145.4); vehicle2 = mice in the group n_2 after the 1st dose of vehicle, (SD = 182.2); vehicle3 = mice in the group n_3 after the 1st dose of vehicle, (SD = 241.1); saline = mice in the group n_1 after the 1st dose of saline, (SD = 379.0); morphine = mice in the group n_2 after the 1st dose of morphine (10.0 mg/kg), (SD = 431.0); ACPA = mice in the group n_3 after the 1st dose of arachidonylcyclopropylamide (1.0 mg/kg), (SD = 301.9); saline/saline = mice in the group n_1 after the challenge dose of saline, (SD = 157.9); morphine/morphine = mice in the group n_2 repeatedly pre-treated with morphine after the challenge dose of morphine (10.0 mg/kg), (SD = 486.0); ACPA/morphine = mice in the group n_3 repeatedly pre-treated with ACPA after the challenge dose of morphine (1.0 mg/kg + 10.0 mg/kg), (SD = 266.9). Statistical significances are as follows: vehicle1 : vehicle2 (non-significant), vehicle2 : vehicle3 (non-significant), vehicle1 : vehicle3 (non-significant); saline : morphine (non-significant), morphine : ACPA (non-significant), saline : ACPA (non-significant); saline/saline : morphine/morphine ($P < 0.05$), morphine/morphine : ACPA/morphine ($P < 0.05$), saline/saline : ACPA/morphine (non-significant); unpaired *t*-test, two tailed, vehicle3 : ACPA ($P < 0.05$); paired *t*-test, two tailed

The challenge dose of morphine evoked a significant increase in Distance Travelled ($P < 0.05$) in animals pre-treated repeatedly with morphine when compared to animals pre-treated with saline after the saline challenge dose (see Figure 1; saline/saline versus morphine/morphine). The challenge dose of morphine administered to animals repeatedly pre-treated with ACPA led to a significant decrease ($P < 0.05$) in Distance Travelled compared to mice pre-treated with morphine after the morphine challenge dose (see Figure 1; morphine/morphine versus ACPA/morphine). No significant difference was found between mice pre-treated repeatedly with saline after the saline challenge dose and mice pre-treated repeatedly with ACPA after the morphine challenge dose (see Figure 1; saline/saline versus ACPA/morphine).

DISCUSSION

Based on results from studies in different animal models and from clinical trials, the existence of functional interactions between endogenous opioid and cannabinoid systems is generally accepted. It is important to determine the conditions, under which these interactions lead to synergistic or antagonistic outcomes because of their consequences for both therapy and addiction.

In the present study we observed the development of behavioural sensitisation to the effects of morphine on mouse locomotor behaviour in the Open Field test after its repeated administration. This corresponds to previously published results (Vanderschuren and Kalivas 2000; Serrano et al. 2002; Singh et al. 2004; Zarrindast et al. 2007; Contet et al. 2008; Azizi et al. 2009; Farahmandfar et al. 2011b; Hofford et al. 2012). We then studied the impact of a possible functional interaction between the behavioural effects of morphine on mouse locomotion and cannabinoid CB_1 receptor activity using administration of ACPA and morphine.

The first dose of ACPA elicited a significant decrease in locomotor behaviour in the present study which is consistent with the results of a previous experiment using the same dose of this substance for evaluation of its influence on the development of metamphetamine behavioural sensitisation (Landa et al. 2014b). However, these findings to some extent run counter to the results of another of our previous studies in which the less selective CB_1 receptor agonist methanandamide (the synthetic analogue of endocannabioid anandamide) did not elicit any changes in mouse locomotion (Landa et al. 2006). It has to be taken into account that in a series of physiological and behavioural assays anandamide was shown to evoke biphasic activity with stimulatory and inhibitory effects at low and high doses, respectively (Sulcova et al. 1998; Katsidoni et al. 2013). It was also suggested that depending on the local concentration of cannabimimetic agents cannabinoid CB_1 receptors are modulated presynaptically at different neurotransmitter pathways, e.g. glutamatergic terminals at low doses and GABAergic at high doses. This explanation is supported by a study in which the CB_1 receptor agonist CP-55,940 elicited anxiolytic-like effects at a low dosing regimen and anxiogenic-like effects after high doses in wild-type mice, but not in mice with brain region-specific CB_1 receptor knockout (Rey et al. 2012). Furthermore, there can be differences in endocannabinoid signalling in different animal lines and between males and females (Keeney et al. 2012) as well as in pharmacokinetic and pharmacodynamic profiles of various cannabinoid receptor agonists. This was reported for example from a comparison of the effects of the cannabimetics HU 210 and CP 55,940 on rat locomotor activities (Bosier et al. 2010). Low doses (0.1 mg/kg) of the herbal cannabinoid THC have also been shown to lead to hyperactivity in the Open Field Test and increase intracranial self-stimulation thresholds, while higher doses (1 mg/kg) elicited hypoactivity and anhedonia. These effects were mediated by stimulation of the CB_1 receptors as they were abolished by co-administration of CB_1 receptor antagonist/inverse agonist rimonabant (Katsidoni 2013).

After repeated administration both cannabinoids and opioids are known to evoke locomotor sensitisation or cross-sensitisation between these two systems; however, in some species differences or discrepancies between pharmacological models are also reported (Robledo et al. 2008). Although the majority of reports speak in favour of cross-sensitisation to opioids after repeated CB_1 receptor agonist administration (Cadoni et al. 2001; Lamarque et al. 2001; Manzanedo et al. 2004) the results presented in this paper suggest inhibition of this phenomenon. In fact, the data obtained in the present study with morphine mirror the results from our previous investigation in which repeated pretreatment with the cannabinoid CB_1 receptor agonist methanandamide elicited cross-sensitisation to the stimulatory drug methamphetamine (Landa

et al. 2006; Landa et al. 2011), whereas the more selective CB_1 receptor agonist ACPA supressed this phenomenon (Landa et al. 2014b).

On the other hand there are also reports supporting the results we describe in this paper. Valverde et al. (2001) treated mice repeatedly over a period of 21 days with THC (10 mg/kg/day, *i.p.*). There were no applications for the next three days and finally, the conditioned place preference produced by different doses of morphine (0.5 or 2 mg/kg, *s.c.*) was evaluated. Administration of morphine after chronic THC treatment did not evoke rewarding responses in the conditioned place preference paradigm and thus Valverde et al. (2001) concluded that chronic use of high doses of cannabinoids presumably does not stimulate psychic dependence on opioids.

Controversial results are also reported from various other studies dealing with the modulatory influence of the endocannabinoid system on the effects of opioids as well as other drugs of abuse. A study on cross-sensitisation between THC and morphine characterised by stereotyped activity in male Sprague-Dawley rats (Cadoni et al. 2001) showed sensitisation to a challenge dose of THC as well as to the synthetic cannabinoid receptor agonist WIN55,212-2; both effects were antagonised by the CB_1 antagonist/inverse agonist rimonabant (SR141716A). Interactions between cannabinoid agonists and antagonists with morphine activity were also demonstrated in another work (Norwood et al. 2003). Hypoactivity during the first hour following morphine administration changed to hyperactivity 14 days after drug administration. An increase in morphine hyperactivity was measured in rats pre-treated with the cannabinoid receptor agonist CP 55,940 or the combination of morphine + CP 55,940, but not in rats administered the antagonist/inverse agonist rimonabant + morphine. These results were believed to support the "gateway theory" of cannabinoid effects for intake of other drugs of abuse in humans.

CB_1 receptor modulation was suggested to be involved in the rewarding effects of morphine which were attenuated in the rat model of conditioned place preference by the antagonist/inverse agonist rimonabant (SR141716). Cannabinoid and opioid cross-sensitisation was also observed in a further study in which heroin increased rat locomotory response after pre-treatment with THC (Singh et al. 2005). On the other hand rats pre-treated with THC (5 mg/kg/day for seven days) did not show any sensitisation to morphine intake under a progressive-ratio schedule in the model of *i.v.* drug self-administration (Gonzales et al. 2005) and in mice THC also reduced the reinforcing effects of morphine in the conditioned place preference test (Jardinaud et al. 2006). These findings resemble to some extent the results of the present study in which we measured a decrease in behavioural sensitisation to the effects of morphine on mouse locomotor behaviour instead of augmentation after pre-treatment with the selective CB_1 receptor agonist ACPA.

Similarly, the motor stimulatory effects measured in mice after acute and repeated low doses of morphine (5 or 7.5 mg/kg) were antagonised by the cannabinoid agonist HU 210 and enhanced by the antagonist/inverse agonist rimonabant (Hagues et al. 2007). Differential neurochemical changes within the brain endocannabinoid system were reported during induction and expression of morphine sensitisation in the rat model of drug-seeking behaviour (Vigano et al. 2004). The levels of endocannabinoids anandamide and 2-arachidonoylglycerol were altered in the brain differentially in these two phases and moreover in opposite ways in specific brain regions. Changes in the activity of CB_1 receptors in the nucleus accumbens were shown to be important for processing of behavioural sensitisation to morphine (Haghparast et al. 2009). Bilateral sub-chronic administration of the CB_1 receptor antagonist/inverse agonist AM 251 into this region caused the development of sensitisation to doses of morphine (0.5 mg/kg) which in intact rats did not produce sensitisation in the conditioned place preference model. Neither saline nor DMSO (dimethyl sulfoxide) used as the solvent led to a similar influence on the sensitising effects of morphine. Later, it was reported (Rezayof et al. 2011) that microinjection of AM 251 into the central amygdala is sufficient to induce the phenomenon of conditioned place preference but inhibits the place preference to morphine. On the other hand, microinjection of ACPA into the central amygdala increased the extent of morphine-induced conditioned place preference. This finding runs counter to our present results where pre-treatment with ACPA led to an inhibition of morphine sensitisation to locomotor effects.

Although the majority of previous reports describe the development of cross-sensitisation to opioids after repeated CB_1 receptor agonist administration, the results presented in this paper suggest an inhibition of this phenomenon. Nevertheless,

these data resemble to some extent our previous results showing a suppression of cross-sensitisation to methamphetamine with the CB_1 receptor agonist ACPA. These discrepancies in results on the involvement the endocannabinoid signalling system in addiction to cannabis, and also to other drugs of abuse including opioids, require further research because more detailed information on the neurobiological basis of cannabinoid-opioid interactions may help to develop novel pharmacotherapeutic interventions in the management of opioid dependence and withdrawal (Gonzales al. 2005; Scavone at al. 2013).

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Review

Implication of NMDA receptors in behavioural sensitization to psychostimulants: A short review

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ABSTRACT

Repeated administration of psychostimulants and other dependence-producing substances induces a substantial increase in behavioural responses, a phenomenon termed as behavioural sensitization. An increased response to the tested drug elicited by previous repeated administration of a different drug is called cross-sensitization. Behavioural sensitization is considered to be a relapse trigger in dependent subjects and animals sensitized by repeated administration of drugs of abuse, thus being considered a suitable model of craving, which is one of the very characteristic features of substance addiction.

It has been described that apart from other actions, drugs of abuse exert their effect on the central nervous system by affecting glutamatergic transmissions, particularly via N-methyl-D-aspartate (NMDA) receptors. Thus, this review presents a brief overview of the impact of inhibition of the NMDA receptor system on sensitization, reflecting particularly on behavioural sensitization to psychostimulants. The text combines up-to-date information with time-proven facts and also compares data from the literature with the authors' recent findings concerning this topic.

Contents

1. Introduction

Behavioural sensitization is a phenomenon that was consistently described in the last decade of the 20th century by Robinson and Berridge (1993). It is typically characterized by an increased behavioural response to a certain drug after conditioning by prior repeated administration of the drug. In contrast to tolerance which develops after the administration of an addictive drug in short intervals or continuously, behavioural sensitization occurs after the administration of the drug in longer intervals—the minimum being a 24-h period. An increased response to the tested drug can also be elicited by prior, repeated administration of a different drug: this phenomenon is called cross-sensitization. Behavioural sensitization observed in laboratory animals is associated with complex neuroadaptation in multiple brain regions, especially in dopamine (DA) and glutamatergic circuits (Tzschentke and Schmidt, 1997; Vanderschuren and Kalivas, 2000), and with reinstatement of drug-seeking behaviour (Steketee and Kalivas, 2011). Although behavioural sensitization is difficult to demonstrate in human subjects, there are reports showing enhanced responses to drugs of abuse after chronic consummation,

Abbreviations: CGS 17,955, Cis-4-[Phosphomethyl]-piperidine-2-carboxylic acid (selfotel); CNS, Central nervous system; CPP, 3-((+/−)-2-carboxypiperazin-4-yl) propyl-1-phosphonic acid; DA, Dopamine; DPA, Dipicrylamine; GABA, Gamma-aminobutyric acid; LTP, Long-term potentiation; MDMA, 3,4-methylenedioxy-methamphetamine (ecstasy); MK-801, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine (dizocilpine); NAc, Nucleus accumbens; NMDA, N-methyl-D-aspartate; PET, Positron emission tomography; THC, Δ9-tetrahydrocannabinol; VTA, Ventral tegmental area

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thus supporting this assumption. Progression of responses was reported after repeated administration of D-amphetamine in healthy human volunteers, reporting higher subjective ratings of vigour and euphoria with a greater impact in women (Steketee and Kalivas, 2011; Strakowski and Sax, 1998; Tzschentke and Schmidt, 1997). Moreover, an open-label clinical study with 1-year follow-up of repeated amphetamine administration in healthy volunteers confirmed behavioural sensitization to psychomotor and alertness responses accompanied with increase in dopamine release measured by the [11C] raclopride PET method (Boileau et al., 2006). Sensitization is considered one of the underlying mechanisms responsible for increased vulnerability, resulting in high rates of relapse to substance addiction in drug-dependent humans. Therefore, further investigation of this phenomenon may facilitate novel findings for the development of new, potential pharmacotherapies of addiction.

Considering the fact that behavioural sensitization is associated with stimulation of dopamine release as well as with an increase in DA synthesis and postsynaptic DA receptors density, and also with a decrease in DA autoreceptors (Robinson and Becker, 1986) and an increase of extracellular glutamate in prefrontal cortex (Abekawa et al., 1994), it was suggested to be a suitable animal model of psychotic disorders development (Nakato et al., 2011). Further research, however, revealed the importance of behavioural sensitization as a model of craving, which is one of the characteristic features of substance addiction. Taking into account that craving probably plays a role as a relapse trigger, studies detecting the neurobiology of this phenomenon are of high importance, providing data for the development of potential pharmacotherapies of addiction (Di Chiara, 1995; Emmett-Oglesby, 1995; Robinson and Berridge, 1993; Steketee and Kalivas, 2011).

The process of behavioural sensitization results from drug-induced neuroadaptive changes in a neural circuit consisting namely of dopaminergic, glutamatergic and GABAergic interconnections between the ventral tegmental area (VTA), nucleus accumbens (NAc), prefrontal cortex, and amygdala (Cador et al., 1999; Carlezon and Nestler, 2002; Kalivas, 2004; Miyazaki et al., 2013; Nestler, 2001; Nordahl et al., 2003; Steketee and Kalivas, 2011; Stephans and Yamamoto, 1995; Vanderschuren and Kalivas, 2000; Zhang et al., 2001).

It has been described that behavioural sensitization as such can be further subdivided into two domains called development and expression, the former being associated with the VTA, whereas the latter being mainly associated with NAc (Kalivas and Duffy, 1993; Kalivas et al., 1993). “Development” or “initiation” involves increasing changes at the molecular and cellular levels that lead to altered processing of environmental and pharmacological stimuli by the CNS; these changes are, however, only temporal and are not detected after longer abstinence. The term “expression” relates to persistent neural changes originating from the process of the development of sensitization (Pierce and Kalivas, 1997).

Behavioural sensitization has been observed after repeated administration of the majority of substances with addictive potential, e.g., ethanol (Bahi and Dreyer, 2012; Pastor et al., 2012), nicotine (Bhatti et al., 2009), THC (Δ9-tetrahydrocannabinol) (Cadoni et al., 2008; Rubino et al., 2003), cocaine (Ramos et al., 2012; Schroeder et al., 2012), methylphenidate (Freese et al., 2012), opioids (Bailey et al., 2010; Farahmandfar et al., 2011; Hofford et al., 2012; Liang et al., 2010), MDMA (3,4-methylenedioxymethamphetamine) (Ball et al., 2011), amphetamine (Costa et al., 2001; Kameda et al., 2011; Wang et al., 2010) or methamphetamine (Horio et al., 2012; Kucerova et al., 2009; Landa et al., 2012b, 2011, 2006a, 2006b) or modafinil (Machalova et al., 2012; Slais et al., 2010). Cross-sensitization was recorded, for example, after repeated pre-exposure with tetrahydrocannabinol to heroin (Singh et al., 2005), between metylphenidate and amphetamine (Yang et al., 2011), with cannabinoid agonist WIN 55,2122 to morphine (Manzanedo et al., 2004) or between nicotine and amphetamine (Santos et al., 2009).

Development of sensitization to methamphetamine effects in mice was induced by pre-treatment with methanandamide (analogous to the endogenous cannabinoid anandamide) and suppressed by CB_1 cannabinoid receptor antagonist AM 251 (Landa et al., 2006a, 2006b). Real-time PCR analyses revealed an increase in CB_1 receptor mRNA expression in mesencephalon after the first dose of methanandamide, followed by a decrease after a combined treatment with a challenge dose of methamphetamine (Landa et al., 2011). These effects were also associated with an increase in D1 and a decrease in D2 densities of the receptor subtypes after the treatment by both methamphetamine and methanandamide (Landa et al., 2012a). Cross-sensitization is also of a great clinical significance as relapses in abstaining users may also be triggered by a different drug (De Vries et al., 2002).

2. Implication of the glutamatergic NMDA receptor system in behavioural sensitization to psychostimulants

It is generally accepted that signalization in a dopaminergic mesocortical pathway is crucial for the rewarding effects of drugs of abuse; however, it is certainly not the only important process involved in substance addiction (Salamone et al., 2005). Glutamate, the main excitatory neurotransmitter in the brain of mammals, is also known for its crucial role in synaptic plasticity. In relation to the activity of the reward pathway, transmissions via dopamine and glutamate are closely connected. It is frequently reported that hyperglutamatergic neurotransmission (namely the activity of NMDA receptor system) is involved in the processes of behavioural sensitization elicited by many drugs of abuse (Cador et al., 1999; Fujio et al., 2005; Kalivas, 2009, 2004; Lee et al., 2011; Miyazaki et al., 2013; Ohmori et al., 1996, 1994; Shirai et al., 1996; Stewart and Druhan, 1993; Subramaniam et al., 1995; Tzschentke and Schmidt, 2003; Wolf, 1998; Zhang et al., 2001).

Because the influx of calcium into the cells via NMDA receptors is the basal mechanism underlying synaptic plasticity, it is speculated that administration of NMDA antagonists may inhibit the formation of drug-associated memories and long-term potentiation (LTP), and thus also the development of sensitization to the effects of a drug (Carmack et al., 2013). Indeed, many articles demonstrate that the administration of NMDA antagonists decreased psychostimulant reward and disrupted the development or the expression of sensitization (Tzschentke and Schmidt, 2003; Wolf, 1998). On the other hand, some experimenters did not find any significant effect of NMDA antagonists on the action of psychostimulants (Wolf, 1998); moreover, there are also reports suggesting that co-administration of NMDA receptor antagonists actually increased the influence of the sensitizing drug (Ito et al., 2006; Tzschentke and Schmidt, 1998).

Extensive research addressing the influence of NMDA ligands on the development and the expression of psychostimulant sensitization was done namely on cocaine and amphetamines. The development of sensitization to psychostimulants was disrupted by a range of noncompetitive, competitive and glycine site NMDA antagonists, including the non-competitive NMDA receptor antagonist, dizocilpine (also known as MK-801) or CGS 19,755, while the expression of sensitization to psychostimulants was not so clearly attenuated by NMDA antagonists, suggesting the involvement of non-NMDA dependent mechanisms (Wolf, 1998).

Ambivalent results were obtained from studies using the competitive antagonist 3-((+/−)-2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP, selfotel). Karler and colleagues performed a series of experiments and suggested that the mechanisms of expression of sensitization differed in cocaine and amphetamine

(Karler et al., 1994, 1991, 1990). CPP as well as the non-competitive antagonist, dizocilpine, also failed to modify the acute effect of methamphetamine on dopamine release in the striatum (Kashihara et al., 1991; Ujike et al., 1992). On the other hand, subsequent experiments with intracerebral applications of CPP showed a dampening effect of CPP on both acute amphetamine effects and the development and expression of sensitization to amphetamine (Bedingfield et al., 1997; Karler et al., 1997). These results were assumed to be caused by the difference between non-specific systemic CPP effects on the entire brain area, and aimed local efficacy in specific brain areas (Wolf, 1998), which was supported by the results of several other studies. For example, a gradual increase in extracellular concentrations of glutamate was found in striatum but not in NAc after a high dose of methamphetamine (Abekawa et al., 1994). An enhanced dopamine and glutamate efflux was also measured by in vivo microdialysis in the prefrontal cortex and striatum in rats sensitized with repeated methamphetamine treatment (Arai et al., 1996; Stephans and Yamamoto, 1995). Regionally specific upregulation of the expression of NMDA receptor following the administration of psychostimulants appeared to be caused by malfunctioning of the glutamatergic system, as demonstrated in several experiments (Kerdsan et al., 2009).

The potency of CPP was also demonstrated in a very recent study, in which the authors compared acute and sensitized responses to cocaine modulated by CPP (Carmack et al., 2013), thus attempting to elucidate some of the above-mentioned discrepancies. Pharmacodynamic interaction between acute cocaine and CPP doses, expressed as a decreased locomotor activity, and the lack of effect on behavioural sensitization were the main findings of this study. As the authors also recorded the development of conditioned place preferences and the impairment in contextual fear learning, they explained the probable effects of NMDA antagonists as an action that dampens the induction of associative memories (Carmack et al., 2013).

Although data on glutamatergic transmission found in animal studies are not uniform and the exact relation between the density of glutamatergic receptors and the processes associated with behavioural sensitization remains unclear (Nelson et al., 2009), the majority of reports in the literature are in agreement with the hypothesis that NMDA receptor antagonists exert inhibitory effects on behavioural sensitization to amphetamines.

Wolf et al. (1995) report that co-administration of dizocilpine with amphetamine prevented the development of behavioural sensitization. In this study, rats were administered water+amphetamine or dizocilpine+amphetamine for six consecutive days. The last dose (challenge) was amphetamine only. The co-administration of dizocilpine led to augmented locomotor response to acute amphetamine administration, whereas repeated pre-treatment with the dizocilpine+amphetamine disrupted the development of sensitization to a subsequent challenge dose of amphetamine. Wolf et al. (1995) also report that co-administration of CGS 19,755 increased the locomotor response to acute amphetamine administration and prevented the development of sensitization after an amphetamine challenge dose (Wolf et al., 1995). Similarly, Carey et al. (1995) demonstrate that an NMDA receptor antagonist increased the behavioural responses provoked by cocaine. The full behavioural profile recorded in rats after applications of cocaine, dizocilpine or their combination was, however, different, and repeated applications resulted in behavioural sensitization to cocaine (Carey et al., 1995).

Amphetamine-sensitized rats that demonstrated apparent, increased locomotion response to the drug also showcased increased dopamine metabolites (3,4-dihydroxyphenylacetic acid and homovanillic acid) and an output of glutamate measured by in vivo microdialysis in ventral pallidum as compared to controls. Pre-treatment by the NMDA receptor antagonist dizocilpine prevented the locomotion hyperactivity in amphetamine-sensitized rats in this study (Chen et al., 2001).

Inhibitory effects of another NMDA receptor antagonist, felbamate, on the development of behavioural sensitization to the amphetamine derivate, methamphetamine, were found in one of our studies (Landa et al., 2012b). In contrast to some previous results (Wolf et al., 1995), acute co-administration of felbamate and methamphetamine did not cause any increase in locomotor behaviour, whereas animals repeatedly co-administered with methamphetamine+felbamate showed development of behavioural sensitization to the methamphetamine stimulatory effects after the methamphetamine challenge dose (Landa et al., 2012b). However, animals repeatedly pre-treated with methamphetamine responded to the challenge of methamphetamine+felbamate by lower locomotor stimulation than animals challenged with methamphetamine alone, thus demonstrating the inhibitory effects of NMDA receptor antagonists on the expression of sensitization to methamphetamine.

In our further experimental testing of the effects of NMDA receptor ligands on behavioural sensitization to the psychostimulant methamphetamine, the NMDA receptor antagonist, memantine, was used (Landa et al., 2012c). The data obtained from this study accorded with our previous felbamate experiment results only to a certain extent. Neither development, nor expression of behavioural sensitization was observed in the group of mice repeatedly pre-treated with methamphetamine after the administration of challenge doses of methamphetamine combined with memantine. This finding was in agreement with the majority of similar experiments, suggesting that NMDA receptor antagonists possess inhibiting effects on the development of sensitization to amphetamines (see above). Carmack et al. (2013) discussed the fact that in the majority of animal studies, CPP diminished the cocaine stimulatory effects acutely but did not prevent the development of sensitization. Our results showed that felbamate and memantine modulated the methamphetamine action after acute administration differentially, and also that the effects of repeated administration were different: while methamphetamine+felbamate challenge dose after repeated methamphetamine pre-treatment resulted in significantly decreased locomotion, in the study involving memantine, mice repeatedly pre-treated with methamphetamine showed an insignificant trend towards locomotion increase after the challenge dose of methamphetamine+memantine. Thus, it seems that the observation made by Carmack et al. (2013) is probably valid only in cases of specific combinations of CPP and cocaine.

The variations between the effects of felbamate and memantine recorded in our animals may favour a hypothesis suggesting that NMDA antagonists influence behavioural sensitization in a substance-dependent way. This is in compliance with, for example, the report of Bespalov et al. (2000) suggesting that cocaine-conditioned behaviours can be selectively modulated by some, but not all NMDA receptor antagonists (Bespalov et al., 2000).

3. Conclusion

Most of the published studies on glutamatergic ligands, NMDA receptor antagonists in particular, and on the ways in which they affect behavioural sensitization to psychostimulants and to other addictive substances suggest that the effects of these substances are largely in favour of their inhibiting effects.

The processes of behavioural sensitization are believed to reflect the neuroadaptive changes involved in addiction. The development of behavioural sensitization to psychostimulants depends on the activity of the dopaminergic mesolimbic pathway, while the glutamatergic-dependent modulation also plays an important role (Degoulet et al., 2013). In pre-clinical models, the ligands of glutamatergic receptors (particularly NMDA receptor antagonists) show a promise for the treatment of addiction

(Bowers et al., 2010), as do the noncompetitive antagonists acting by voltage-dependent mechanisms, e.g., hydrophobic anion dipicrylamine (DPA) (Bisaga et al., 2000), and agents with indirect mechanisms for altering the NMDA receptor function (Tomek et al., 2013). Despite the somewhat controversial data in literature, the use of NMDA receptor antagonists is considered by many authors to be a potential tool for the inhibition of behavioural sensitization, which would decrease the risks of relapsing in ex-addicts (Bisaga et al., 2010; David et al., 2006; Kalivas, 2009; Sani et al., 2012; Steketee and Kalivas, 2011; Tomek et al., 2013). The exact interaction of psychostimulant drugs and NMDA receptor antagonists, however, depends on the particular psychostimulant and on the modulator of the NMDA receptor activity involved, and also probably on the experimental procedure and other conditions, such as the species of animals used. Nevertheless, further investigation evaluating these effects is worthwhile for the purposes of future clinical testing of NMDA receptor modulators in the treatment of human addicts.

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The use of cannabinoids in animals and therapeutic implications for veterinary medicine: a review

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ABSTRACT: Cannabinoids/medical marijuana and their possible therapeutic use have received increased attention in human medicine during the last years. This increased attention is also an issue for veterinarians because particularly companion animal owners now show an increased interest in the use of these compounds in veterinary medicine. This review sets out to comprehensively summarise well known facts concerning properties of cannabinoids, their mechanisms of action, role of cannabinoid receptors and their classification. It outlines the main pharmacological effects of cannabinoids in laboratory rodents and it also discusses examples of possible beneficial use in other animal species (ferrets, cats, dogs, monkeys) that have been reported in the scientific literature. Finally, the article deals with the prospective use of cannabinoids in veterinary medicine. We have not intended to review the topic of cannabinoids in an exhaustive manner; rather, our aim was to provide both the scientific community and clinical veterinarians with a brief, concise and understandable overview of the use of cannabinoids in veterinary medicine.

Keywords: cannabinoids; medical marijuana; laboratory animals; companion animals; veterinary medicine

Abbreviations

AEA = anandamide (N-arachidonoylethanolamine, $CB_{1, 2}$ receptor agonist), **2-AG** = 2-arachidonoylglycerol (CB_1 receptor agonist), **2-AGE** = 2-arachidonyl glyceryl ether (noladin ether, CB_1 receptor agonist), **AM 251** = N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (synthetic CB_1 receptor antagonist/inverse agonist), **CB_1** = cannabinoid receptor type 1, **CB_2** = cannabinoid receptor type 2, **CP-55,940** = (–)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (mixed $CB_{1, 2}$ receptor agonist), **FAAH** = fatty acid amide hydrolase, **GABA** = gamma-amino butyric acid, **GPR18** = G-protein coupled receptor 18, **GPR55** = G protein-coupled receptor 55, **GPR119** = G protein-coupled receptor 119, **HU-210** = (6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d] pyran-9-methanol (synthetic mixed $CB_{1, 2}$ receptor agonist), **HU-308** = [(1R,2R,5R)-2-[2,6-dimethoxy-4-(2-methyloctan-2-yl)phenyl]-7,7-dimethyl-4-bicyclo[3.1.1]hept-3-enyl] methanol (highly selective CB_2 receptor agonist), **IgE** = immunoglobulin E, **MGL** = monoacylglycerol lipase, **NADA** = N-arachidonoyl dopamine (CB_1 receptor agonist), **PEA** = palmitoylethanolamide, **SR144528** = N-[(1S)-endo-1,3,3-trimethylbicyclo [2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1H-pyrazole-3-carboxamide (CB_2 receptor antagonist/inverse agonist), **THC** = delta-9-tetrahydrocannabinol (mixed $CB_{1, 2}$ receptor agonist), **TRPV1** = transient receptor potential cation channel subfamily V member 1, **WIN 55,212-2** = (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone (synthetic $CB_{1, 2}$ receptor agonist)

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Contents

1. Introduction

Cannabinoids have been used in traditional medicine for thousands of years. There are reports going back to ancient China (Unschuld 1986; Zuardi 2006), medieval Persia (Gorji and Ghadiri 2002) or in Europe to the 19th century (following the Napoleonic invasion of Egypt) (Kalant 2001). It is important to emphasise that the use of cannabinoids in ancient or medieval cultures was not only because of the psychoactive effects of these substances; treatment was largely aimed at various somatic disorders including headache, fever, bacterial infections, diarrhoea, rheumatic pain or malaria (Kalant et al. 2001; Gorji and Ghadiri 2002; Zuardi 2006). Despite this fact, the use of cannabinoids is still illegal in many countries due to their psychoactive effects and addictive potential. Attempts by pharmaceutical companies in the sixth decade of the twentieth century to produce cannabinoids with pharmacological effects and without psychotropic activity were not successful (Fisar 2009; Pertwee 2009), although cannabinoids with very weak or no psychotropic activity are known (e.g. cannabidiol, cannabigerol, cannabichromene) (Izzo et al. 2009; Hayakawa et al. 2010).

Although cannabinoids have been attracting attention for many years, the last four decades have brought completely new and scientifically well-founded insights into their therapeutic potential. Since 1975 more than 100 controlled clinical trials with cannabinoids (or whole-plant preparations) for several indications have been carried out and the results of these studies have led to the approval of cannabis-based medicine in various countries (Grotenhermen and Muller-Vahl 2012). Consequently, there is increasing interest, particularly in companion animal owners, regarding the possible use of cannabinoids in veterinary medicine.

In order to cover this broad theme in a concise manner the text will first be focused on the classification of cannabinoids and cannabinoid receptors. Attention will then be turned to the therapeutic potential of cannabinoids with regard to veterinary medicine.

2. The endocannabinoid system and classification of cannabinoids

The endocannabinoid system consists of several subtypes of cannabinoid receptors (the best characterised are subtypes CB_1 and CB_2), endocannabinoids (endogenous substances that bind to the receptors) and enzymes involved in endocannabinoid biosynthesis through phospholipases or degradation: post-synaptically by FAAH (fatty acid amide hydrolase) and pre-synaptically by MGL (monoacylglycerol lipase) (Pertwee 2005; Muccioli 2010; Battista et al. 2012). This system represents a ubiquitous lipid signalling system (that appeared early in evolution), which plays important regulatory roles throughout the body in all vertebrates (De Fonseca et al. 2005). Below, we will focus on the cannabinoid receptors and their ligands (cannabinoids) because of their principal therapeutic significance.

Cannabinoids are chemical substances which act primarily on specific cannabinoid receptors and are basically divided into three groups; beside endogenous cannabinoids (endocannabinoids) also herbal cannabinoids (phytocannabinoids) and synthetic cannabinoids have been described (Fisar 2009).

Endocannabinoids are endogenously formed from membrane phospholipids in response to increases in intracellular calcium; they are immediately released and act as ligands of cannabinoid receptors (Miller and Devi 2011). The first endogenous ligand, N-arachidonoylethanolamine, was identified in 1992 from porcine brain (Devane et al. 1992). It was named anandamide (AEA) based on the Sanskrit word 'ananda' which means 'internal bliss'. Other endogenous cannabinoids include 2-arachidonoylglycerol (2-AG), 2-arachidonyl glyceryl ether (2-AGE, noladin ether) (Hanus et al.

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2001), O-arachidonoylethanolamine (virhodamine) (Porter et al. 2002) and N-arachidonoyldopamine (NADA) (Bisogno et al. 2000; Gaffuri et al. 2012; Mechoulam et al. 2014). Within the nervous system endocannabinoids are released from post-synaptic neurons (retrograde neurotransmission) and they bind to presynaptic CB_1 receptors (see below) which results particularly in inhibition of GABA or glutamate release (Heifets and Castillo 2009). In neuron-astrocyte signalling cannabinoids released from post-synaptic neurons stimulate astrocytic CB_1 receptors, thereby triggering glutamatergic gliotransmission (Castillo et al. 2012).

Phytocannabinoids are chemicals produced especially by female plants of *Cannabis sativa* and are present in the resin of the herb. It has been found that these plants contain over 100 phytocannabinoids (Hill et al. 2012). The most studied cannabinoids from *Cannabis sativa* include e.g. delta-9-tetrahydrocannabinol (THC), cannabidiol, tetrahydrocannabivarin, tetrahydrocannabiorcol, cannabichromene and cannabigerol (Maione et al. 2013). THC was first isolated in 1964 (Gaoni and Mechoulam 1964) and the majority of the herbal cannabinoids soon after.

Synthetic cannabinoids are manufactured compounds which bind to cannabinoid receptors (with either agonistic or antagonistic activity) and many of them were originally synthesised for research purposes in University scientific departments or pharmaceutical companies. The most frequently reported series are represented by JWH (John W. Huffman, Clemson University), CP (Pfizer), HU (Hebrew University), AM (Alexandros Makriyannis, Northeastern University), WIN (Sterling Winthrop) and RCS (Research Chemical Supply) (Presley et al. 2013). Both phytocannabinoids and synthetic cannabinoids mimic the effects of endocannabinoids (Grotenhermen 2006).

Two cannabinoid receptors were initially recognised, CB_1 and CB_2. Both these subtypes belong to the large family of receptors that are coupled to G proteins (Svizenska et al. 2008). Cannabinoid CB_1 receptors are among the most plentiful and widely distributed receptors coupled to G proteins in the brain (Grotenhermen 2006). The CB_1 receptor was cloned in 1990 (Matsuda et al. 1990) and CB_2 in 1993 (Munro et al. 1993). CB_1 receptors are present primarily in the central nervous system in regions of the brain that are responsible for pain modulation (certain parts of the spinal cord, periaqueductal grey), movement (basal ganglia, cerebellum) or memory processing (hippocampus, cerebral cortex) (Grotenhermen 2006). To a lesser extent, they can also be found in some peripheral tissues such as pituitary gland, immune cells, reproductive tissues, gastrointestinal tissues, sympathetic ganglia, heart, lung, urinary bladder and adrenal gland (Pertwee 1997).

CB_2 receptors are particularly expressed in the periphery, in the highest density on immune cells, especially B-cells and natural killer cells (Pertwee 1997) and also in tonsils or spleen (Galiegue et al. 1995); nevertheless, their presence has also been described in the CNS (Van Sickle et al. 2005). The frequently discussed psychotropic effects of cannabinoids are mediated only by the activation of CB_1 receptors and not of CB_2 receptors (Grotenhermen and Muller-Vahl 2012).

Endocannabinoids have also been shown to act on TRPV1 receptors (transient receptor potential cation channels subfamily V member 1, also known as the "capsaicin receptor" and "vanilloid receptor" 1) (Ross 2003). The existence of other G-protein cannabinoid receptors has also been suggested. These proposed receptors (also called putative or non-classical cannabinoid receptors) include GPR18, GPR55 and GPR119 that have structural similarity to CB_1 and CB_2 (Alexander et al. 2013; Zubrzycki et al. 2014).

3. The use of cannabinoids in animals

It has been shown that the mechanism of action of cannabinoids is very complex. The activation of cannabinoid CB_1 receptors results in retrograde inhibition of the neuronal release of acetylcholine, dopamine, GABA, histamine, serotonin, glutamate, cholecystokinin, D-aspartate, glycine and noradrenaline (Grotenhermen and Muller-Vahl 2012). CB_2 receptors localised mainly in cells associated with the immune system are involved in the control of inflammatory processes. Their activation results in, among other effects, inhibition of pro-inflammatory cytokine production and increased release of anti-inflammatory cytokines (Zubrzycki et al. 2014). In addition, some cannabinoids were shown to act not only at cannabinoid receptors but also at vanilloid or serotonin 5-HT_3 receptors (Contassot et al. 2004; Grotenhermen and Muller-Vahl 2012). This complexity of interactions explains both the

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large number of physiological effects of cannabinoids and the pharmacological influences of cannabinoid preparations (Grotenhermen and Muller-Vahl 2012).

There are a huge number of reports on the possible beneficial effects of cannabinoids in human medicine. Their therapeutic potential has been demonstrated in the treatment of many disorders including pain, inflammation, cancer, asthma, glaucoma, spinal cord injury, epilepsy, hypertension, myocardial infarction, arrhythmia, rheumatoid arthritis, diabetes, multiple sclerosis, Parkinson's disease, Alzheimer's disease, depression or feeding-related disorders, and many others (e.g. Porcella et al. 2001; Robson 2001; Rog et al. 2005; Blake et al. 2006; Pacher et al. 2006; Russo 2008; Scheen and Paquot 2009; Karst et al. 2010; Lynch and Campbell 2011; Caffarel et al. 2012; Grotenhermen and Muller-Vahl 2012; Hill et al. 2012; Maione et al. 2013; Lynch et al. 2014; Serpell et al. 2014; Lynch and Ware 2015).

Information concerning the effects of cannabinoid on animals can be found on the experimental level and were obtained during the pre-clinical testing of individual substances in mice, rats and guinea pigs (i.e. laboratory rodents). Beneficial effects of cannabinoids in these animals have been reported e.g. for disorders of the cardiovascular system, cancer treatment, pain treatment, disorders of the respiratory system or metabolic disorders, and suggest the usefulness of further research in this direction. Examples are summarised in Table 1.

For many further examples see the following reviews: Croxford (2003), Guzman (2003), Croxford and Yamamura (2005), Mendizabal and Adler-Graschinsky (2007), Sarfaraz et al. (2008), Nagarkatti et al. (2009), Steffens and Pacher (2012), Velasco et al. (2012), Han et al. (2013), Massi (2013), Stanley et al. (2013), Kucerova et al. (2014), Pertwee (2014), Kluger et al. (2015).

Compared to reports from laboratory rodents, there are a much smaller number of published papers dealing with pre-clinical testing of cannabinoids in other species (rabbits, ferrets, cats, dogs), and an even smaller number of reliable sources are available to date concerning the clinical use of cannabinoids in veterinary medicine for both companion and large animals. Indeed, the majority of articles concerns actually marijuana poisoning and its treatment rather than therapeutic applications (Girling and Fraser 2011; Meola et al. 2012; Fitzgerald et al. 2013).

It is therefore interesting that Mechoulam (2005) reported the use of cannabinoid acids (which are precursors of the neutral cannabinoids, such as THC and cannabidiol) for veterinary purposes in Czechoslovakia already in the 1950s because of their antibiotic properties. The use of cannabinoids as antibiotic drugs, however, was not further investigated, although it has been shown that cannabinoids exert antibacterial activity (Appendino et al. 2008; Izzo et al. 2009).

The most frequently reported use of cannabinoids in companion animals (on a pre-clinical basis) is in association with the topical treatment of glaucoma. Pate et al. (1998) administered AEA, its R-alpha-isopropyl analogue, and the non-classical cannabinoid CP-55,940 into the eyes of normotensive rabbits. These substances were dissolved in an aqueous 10–20% 2-hydroxypropyl-beta-cyclodextrin solution (containing 3% polyvinyl alcohol). The doses were 25.0 µg for CP-55,940 and 62.5 µg for AEA and R-alpha-isopropyl anandamide. The low solubility of the cannabinoids in water was modified with cyclodextrins. It was shown that CP-55,940 had considerable ocular hypotensive effects, R-alpha-isopropyl anandamide exerted these effects to a smaller extent and AEA caused a typical bi-phasic initial hypertension and subsequent decrease in intraocular pressure (Pate et al. 1998). Song and Slowey (2000) administered the substance WIN 55212-2 ($CB_{1,2}$ receptor agonist) topically into the eyes of healthy rabbits at doses of 4, 20 and 100 µg. WIN 55212-2 at a dose of 100 mg significantly reduced intraocular pressure at 1, 2, and 3 h after application. The effects of the substance peaked between 1 and 2 h after administration and intraocular pressure returned to control levels at 4 h after application. The effects of WIN 55212-2 on intraocular pressure were dose-dependent. Twenty mg of the substance produced a smaller effect than 100 mg and 4 mg of the drug elicited non-significant lowering effects (Song and Slowey 2000). Fischer et al. (2013) tested the effects of topical administration of an ophthalmic solution containing THC (2%) on aqueous humour flow rate and intraocular pressure in 21 clinically normal dogs. Topical administration of THC ophthalmic solution led to a moderate reduction in mean intraocular pressure in these animals. Chien et al. (2003) used cannabinoids in both normotensive and glaucomatous monkeys (*Macaca cynomolgus*). WIN 55212-2 ($CB_{1,2}$ receptor agonist) dissolved in 45% 2-hydroxylpropyl-β-cyclodextrin was administered at concentrations of 0.07%, 0.2%, and 0.5%

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Table 1. Examples of cannabinoid use in rodent models

Cardiovascular disorders	Slavic et al. (2013) – blockade of CB_1 receptor with rimonabant (CB_1 receptor antagonist/inverse agonist) improved cardiac functions after myocardial infarction and reduced cardiac remodelling Di Filippo et al. (2004) – administration of WIN 55,212-2 (synthetic $CB_{1, 2}$ receptor agonist) significantly decreased the extent of infarct size in the area at risk in a model of mouse myocardial ischaemia/reperfusion Batkai et al. (2004) – endocannabinoids tonically suppressed cardiac contractility in hypertension in rats Mukhopadhyay et al. (2007) – treatment with rimonabant significantly improved cardiac dysfunction and protected against doxorubicin-induced cardiotoxicity in mice Steffens et al. (2005) – oral administration of THC ($CB_{1, 2}$ receptor agonist) inhibited atherosclerosis in mice
Cancer	Grimaldi et al. (2006) – metabolically stable anandamide analogue, 2-methyl-2V-F-anandamide (CB_1 receptor agonist) significantly reduced the number and dimension of metastatic nodes in mice Guzman (2003) – *in vivo* experiments revealed that cannabinoid treatment of mice slowed down the growth of various tumour xenografts, including lung carcinomas, gliomas, thyroid epitheliomas, skin carcinomas and lymphomas
Pain	Luongo et al. (2013) – chronic treatment with palmitoylethanolamide (endogenous cannabinoid-like compound in the central nervous system) significantly reduced mechanical allodynia and thermal hyperalgesia Pascual et al. (2005) – WIN 55,212-2 (synthetic $CB_{1, 2}$ receptor agonist) reduced neuropathic nociception induced by paclitaxel in rats Hanus et al. (1999) – HU-308 (highly selective CB_2 receptor agonist) elicited anti-inflammatory and peripheral analgesic activity Xiong et al. (2012) – administration of cannabidiol (indirect antagonist of CB_1 and CB_2 receptor agonists) significantly suppressed chronic inflammatory and neuropathic pain in rodents
Asthma	Jan et al. (2003) – THC and cannabinol exhibited potential therapeutic utility in the treatment of allergic airway disease by inhibiting the expression of critical T cell cytokines and the associated inflammatory response in an animal model of mice sensitised with ovalbumin Giannini et al. (2008) – CP-55,940 ($CB_{1, 2}$ receptor agonist) showed positive effects on antigen-induced asthma-like reaction in sensitised guinea pigs and conversely, both SR144528 (CB_2 receptor antagonist/inverse agonist) and AM 251 (CB_1 receptor antagonist/inverse agonist) reverted these protective effects
Vomiting	Darmani et al. (2001a) – THC and CP-55,940 (synthetic agonist at CB_1 and CB_2 receptors) prevented emesis produced by SR 141716A (CB_1 receptor antagonist/inverse agonist) in in the least shrew (*Cryptotis parva*) Darmani (2001b) – THC reduced the percentage of animals vomiting and the frequency of vomits provoked by cisplatin in the same animal species Parker et al. (2004) – THC and cannabidiol (indirect antagonist of CB_1 and CB_2 receptor agonists) reduced lithium-induced vomiting in the house musk shrew (*Suncus murinus*)
Diabetes	El-Remessy et al. (2006) – cannabidiol (indirect antagonist of CB_1 and CB_2 receptor agonists) reduced neurotoxicity, inflammation, and blood-retinal barrier breakdown in streptozotocin-induced diabetic rats Weiss et al. (2006) – cannabidiol significantly reduced the incidence of diabetes in young non-obese diabetes-prone female mice Weiss et al. (2008) – cannabidiol ameliorated the manifestations of diabetes in non-obese diabetes-prone female which were either in a latent diabetes stage or with initial symptoms of the disease
Retinitis pigmentosa	Lax et al. (2014) – HU-210 ($CB_{1, 2}$ receptor agonist) preserved cone and rod structure and function, thus showing neuroprotective effects on retinal degeneration in a rat model for autosomal dominant retinitis pigmentosa
Food intake, body weight	Hildebrandt et al. (2003) – AM 251 (CB_1 receptor antagonist/inverse agonist) reduced inguinal subcutaneous, retroperitoneal and mesenteric adipose tissue mass in Western diet-induced obese mice. Anorectic effects of AM 251 were also reported by e.g. Slais et al. (2003), Chambers et al. (2006) and Tallett et al. (2007)

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Five normal monkeys received 50 µl (2 × 25 µl) of WIN 55212-2 to the right eye, and an equal volume of the vehicle to the left eye. In glaucomatous monkeys, 50 µl of WIN 55212-2 was administered to the glaucomatous eye only. Moreover, a multiple-dose study was carried out in 8 monkeys with unilateral glaucoma. WIN 55212-2 (0.5%) was administered to the glaucomatous eye twice daily at 9:30 AM and 3:30 PM for five consecutive days. It was shown that in the five normal monkeys unilateral application of the substance significantly decreased intraocular pressure for up to 4, 5, and 6 h following administration of the 0.07%, 0.2%, and 0.5% concentrations, respectively. The maximum changes in intraocular pressure were found at 3 h after drug application. In the eight glaucomatous monkeys the administration of WIN 55212-2 also resulted in a significant decrease in intraocular pressure (Chien et al. 2003).

Other potential and promising indications for cannabinoid use in veterinary medicine include inflammation and pain treatment as well as possible applications in dermatology and oncology. With respect to inflammation and pain, Re et al. (2007) authored a review in which they focused on the role of an endogenous fatty acid amide analogue of the endocannabinoid AEA – termed palmitoylethanolamide (PEA) – in tissue protection. PEA does not bind to CB_1 and CB_2 receptors but has affinity for the cannabinoid-like G-coupled receptors GPR55 and GPR119. It acts as a modulator of glia and mast cells (Keppel Hesselink 2012), and has been shown to enhance AEA activity through a so-called "entourage effect" (Mechoulam et al. 1998). Re et al. (2007) concluded that the use of natural compounds such as PEA influences endogenous protective mechanisms and can represent an advantageous and beneficial novel therapeutic approach in veterinary medicine. Regarding dermatology, Scarampella et al. (2001) administered the substance PLR 120 (an analogue of PEA) to 15 cats with eosinophilic granulomas or eosinophilic plaques. Clinical improvements of signs and lesions were evident in 10 out of 15 cats, suggesting that PLR-120 could be a useful drug for the treatment of these disorders (Scarampella et al. 2001). Similarly, Cerrato et al. (2010) isolated mast cells from the skin biopsies of 18 dogs, incubated these cells with IgE-rich serum and challenged them with anti-canine IgE. Subsequently, histamine, prostaglandin D2 and tumour necrosis factor-alpha release was measured in the presence and absence of increasing concentrations of palmitoylethanolamide. The authors found that histamine, prostaglandin D2 and tumour necrosis factor-alpha release induced by canine anti-IgE were significantly inhibited in the presence of PEA. Thus, it can be concluded that PEA has therapeutic potential in the treatment of dermatological disorders involving mast cell hyperactivity (Cerrato et al. 2010). Moreover, Cerrato et al. (2012) evaluated the effects of PEA on the cutaneous allergic inflammatory reaction induced by different immunological and non-immunological stimuli in six spontaneously *Ascaris*-hypersensitive Beagle dogs. These dogs were challenged by intradermal injections of *Ascaris suum* extract, substance P and anti-canine IgE, before and after PEA application (orally at doses of 3, 10 and 30 mg/kg). The results have shown that PEA was effective in reducing immediate skin reaction in these dogs with skin allergy (Cerrato et al. 2012). With respect to oncology, Figueiredo et al. (2013) found that the synthetic cannabinoid agonist WIN-55,212-2 was effective as a potential inhibitor of angiogenesis in a canine osteosarcoma cell line. Although further *in vivo* research is certainly required, the results thus far indicate that the use of cannabinoid receptor agonists as potential adjuvants to chemotherapeutics in the treatment of canine cancers could be a promising therapeutic strategy. Looney (2010) reported the use of cannabinoids for palliative care in animals suffering from oncological disease to stimulate eating habits. Finally, McCarthy and Borison (1981) reported antiemetic activity of nabilone (synthetic $CB_{1,2}$ agonist) in cats after cisplatin (anti-cancer drug) treatment and similarly Van Sickle et al. (2003) reported that THC (0.05–1 mg/kg *i.p.*) reduced the emetic effects of cisplatin in ferrets.

4. Prospective veterinary use of cannabinoids

As can be seen from the above instances, cannabinoids have a myriad of pharmacological effects and the beneficial impact of different cannabinoids has been proven and documented many times in various laboratory/companion animals. It has been shown that the same cannabinoid drug can elicit divergent responses in humans and animals. For example, Jones (2002) reported increased heart rate and slightly increased supine blood pressure after THC administration in humans, whereas the cardiovascular effects in animals were different, with bradycardia and hypotension (Jones 2002). Thus, a definite advantage of the use of cannabinoids in

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animals is that the research and pre-clinical testing was carried out on various animal species and these categories can now represent target species in the case of veterinary use. In other words, the risk of divergent responses to the same drug, which has been described for humans and animals, is much lower.

It should also be taken into account that the majority of cannabinoids possess psychotropic properties which may change the behaviour of animals (e.g. locomotion) and that these substances have addictive potential (Fattore et al. 2008; Landa et al. 2014a; Landa et al. 2014b). On the other hand, other drug classes with even stronger effects on the CNS and addictive properties have been used therapeutically in both humans and veterinary medicine for centuries (e.g. opioids) because their benefit outweighs the risks.

Cannabis-based medical products were introduced to human medicine in the last years in many countries (among others Austria, Canada, Czech Republic, Finland, Germany, Israel, Italy). Preparations approved for use in human medicine include Cesamet, Dronabinol, Sativex, Bedrocan, Bedrobinol, Bediol, Bedica or Bedrolite. For dogs and cats, the veterinarian-recommended, ready-made hemp based supplement Canna-Pet is presently available (containing non-psychoactive cannabidiol). PEA can at present be used to restore skin reactivity in animals in a veterinary medication sold under the trade name Redonyl (LoVerme et al. 2005). It is therefore not surprising that owners of animals are also exhibiting increasing interest in the possible use of cannabinoids/medical marijuana in veterinary medicine as can be seen by the number of internet forums concerned with this issue (e.g. dvm360 magazine, Cannabis Financial Network or Medical Daily). In the Journal of the American Veterinary Medical Association, Nolen (2013) reported anecdotal evidence from pet owners describing beneficial effects of marijuana use in dogs, cats and horses and, moreover, also the opinions of professionals who believe in the potential usefulness of cannabis use in veterinary medicine. The reluctant attitude of veterinarians towards the use of cannabinoids/medical marijuana in animals could be associated with the risk that owners will make attempts to treat their animals using cannabis-based products, which can lead to intoxication. In the article by Nolen (2013), Dr. Dawn Boothe (Clinical Pharmacology Laboratory at Auburn University College of Veterinary Medicine) concluded that veterinarians should be part of the debate about the use of cannabinoids/medical marijuana, e.g. by means of a controlled clinical trial dealing with the use of marijuana to treat cancer pain in animals.

5. Conclusions

The isolation of THC in 1964 represented a breakthrough in research progress concerning cannabinoids. The discovery of the cannabinoid receptors and their endogenous ligands, definition of the endocannabinoid system and description of other cannabinoid substances elicited increased interest in this research and in the possible therapeutic potential in animal models. The results from this basic research finally led to the addition of cannabinoids/medical marijuana to the spectrum of therapeutic possibilities for various disorders in humans. The therapeutic effects of cannabinoids/medical marijuana on companion animals are now the subject of discussion in numerous internet forums and such debate could result in attempts at treatment using cannabinoids without the necessary safety precautions. Thus, the prospective use of cannabinoids for veterinary purposes needs to be taken seriously; this could decrease the risk of attempts at unauthorised and non-professional treatment by animal owners. Legislative regulations may differ in various countries and the use of cannabinoids/medical marijuana must be in accordance with the respective rules.

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Medical cannabis in the treatment of cancer pain and spastic conditions and options of drug delivery in clinical practice

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The use of cannabis for medical purposes has been recently legalised in many countries including the Czech Republic. As a result, there is increased interest on the part of physicians and patients in many aspects of its application. This mini review briefly covers the main active substances of the cannabis plant and mechanisms of action. It focuses on two conditions, cancer pain and spasticity in multiple sclerosis, where its effects are well-documented. A comprehensive overview of a few cannabis-based products and the basic pharmacokinetics of marijuana's constituents follows. The review concludes with an outline for preparing cannabis (dried inflorescence) containing drug dosage forms that can be produced in a hospital pharmacy.

Key words: cannabis, pain, cancer, spasms, multiple sclerosis, mechanism of action, THC, cannabinoids

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INTRODUCTION

Cannabis is an annual dioecious plant containing over 1,300 natural compounds[1]. It diverged around 27.8 million years ago from *Humulus*, the hop plant[2] and botanic taxonomy classifies cannabis as follows: order *Urticales*, family *Cannabaceae*, genus *Cannabis* (hemp), species *sativa* Linné[3]. There is continuing debate whether cannabis is one species (*Cannabis sativa*, with several subspecies and varieties) or if there are several distinct species (*Cannabis sativa*, *Cannabis indica* and *Cannabis ruderalis*) (ref.[2]). Marijuana was domesticated thousands of years ago and the two most frequently cited hypotheses on the origin of cannabis domestication locate the centre to either China or Central Asia[4,5].

Cannabis was used therapeutically for almost 5.000 years (first noted in China 2737 B.C.) (ref.[6,7]). In ancient and medieval cultures it was predominantly used for the treatment of various somatic disorders including headache, fever, bacterial infections, diarrhoea, rheumatic pain and malaria, apart from its psychoactive uses[6,8,9]. Western medicine also used cannabis, particularly in the 19th century. It was a common analgesic drug before the introduction of Aspirin[10].

Naturally occurring phytochemicals of the species *Cannabis sativa*, *Cannabis indica* and *Cannabis ruderalis* comprise nearly 1,300 chemical entities. Of these, more than 140 are classified as phytocannabinoids[11] – substances able to bind to cannabinoid receptors. These compounds are present in the highest amounts in the viscous resin produced by the glandules of female cannabis inflorescence[12]. Eleven chemical classes of phytocannabinoids were defined by Elsohly et al.[12]. These include: 1) cannabigerol type, 2) cannabichromene type, 3) cannabidiol type, 4) (-)-Δ9-trans-tetrahydrocannabinol type, 5) (-)-Δ8-trans-tetrahydrocannabinol type, 6) cannabicyclol type, 7) cannabielsoin type, 8) cannabinol type, 9) cannabinodiol type, 10) cannabitriol type, and 11) miscellaneous type. The (-)-Δ^9-trans-tetrahydrocannabinol type, cannabinol type, and cannabidiol type are the most abundant and best known. They are also the most studied and used/tested in clinical trials as therapeutic agents.

The main psychoactive ingredient is Δ^9-tetrahydrocannabinol (THC) (ref.[13]). The other compounds of non-cannabinoid nature involve among others, nitrogenous compounds, amino acids, proteins, enzymes, glycoproteins, sugars, alcohols, aldehydes, ketones, fatty acids, esters, lactones, steroids and terpenes, thus the profile of the *Cannabis* plant is very complex[12]. Its characteristic aroma is due to volatile terpenoids, not cannabinoids[14].

From the medical point of view, cannabinoids are the best studied components of cannabis. Herbal cannabinoids or phytocannabinoids are compounds produced especially by female plants of *Cannabis sativa* and found in the resin of the herb. The first substance isolated from *Cannabis sativa* was cannabinol at the end of the 19th century[15]. The major psychoactive substance is THC, which was isolated in 1964 (ref.[16-18]) and the majority of the phytocannabinoids were isolated shortly afterwards. The best explored phytocannabinoids are THC, cannabidiol (CBD), tetrahydrocannabivarin, tetrahydrocannabiorcol, cannabichromene and cannabigerol[19,20]; the first real cannabinoid compound in cannabis plant (cannabidiolic acid) was isolated and identified by Krejci and Santavy

in 1955 (ref.[20]). Cannabis plant varieties differ greatly in their content of THC. The concentration of THC in industrial hemp is less than 0.3% and according to legal bodies is not considered a substance of abuse and thus its possession is not restricted in the Czech Republic. On the other hand, strains producing higher amounts of THC or CBD have been recently cultured and these may contain up to 25% of THC in the dried inflorescence[21].

The effects of phytocannabinoids are mostly associated with their ability to influence the function of the endocannabnoid system. This consists of endocannabinoids, cannabinoid receptors and enzymes involved in endocannabinoid biosynthesis and degradation[22]. Thus, actions of both endocannabinoids and phytocannabinoids are mediated by cannabinoid receptors CB_1 and CB_2. Both types of receptors are coupled with the G protein[23]. CB_1 receptors are found particularly in the central nervous system (CNS) in regions of the brain responsible for movement, pain modulation, and memory[24,25]. They are also expressed in smaller amounts in some peripheral tissues such as immune cells, reproductive tissues, pituitary gland, gastrointestinal tissues, sympathetic ganglia, heart, lung, urinary bladder and adrenal gland[26]. In contrast, CB_2 receptors are found especially in the peripheral tissues, with the highest density in immune cells[26], tonsils and spleen[27]; however, they have also been found within the CNS (ref.[28]). Besides the two "classical" receptors, cannabinoids can act on $TRPV_1$ receptors (transient receptor potential cation channels subfamily V member 1, also known as the "capsaicin receptor" and "vanilloid receptor" 1) (ref.[29]) and the existence of other G-protein cannabinoid receptors (putative cannabinoid receptors) has also been suggested – GPR12, GPR18, GPR55 and GPR119 (ref.[30-32]).

APPROVED CANNABIS-BASED PRODUCTS AVAILABLE IN THE CZECH REPUBLIC AND EUROPE

Two categories of cannabinoid medicines are currently approved in the Czech Republic: ready-made products containing standardized extract of cannabis sold under the trade name Sativex® and crude medical cannabis (marijuana) available as a pharmaceutical compound with a standardized content of 19% THC and 6% CBD, 16% THC and 0.1% CBD or 10% THC and 10% CBD. Crude medical cannabis is intended for use as individually prepared preparations. Sativex® (GWPharmaceuticals, Salisbury, Wiltshire, UK) is an oromucosal spray containing 38-44 mg/mL and 35-42 mg/mL of two extracts (as soft extracts) of *Cannabis sativa, folium cum flore* (Cannabis leaf and flower) corresponding to 2.7 mg Δ^9-tetrahydrocannabinol and 2.5 mg cannabidiol per mL. According to SmPC, Sativex® is indicated for the treatment of adult patients with moderate to severe spasticity due to multiple sclerosis (MS) who have not responded to other anti-spasticity medication and who showed clinically significant improvement in spasticity related symptoms during an initial treatment trial.

The term "medical marijuana" is in general related to the cannabis that healthcare providers recommend for therapeutic purposes[33]. The State Agency for Medical Cannabis in the Czech Republic defines medical cannabis as dried female flowers of *Cannabis sativa* L. or *Cannabis indica* Lam. plants. It contains a range of active substances, among others Δ^9-tetrahydrocannabinol and cannabidiol. Cannabis issued in pharmacies meets qualitative requirements defined in the Decree No 236/2015 Coll. It is indicated as supportive treatment to moderate symptoms accompanying serious diseases. According to the definition, the expressed content of THC as percentage is in the range 0.3% - 21.0% and expressed content of CBD as percentage is in the range 0.1% - 19.0%. The actual content of both THC and CBD in medical cannabis must not differ more than ± 20% from the value given by the producer. As mentioned above, currently available medical cannabis contains 19% of Δ^9-THC and 6% of CBD, 10% Δ^9-THC and 10% CBD or 16% Δ^9-THC and 0.1% CBD.

It can be seen, that despite the tremendous number of compounds present in cannabis, the greatest attention is being paid to two substances – THC and CBD. Δ^9-tetrahydrocannabinol, the main psychotropic substance in cannabis is a partial agonist of cannabinoid CB_1 and CB_2 receptors. Cannabidiol (possessing no psychotropic effects) is referred to as an antagonist of CB_1/CB_2 receptor agonists in CB_1- and CB_2- receptor expressing cells or tissues[26]. This mechanism could lead to the assumption that CBD decreases the effects of THC, however it has been shown that it may conversely potentiate the pharmacological effects of THC via a CB_1 receptor-dependent mechanism – by increasing CB_1 receptor density[34]. Moreover, it has been also shown that CBD can stimulate vanilloid pain receptors (VR1), inhibit uptake of anandamide, and weakly inhibit its degradation[35].

In compliance with the legal rules mentioned, medical cannabis can be prescribed by physicians of the following professional competences: clinical oncology, radiation oncology, neurology, palliative medicine, pain treatment, rheumatology, orthopaedics, infectious medicine, internal medicine, ophthalmology, dermatovenerology, geriatrics and psychiatry. Indications involve among others: chronic persistent pain – especially in association with cancer, neuropathic pain, pain associated with glaucoma, pain associated with degenerative disease of the musculoskeletal system, spasticity and pain in multiple sclerosis, tremor caused by Parkinson's disease, nausea and vomiting particularly following cancer treatment, stimulation of appetite in cancer and HIV patients, Tourette syndrome and superficial treatment of dermatosis and mucosal lesions.

Besides Sativex® and medical cannabis, there are two other cannabinoids approved for use in other countries – nabilone (Cesamet®, Cesamet, Valeant Pharmaceuticals, Aliso Viejo, CA, USA) and dronabinol (Marinol®, Solvay Pharmaceuticals, Brussels, Belgium) (ref.[36]). Both nabilone and dronabinol are available in capsules and are used to treat chemotherapy-induced nausea and vomiting, particularly in oncologic patients who have not responded

to standard means for control of these conditions[37]. Dronabinol is also used to treat anorexia associated with AIDS (ref.[38]). Dronabinol is synthetic THC, nabilone is synthetic THC analogue; each of these substances has partially agonistic effect at the cannabinoid CB_1 and cannabinoid CB_2 receptors[37].

CLINICAL EXPERIENCE WITH CANNABIS IN CANCER PAIN AND MULTIPLE SCLEROSIS

Cancer pain

It is generally accepted that smoking cannabis ameliorates the perception of pain in healthy volunteers[39-41]. However, it is questionable, whether the effect is of antinociceptive or rather psychotropic nature and possibly both components may play a role[42]. From the pathophysiological point of view, cancer pain comprises both nociceptive and neuropathic components. Hence, all the clinical studies assessing the role of cannabis in this condition are relevant. Unfortunately, there is a lack of studies evaluating the therapeutic effectiveness of pure cannabis in cancer pain exclusively.

Importantly, the analgesic efficacy of cannabinoids (THC 5–20 mg orally and levonantradol 1.5–3.0 mg i.m.) was confirmed in a meta-analysis of 9 clinical trials, where these substances were administered in patients with cancer, chronic non-cancer, and acute postoperative pain (total n = 222). Their effects were very similar to codeine (50–120 mg), which is commonly referred to as a weak opioid analgesic (The Number Needed to Treat, NNT for codeine 60 mg for acute pain: 16.7; 11.0–48.0) (ref.[43]). This conclusion is somewhat contradictory as NNT values for THC in the treatment of distal sensory predominant polyneuropathy are 3.5 and 3.6 according to Abrams et al.[44] and Ellis et al.[45], respectively.

Considering cannabis smoking effects in pain, several studies have been published so far. Abrams et al. observed the superior effectiveness of smoking cannabis over placebo (three times a day for 5 days) in experienced smokers suffering from the neuropathic pain of HIV-associated sensory neuropathy (n = 50). When compared to placebo, it significantly reduced daily pain (-34% vs. -17%; P = 0.03). Reduction in pain greater than 30% was achieved in 52% and 24% subjects on cannabis and placebo, respectively (P = 0.04). Importantly, smoked cannabis (3.56 % tetrahydrocannabinol) also reduced hyperalgesia to both brush and von Frey hair stimuli ($P \leq 0.05$) (ref.[43]). The beneficial effects of smoked cannabis in HIV-patients with distal sensory neuropathy (both in terms of the total pain relief and the proportion of patients with at least 30% pain relief versus placebo) were additionally achieved in another placebo-controlled trial (n = 28) (ref.[45]).

Also, the other published trials on smoked cannabis assessed its effects in neuropathic pain. Wilsey et al. in his double-blinded, placebo-controlled, cross-over study evaluated its effects in thirty-eight patients with central and peripheral neuropathic pain. An analgesic response to cannabis with good tolerance was achieved[46]. Smoked cannabis of four different potencies (0%, 2.5%, 6% and 9.4% tetrahydrocannabinol) was additionally evaluated in 21 adults with post-traumatic or postsurgical neuropathic pain over four 14-day periods in a double-blind, placebo-controlled, four-period crossover trial. Only cannabis with a potency of 9.4% THC administered three times a day for five days significantly reduced the intensity of pain, improved sleep and was well tolerated[47]. Lower doses possessed only insignificant trend. All these studies are reflected in the latest guideline for the treatment of neuropathic pain published by NICE (ref.[48]).

Smoking cannabis was reported to be effective also in patients with non-cancer pain (i.e. post-traumatic pain, osteoarthritic pain etc.) as presented and discussed in several papers[49-52], however, the extent of its use in this indication might be limited by adverse effects[53].

To our knowledge, there has been no well-designed clinical trial with cannabis monotherapy in the treatment of cancer pain. It is also demanding to perform such a study and thus, the evidence of antinociceptive effect of cannabis is only indirect. Nonetheless, as mentioned above, some its antinociceptive effects were recorded in various types of pain. Importantly, cannabis/cannabinoids are well recognized antiemetic agents, hence an additional benefit of their use in oncologic patients undergoing chemotherapy might be expected[54].

Multiple sclerosis

The therapeutic efficacy of medical cannabis in managing symptoms of MS was evaluated in several case-studies and clinical trials with relatively heterogeneous results. Probably in the first clinical trial evaluating 10 adults with spasticity and 10 healthy volunteers found that smoking cannabis impairs posture and balance in patients with spasticity[55] During the late 90's., Schon et al. described beneficial effect of smoking cannabis resin in one patient with MS. He substantially improved in terms of dramatic suppression of acquired pendular nystagmus. Surprisingly, he did not respond adequately either to oral nabilon or capsules containing cannabis oil[56]. The typically mentioned problem with the use of cannabis in any therapeutic indication, including MS, is the side-effects (namely, central nervous system disorders – drowsiness, anxiety, paranoia etc.) as well as the attitudes of the society to any use of marijuana. Therefore, the evaluation of attitudes of MS-patients in this relation was very important to establish. Page et al. published a work, where MS-patients were asked to describe their own beliefs with cannabis (n = 420). The majority of them (96%) considered cannabis as potentially useful. Forty-three percent had their own experience with this plant, however, only 16% of these cases were related to MS. These patients reported especially an improvement in general symptoms of MS (e.g. anxiety/depression, spasticity and chronic pain) (ref.[57]). This corresponds with results published by Clark et al. one year later, where stress, sleep, mood, stiffness/spasm, and pain were substantially improved in medicinal cannabis users (n = 34; orally or smoked; THC content not specified; the single dose size varied from 1–2 puffs to the entire joint in smokers and mostly up to 1 g when given orally) trying to alleviate their MS-related symptoms[58].

Since then, several studies were published. Fox et al. did not observe any significant improvement in any of the objective measures of upper limb tremor with oral cannador (cannabis extract) at the mean dose of 0.107 mg/kg twice a day of THC compared to placebo in 14 patients with MS; only a weak subjective relief of symptoms was reported[59]. Vaney et al. also provided no convincing evidence of cannabis benefits in this illness. In total, 50 subjects were involved in a prospective, randomized, double-blind, placebo-controlled cross-over study, where cannabis-extract capsules, standardized to 2.5 mg tetrahydrocannabinol and 0.9 mg cannabidiol (maximal daily dose was 30 mg of THC after dose-escalation phase) were used. Only a statistically insignificant trend in favour of these capsules was observed in terms of improving spasm frequency, mobility and getting to sleep in the intention-to-treat analysis. However, as shown in per-protocol analysis (n = 37), a significant improvement in spasm frequency ($P = 0.01$), and mobility was recorded. Hence, the authors conclude that a standardized cannabis extract might lower spasm frequency and increase mobility with tolerable side effects in patients with persistent spasticity not responding to other drugs[60]. Nevertheless, the MUSEC trial shows significant superiority of oral cannabis extract to placebo (n = 279) in terms of muscle stiffness after twelve weeks of administration. Similar results were also obtained after four and eight weeks of the treatment[61]. The most recently published review covering the role of encodannabinoid system in the multiple sclerosis was presented by Chiurchiu et al.[62].

PHARMACOKINETICS OF MEDICAL CANNABIS CONSTITUENTS

The medical use of cannabis exploits oral and inhalation routes of administration. Both have considerable benefits and also pitfalls, with different pharmacokinetic features as clinically the most relevant consequence. There are available registered products with synthetic THC and/or CBD, such as synthetic THC (dronabinol – Marinol, Syndros) or nabilone – synthetic analogue of THC (Cesamet). Other options of oral use include standardized extracts and cannabis-derived formulations with content of both THC and CBD (Sativex, Cannador). This combination is claimed to improve tolerability for medical uses by reducing the psychoactive effects of THC (ref.[63]). There may be considerable differences between pharmacokinetics of pure THC and/or CBD in tablets, extracts and raw material in oral drug dosage forms - possibly due to matrix effects on absorption.

The general features of cannabinoids, such as protein binding and volume of distribution are apparently little influenced by the route of administration. The protein binding of THC is reported to be 95-99% and volume of distribution of 5.7-10.0 L/kg. The volume of distribution is reported to increase with chronic administration[64].

Inhalation

Medical cannabis may be principally smoked or vaporized and inhaled. Vaporization or smoking of medical cannabis apparently seems to be most effective way of administration. The main reasons for greater bioavailability are the lipophilic nature of major constituents (partition coeff. octanol/water between $6x10^3$ and $9 x10^6$) and effective conversion of THC-A and CBD-A to their decarboxylated forms when smoked. In contrast, smoking is also not recommended due to the adverse effects of smoking due to the possible toxic effects of other compounds formed at high temperatures of raw material[63].

The technique of smoking considerably affects the absorption and therefore there is great variability in bioavailability, estimated as 2–56%. Absorption is very fast, with C_{max} reached within several minutes[65]. To the best of our knowledge, no pharmacokinetic studies with vaporization of medical cannabis have been published.

Oral ingestion

Oral administration comprises variety of oral drug dosage forms with the oromucosal spray and tablets as the most used. Others include crude medical cannabis in capsules, chocolate bars, cookies, but also herbal infusions, tinctures and oils. Various kinds of extracts may be encapsulated (dry extracts) or used as oral liquid ethanolic or oily extracts. The pharmacokinetic features of THC and CBD after oral intake may be greatly influenced by the drug dosage form, excipient, intake with/without food, physiological factors (motility, constipation), pathophysiology (liver functions) and co-medication (e.g. administration of antiemetics – metoclopramide, itopride) (ref.[64]).

Even though the oral route may look safer than inhalation (toxic compounds originated during cannabis combustion, more precise dosing), it may result in more frequent central adverse effects[66-68] possibly due to greater proportion of active metabolite 11-OH THC to parent THC (ref.[69,70]).

The absorption of THC and CBD is very rapid upon oromucosal administration with T_{max} reported to vary from 15 min to 1 h, and variable half-life increasing with the dose – from 1.9 to 3.72 and 5.2 h in case of THC and from 5.3 to 6.4 and 9.4 h in case of CBD after 2,4 and 8 inhalations, which corresponds to 5.4 mg THC/5.0 mg CBD, 10.8 mg THC/10.0 mg CBD and 21.6 mg THC/20.0 mg CBD, respectively[71,72]. There is huge inter- and intra-individual variability of the pharmacokinetic parameters with CV ranging from 57 to 74% (ref.[71]). No significant drug accumulation was found after repeated doses (up to 21.6 mg THC and 20.0 mg CBD) (ref.[72]).

The pharmacokinetics of THC and CBD in oral tablets shows lower absorption rate with THC T_{max} of approx. 0.6 to 2.6 h after ingestion, depending on the dose and drug dosage form[66,73,74]. Interestingly, slower absorption was reported in sublingual (crushed tablet) administration of 5 mg THC than after normal oral use, in study of Klumpers et al.[74]. The elimination of cannabinoids after conventional oral administration is believed to be biphasic, with a distribution half-life of about 4 hours and terminal elimination half-life of 24 to 38 h[71,75], which may even be prolonged in chronic users[64]. Elimination parameters do not seem to be affected by the route of administration,

with terminal half-life of 24-36 h observed after inhalation[76], which is comparable to half-life reported by Ahmed and Heuberger after oral, inhalation or intravenous administration[73,75]. Interestingly, a close correlation of serum ALT levels and elimination rate constant was found[76], which could make ALT an important predictive marker of THC elimination and co-variate in pharmacokinetic models. Similar profile as THC show also major metabolites 9-hydroxy- and 1-hydroxy-THC (ref.[64,77]). On contrary, the plasmatic levels of major secondary metabolite, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) exerts much slower increase (T_{max} approx. 3 h) (ref.[66]) with sustained plasmatic levels, especially in chronic administration[64,77,78]. Overall bioavailability after oral administration varies from 4 to 20% (ref.[64]). Interestingly, some studies reported two peaks in plasma after single dose administration which occurs due to entero-hepatic circulation[64,65]. In older subjects, there could be observed greater bioavailability due to decreased liver metabolic activity and also lower elimination rate due to larger volume of distribution[79]. In general, oral administration exerts slower absorption, lower bioavailability and delayed peak in plasma compared to inhalation[64].

To date, there were not published pharmacokinetic studies with non-extracted medical cannabis in capsules.

There are few studies on rectal administration of cannabinoids. T_{max} of THC after rectal administration of 2.5 and 5 mg of THC were 2−8 h. The bioavailability of THC after rectal administration was considerably higher (about twice) than after oral route, possibly due to greater extent of absorption and lower pre-systemic elimination[80]. Recently, the pharmacokinetic interactions of cannabinoids, including THC and CBD, have been reviewed elsewhere[81].

PRODUCTION OF INDIVIDUALLY PREPARED PREPARATIONS WITH CANNABIS IN ST. ANNE'S FACULTY HOSPITAL PHARMACY IN BRNO

Following legalisation of medical cannabis use in the Czech Republic since 2013, pharmacists had to solve the issue, what drug dosage forms are suitable for production of customised preparations. It has been shown that for oral use, capsules are very convenient and this final section briefly describes production of casules in St. Anne's Faculty Hospital. The main reasons for the issue of individual preparationswere: cancer pain, spasticity and antiemetic purposes.

Cannabis is supplied to the pharmacy in the form of dried female flowers. It is well known, that apart from THC and CBD, carboxylated forms (tetrahydrocannabinolic acid, THC-A and cannabidiolic acid, CBD-A) are also present in significant amounts in raw plant material. These carboxylated cannabinoids are spontaneously converted to THC and CBD at high temperatures (approx. 100-140 °C). Moreover, THC-A may be converted to cannabinolic acid (CBN-A) when exposed for long time to oxygen in the air. CBN-A may be also decarboxylated to CBN at high temperatures[82,83].

Thus, in order to increase the effect of oral ingestion the first step involves cannabis decarboxylation. The plant is first of all weighed out into suitable containers. Decarboxylation is carried out using a sterilisation procedure: temperature 121 °C for 30 min. After this, the material must be allowed to cool down. Cannabis is then treated in a splintery grinder and homogenized. Following homogenization, adjuvant substances are added (suitable filling mass such as lactose or starch) and finally the required volume is produced. This mass is subsequently adjusted to gelatinous capsules; size 2 is commonly used. The amount of dried cannabis is usually 125 mg per capsule, but 250 and 375 mg per capsule are also produced.

Raw medical cannabis, even if available as standardized extract, is considered instable and the content of active components can vary with storage condition. Hence, capsules are stored in tightly closed plastic containers kept at – 18 °C to prevent excessive evaporation of volatile oils.

Capsules containing medical cannabis of Czech origin (Elkoplast) were produced in the pharmacy of St. Anne's Faculty Hospital from April 2016 until February 2017. Cannabis was not available from March 2017 till June 2017. Capsules were produced again from July 2017 to August 2017 and contained cannabis of Dutch origin (Bedrocan). Currently, there is available cannabis of Canadian origin with 16% of THC and 0.1% CBD or with 10% of both THC and CBD, and of Czech origin containing 19% of THC and 6% of CBD.

Capsules with medical cannabis produced in the period April 2016-August 2017 were prepared for approximately 20 patients predominantly from Southern and Northern Moravia. These patients described in general, pain relief and consequent improvement of sleep.

CONCLUSION

Despite the long history, the current use of cannabis in practical medicine is still rather limited. This situation however soon became subject to change. Interestingly, despite the huge number of substances that have been identified in the plant, attention is only paid to THC and CBD, and other compounds that could also play a role in the mechanism of action of medical cannabis are not in the centre of interest. Studies declare only amounts of THC and CBD, and regulatory authorities control medical cannabis for the content of these two substances. Recently however, promising neuroprotective properties of cannabigerol (CBG) in Huntington's disease have been reported[84]. Thus, it can be concluded, that further research will provide other facts and this will contribute to larger introduction of medical cannabis into practical use.

Search strategy and selection criteria

Literature was searched using the databases: Medline, EBSCO, EMBASE, Cochrane Library, and OVID. The mesh words used during searching were: "cannabis"/ "cannabinoid"/"nabilon"/"dronabinol" both alone and

in combination with words "pharmacokinetics", "pharmacodynamics", "pain", "analgesia", "cancer pain", "spasticity", "multiple sclerosis", "toxicity", "safety", "interactions", "effectiveness" and "efficacy". The most relevant published studies are discussed in the presented article.

ABBREVIATIONS

ALT, alanine aminotransferase; CB, cannabinoid; CBD, cannabidiol; CBN-A, cannabinolic acid; CBG, cannabigerol; CNS, central nervous system; THC, Δ^9-tetrahydrocannabinol; HIV, human immunodeficiency virus; MS, multiple sclerosis; NICE, National institute for health and care excellence; NNT, the number needed to treat; $TRPV_1$, transient receptor potential cation channels subfamily V member 1; VR1, vanilloid pain receptors.

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