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The role of ghrelin signalling in the neurobiological mechanisms of rewarding effects of cannabinoids and opioids

Úloha ghrelinové signalizace v neurobiologických mechanismech odměňovacích účinků kanabinoidů a opioid

Dissertation research thesis

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Praha, 2022

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TABLE OF CONTENTS

ABSTRACT 7

ABSTRAKT 9

I. INTRODUCTION 11

II. THEORITICAL PART 13

1. Introduction to the problematic of addictive substances and addiction 13

1.1. The public health significance of the problematic use of psychoactive drugs 13

1.2. Regulation, policies, and the impact in the field of psychoactive drug misuse/overuse 14

1.3. Use of psychoactive drugs among children, adolescents, and the adult population .. 15

2. Cannabinoids 18

2.1. Current situation – spectrum of use and abuse cannabinoids in the Czech Republic and Europe 18

2.2. Neurobiological mechanisms of action and risks 19

2.3. Treatment approaches and limitations in cannabis/cannabinoid addiction 20

3. Opioids 22

3.1. Current situation – spectrum of abused opioids in the Czech Republic and Europe 22

3.2. Neurobiological mechanisms of action and risks 22

3.3. Treatment approaches and limitations in opioid addiction 24

4. Ghrelin and GHS-R1A - their role in mechanisms of addiction 26

4.1. Current knowledge of complex ghrelin/GHA-R1A effects and potential use in addiction therapy 26

4.2. Role of ghrelin/GHS-R1A in the cannabinoid addiction 28

4.3. Role of ghrelin/GHS-R1A in the opioid addiction 29

5. Experimental models used in the preclinical addiction research 31

5.1. Microdialysis in vivo 31

5.2. LABORAS 32

III. EXPERIMENTAL PART 34

6. Hypothesis and Aim 34

7. Methods 36

7.1. Animals 36

7.2. Drugs and Chemicals 36

7.3. In-vivo microdialysis and chemical analysis assay 39

7.4. Behavioural Testing in LABORAS 40

7.5. Statistical Analysis 41

8. Results 42

8.1. The JMV2959 effects on the WIN55,212-2-induced increase of dopamine and its metabolites in the NACSh 42

8.2. The JMV2959 effects on the WIN55,212-2-induced extracellular turnover of dopamine in the NACSh 44

8.3. The JMV2959 effects on the WIN55,212-2-induced changes of anandamide, 2-AG and GABA in the NACSh 46

8.4. The JMV2959 effects on the μ-selective opioid fentanyl-induced changes of anandamide, 2-AG and GABA in the NACSh 49

8.5. The JMV2959 effects on the μ-selective opioid fentanyl-induced changes of accumbens anandamide and 2-AG when administered into the VTA or NACSh 52

8.6. JMV2959 effects on THC/WIN55,212-2 -induced behavioural stimulation 53

9. Discussion 57

IV. CONCLUSION 65

LIST OF FIGURES 67

LIST OF PUBLISHED ARTICLES 68

LIST OF ABBREVIATIONS 69

LITERATURE 71

ABSTRACT

Background: Ghrelin, an orexigenic appetite stimulating peptide, in addition to promoting energy balance, contributes to the rewarding effects associated with overeating. It also seems to play an important role in the rewarding/reinforcing effects of alcohol and addictive stimulants. The involvement of the ghrelin mechanisms in cannabinoid and opioid misuse and addiction have been under-researched.

Aims: The principal aim of this research thesis was to investigate whether the pretreatment with the growth hormone secretagogue receptor 1A (GHS-R1A) antagonist (JMV2959) could reduce the cannabinoid receptor type 1 (CB1R) agonist WIN55,212-2–induced dopamine efflux in the nucleus accumbens shell (NACSh), which is considered a crucial trigger impulse of the addiction process. Also, test whether JMV2959 can influence the WIN55,212-2 and fentanyl-induced effects on the endocannabinoids N-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) and the γ-Aminobutyric(GABA) content in the NACSh, and in extend, to specify the involvement of GHS-R1A located in the ventral tegmental area (VTA) and the NACSh in the observed accumbens changes. Furthermore, to test whether the JMV2959 pretreatment could reduce the cannabinoid [tetrahydrocannabinol (THC) and WIN55,212-2] induced behavioural stimulation.

Methods: In vivo microdialysis was used to determine the changes of dopamine and its metabolites in the NACSh in rats following the synthetic aminoalklylindol cannabinoid WIN55,212-2 administration into the posterior VTA with and without the ghrelin antagonist pretreatment (JMV2959, 3 mg/kg i.p. 20 min before WIN55,212-2 administration) and also to determine the WIN55,212-2 and fentanyl effects on anandamide, 2-AG and GABA accumbens content. The behavioural changes in rats were observed on the fully automated behaviour recognition system (LABORAS) apparatus which monitored the effects of JMV2959 on the THC and WIN55,212-2.

Findings: The WIN55,212-2 administration induced significant accumbens dopamine release, which was significantly reduced by the 3 mg/kg i.p. JMV2959 pretreatment. Simultaneously, the cannabinoid-increased accumbens dopamine metabolic turnover was significantly augmented by the JMV2959 pretreatment. The intracerebral WIN55,212-2 administration also increased the endocannabinoid anandamide and the 2-AG extracellular levels in the NACSh, which was moderately but significantly attenuated by the JMV2959 pretreatment. Moreover, the cannabinoid-induced decrease in accumbens GABA levels was reversed by the JMV2959 pretreatment. The pretreatment with JMV2959 (administered systemically, into the NACSh or

VTA) reversed the dose dependent fentanyl-induced anandamide increase in the NACSh, resulting in a significant anandamide decrease and intensified the fentanyl-induced decrease in accumbens 2-AG levels. The behavioural study in the LABORAS apparatus showed that JMV2959 pretreatment significantly and dose-dependently reduced the systemic THC/WIN55,212-2-induced behavioural stimulation in rats.

Conclusions: The overall findings on this research documented the significant contribution of ghrelin / GHS-R1A in the cannabinoid's and opioid's pro-addictive effects and supported further research into ghrelin antagonism as a potential new therapeutic direction in these addictions.

Key words

cannabis – THC - WIN55,212-2 - opioids – fentanyl - ghrelin – GHS-R1A - JMV2959 – NACSh - VTA

ABSTRAKT

Úvod: Orexigenní peptid stimulujícího chuť k jídlu, vedle podpory energetické rovnováhy také navozuje odměňující účinky spojené s přejídáním. Mimo jiné se ukazuje, že ghrelin hraje důležitou roli v odměňujících/posilujících účincích alkoholu a návykových stimulantů. Nicméně zapojení ghrelinového mechanismu ve zneužívání a závislosti na opioidech a kanabinoidech není dosud dostatečně prozkoumáno.

Cíle: Hlavním cílem této výzkumné práce bylo zjistit, zda premedikace antagonistou GHS-R1A (JMV2959) může snížit vyplavení dopaminu z nucleus accumbens shell (NACSh), indukovaný agonistou CB1R (kanabinoidní receptor typu 1) WIN55,212-2, což je považováno za klíčový impuls procesu závislosti. Bylo také otestováno, zda JMV2959 může ovlivnit účinky WIN55,212-2 a fentanylu na endokanabinoidy N-arachidonoylethanolamin (anandamid) a 2-arachidonoylglycerol (2-AG) a obsah kyseliny gama-aminomáselné (GABA) v NACSh a dále specifikovat zapojení GHS-R1A umístěného ve ventrální tegmentální oblasti (VTA) a NACSh do pozorovaných změn v NAc. Dále bylo testováno, zda premedikace JMV2959 může snížit kanabinoidy (tetrahydrokanabinol/THC a WIN55,212-2) indukovanou behaviorální stimulaci.

Metody: In vivo mikrodialýza byla použita ke stanovení změn dopaminu a jeho metabolitů v NACSh u potkanů po podání syntetického aminoalklylindolového kanabinoidu WIN55,212-2 do zadní VTA s premedikací antagonisty ghrelinu (JMV2959, 3 mg/kg i.p., 20 min před WIN55,212-2) a bez ní. Stejná metodika byla použita za účelem stanovení účinků WIN55,212-2 a fentanylu na obsah anandamid, 2-AG a GABA v NAc. K pozorování účinků JMV2959 na změnu chování potkanů způsobené THC a WIN55,212-2 bylo použito automatizované zařízení pro rozpoznávání chování (LABORAS).

Zjištění: Podání WIN55,212-2 vyvolalo významné uvolnění dopaminu v NAc, které bylo významně sníženo premedikací JMV2959 v dávce 3 mg/kg i.p. Současně byl metabolický zvrat dopaminu v NAc, který byl zvýšen kanabinoidy, významně zvýšen premedikací JMV2959. Intracerebrální podání WIN55,212-2 zvýšilo anandamid a extracelulární hladiny 2-AG v NACSh, což bylo mírně, ale významně sníženo premedikací JMV2959. Mimo to byl kanabinoidy indukovaný pokles hladin GABA v NAc zvrácen premedikací JMV2959. Premedikace JMV2959 (podávaný systémově i do NACSh nebo VTA) zvrátila na dávce závislé zvýšení anandamid vyvolané fentanylem v NACSh, což vedlo k významnému poklesu anandamid a zesílilo fentanylem vyvolaný pokles hladin 2-AG v NAc. Behaviorální studie pomocí LABORAS ukázala, že premedikace JMV2959 významně a v závislosti na dávce snížila systémovou stimulaci chování vyvolanou THC/WIN55,212-2.

Závěry: Výsledky ukázaly významný vliv ghrelinu/GHS-R1A na závislostních účincích opioidů a kanabinoidů a podporují další výzkum ghrelinového antagonismu jako potenciálního nového terapeutického směru u těchto závislostí.

Klíčová slova

konopí - THC - WIN55,212-2 - opioidy - fentanyl - ghrelin - GHS-R1A - JMV2959 - NACSh - VTA

I. INTRODUCTION

Addiction is a mental and physical condition, chronic mental disease, characterized by the loss of control of the individual over a certain type of behaviour. It is a chronic, relapsing disease/disorder with complex negative effects on individuals and in extend, on society. Substance dependence involves the urge to use the substance/drug repeatedly (constantly or intermittently) to achieve the expected psychological effects (excessive satisfaction/well-being/reward) or to prevent the occurrence of unpleasant conditions that arise in the absence of the substance/drug in the body (withdrawal symptoms); the substance/drug use occur despite clear evidence of harmful consequences (NIDA 2018). Dopamine is a key component in drug reward (Di Chiara and Imperato 1988; Koob and Bloom 1988). The acute intake of all substances that are known to cause addiction increase the extracellular dopamine concentration in the nucleus accumbens (NAc) (Weiss et al. 1992). All addictive drugs significantly activate dopaminergic transmission in the NACSh, which is considered an important initial impulse of the addiction processes, linked with reward, reinforcement, and disruption of salience attribution (Hyman, Malenka, and Nestler 2006; Koob and Volkow 2010; Nestler 2005a). The addictive drug-induced dopamine efflux in the NAc triggers consequent conditioning processes in the brain which form associations of drug reward with particular conditions/cues and reinforce the drug-seeking behaviour (Adinoff 2004).

Cannabis/cannabinoids are the most used and widely available illicit drug in Europe. However, an effective treatment for cannabinoid-associated use disorders and dependence is still lacking. GHS-R1A antagonism was recently suggested as a promising mechanism for drug dependence treatment. Nevertheless, the role of GHS-R1A and its endogenous ligand ghrelin in cannabinoid abuse remains unclear. In drugs of abuse, the cannabinoid-induced behavioural stimulation is suggested to be a sign of dopaminergic nigrostriatal pathway activation that contributes to drug dependence (Polissidis et al. 2013; Koob and Volkow 2016)

This dissertation research thesis summarises the important findings of a neurobehavioral research of the mesolimbic ghrelin signalling involvement in the cannabinoids (e.g., THC and WIN55,212-2) pro-addictive effects. Furthermore, the endocannabinoid changes induced by opioids (fentanyl) in the rat NAc, with or without the GHS-R1A antagonist pretreatment, were rigorously described as well. Besides other findings, these achieved innovative results, which were also published in international journals with high IF (average IF=5.924), demonstrated that the ghrelin/GHS-R1A system importantly participates in the accumbens-neurotransmitter and exhibit behavioural changes that are associated with the

rewarding/reinforcing effects of cannabinoids as well as opioids. This encourages further research of the GHS-R1A antagonism as a potential novel approach to cannabinoid and opioid addiction treatment and the possibility to decrease the craving and in extent, relapse.

The theoretical part of this thesis documents a reflection of the problematic around the use of addictive substances around the globe and suggests how it affects the human brain in a physiological and molecular point of view. The practical part of thesis summarizes the most important experimental findings of the ghrelin/GHS-R1A-addiction relationships, which were obtained during this rigorous investigation.

II. THEORITICAL PART

1. Introduction to the problematic of addictive substances and addiction

1.1. The public health significance of the problematic use of psychoactive drugs

Abuse or overuse of psychoactive drugs affects a substantial part of the population and is the cause of health disorders and causes negative social impacts on users and their surroundings. The overarching definition of drug abuse includes any use of a drug that is shown to be problematic. For example, obtained with or without a prescription, but outside of accepted medical procedures or instructions, for recreational purposes (due to the intoxicating effects of treatment) or as part of self-medication, where the risks and problems associated with the use outweigh its potential benefits. Specific forms of drug abuse could be their use as a source or as a precursor to create other drugs or the administration of a drug to another person to involuntarily drug them. In principle, the two main groups of people that abuse psychoactive drugs are those suffering from various health problems whose primary motive is the treatment of those problems, who lose control over the use of these drugs and people that are addicted to drugs, including alcohol and/or illicit drugs, who use psychoactive drugs as a substitute or supplement for other drugs or to alleviate withdrawal symptoms (Chomynová and Mravčík 2021). The impact of the long-term overuse of psychoactive drugs is the emergence of addiction. Addiction is often of iatrogenic origin in the inappropriate indication and treatment of various problems, when the risk of addiction is insufficiently controlled by the doctor (Peltz and Sudhof 2018).

The source of psychoactive drugs is primarily available from the official health system; people who abuse drugs by obtaining them from doctors of various specialties (so-called doctor shopping). If it is impossible to obtain psychoactive drugs from a standard medical source, the drugs are obtained from family or acquaintances, on the internet, or on the illegal drug market. A specific way of obtaining psychoactive drugs is to falsify prescriptions or obtain used drugs from medical or household waste. The current psychoactive substance market consists of many substances, with new compounds being introduced continually, at a rate of more than 50 new drugs per year since 2012 (EMCDDA 2020, 2021). Psychoactive drugs are often overused in the context of self-medication for pain, anxiety, etc., and users are often unaware of the addictive potential of the drugs they use.

1.2. Regulation, policies, and the impact in the field of psychoactive drug misuse/overuse

The basic legislative framework involving the abuse of psychoactive drugs is provided by the Act No. 378/2007 Coll. on medicinal products. According to this Act, misuse of medicinal products means intentional overuse of medicinal products or intentional use of medicinal products in a manner that is contrary to the intended purpose of use, even after their further processing, accompanied by harmful effects on the organism, including harmful effects on its psyche.

The policy involving the overuse of psychoactive drugs in the Czech Republic is part of the policy in the field of addiction, which since 2014 has integrated the topics of legal and illegal addictive substances and non-substance addictions. The government is responsible for the creation and its implementation. The Government Council for Drug Policy Coordination (GCDPC) is the government's coordinating and advisory body on addiction issues. The area of health protection against the harmful effects of addictive substances, including psychoactive drugs, as well as drug supervision falls under the responsibility of the Ministry of Health including the State Institute for Drug Control (SÚKL), which is also responsible for the drug misuse. SÚKL coordinates the pharmacovigilance system of medicinal products for human use, e.g., the system of supervision over the safety of medicinal products after their registration, which also includes the reporting of inappropriate use or misuse of drugs. The basic measure against the misuse of medicinal products is prescription or dispensing (either with or without a prescription) with restrictions. The Restricted Register of Medicinal Products is a practical tool that ensures that the prescription and dispensing of a medicinal product is limited to the appropriate amount and time. Another measure against misuse of medicines is the dispensing of medicines in the pharmacy. In case there is a doubt that the person dispensing the medicine to is not able to guarantee the correct use of the medicine or can misuse it, it might not be dispensed by the pharmacist (EMCDDA 2014).

The number of people with problematic consumption of psychoactive drugs, with addiction to psychoactive drugs, can be estimated at up to 1 million people. In 2020, a total of 96 fatal overdoses with illegal drugs, volatile substances, and psychoactive drugs (84 in 2019) were reported in the National Register of Autopsies and Toxicological Examinations (NRPATV) performed at the Department of Forensic Medicine, of which 38 were fatal overdoses from psychoactive drugs. It is documented that in opioids, a total of 28 cases in 2020 included overdoses with opioid analgesics such as fentanyl, codeine, dihydrocodeine, hydromorphone, oxycodone. In addition, 150 deaths under the influence of illegal drugs and

psychoactive drugs from causes other than overdose were identified in the NRPATV in 2020 (133 in 2010), most of them as in the past due to illness, accidents and suicides. Approximately 150-200 people are hospitalized annually for injuries under the influence of psychoactive drugs (UZIS 2020).

1.3. Use of psychoactive drugs among children, adolescents, and the adult population

Monitoring the extent of psychoactive drug use among children, adolescents and adults is currently not comprehensively conceptualized. Different studies look at differently defined categories of psychoactive drugs. In the European School Survey Project on Alcohol and Other Drugs study (EMCDDA 2019), a total of 5.8% of 16-year-olds have used psychoactive drugs in combination with alcohol (in order to get in the mood) at some point in their lives. A total of 14.4% of students reported having drug abuse at least once in their life, while cannabinoids were the most frequently used illegal substances (e.g., medicines used without a prescription) of which 5.2% reported drug abuse repeatedly (e.g., 3 or more times). The use of prescription and over-the-counter drugs, including opioids, is more often reported by girls and students at schools (NMS 2021).

Czech students do not deviate from the European average in the use of prescription drugs in life, but more often they report the use of medicines for pain (in order to get in the mood) and the use of alcohol together with medication. 10.3% of 11–15-year-olds and 17.8% of 15–19-year-olds had experience with the use of drugs for no reason (UPOL study) (NMS 2021). A total of 15.8% of adults have used psychoactive drugs (prescription and over the counter) in the last 12 months (National Research 2020)(Chomynová and Mravčík 2021). The use of psychoactive drugs is approximately twice as common among women and increases with the age of the respondents. The use of opioid analgesics predominates in the younger age categories. Approximately 12% of users of psychoactive drugs obtained them other than on prescription (from acquaintances, in a pharmacy without a prescription or over the Internet) (NMS 2021). When extrapolated to the entire population of the Czech Republic, aged 15+ of a total 1.35 million people showed signs of problematic use with psychoactive drugs of which 430 thousand were men and 900 thousand were women (National Research 2020) (Chomynová and Mravčík 2021).

1.4. General basal principles of mechanisms of drug addiction

Addiction is a mental and physical condition, chronic mental disease, characterized by the loss of control of the individual over a certain type of behaviour. Addiction to substances is classified by the World Health Organization (WHO) within the International Classification of Diseases (ICD/ICD-10), as F10-F19 - Mental and behavioural disorders caused by psychoactive substances. Dopamine is a key component in drug reward (Di Chiara and Imperato 1988; Koob and Bloom 1988). The acute intake of all substances that are known to cause addiction increase the extracellular dopamine concentration in the NAc (Weiss et al. 1992). All addictive drugs markedly activate dopaminergic transmission in the NACSh, which is considered an important initial impulse of the addiction processes, linked with reward, reinforcement and disruption of salience attribution (Hyman, Malenka, and Nestler 2006; Koob and Volkow 2010) (Nestler 2005b). The addictive drug-induced dopamine efflux in the NAc triggers consequent conditioning processes in the brain which form associations of drug reward with the particular conditions/cues and reinforce the drug-seeking behaviour (Adinoff 2004).

Mesolimbic dopaminergic pathways between the VTA and NAc and other junctions with medial prefrontal cortex (mPFC) and possibly other structures are components of the brain reward system (Di Chiara and Imperato 1988; Koob and Bloom 1988). These structures are essential for feeling satisfaction, reward, reinforcement, and they play a role in substance abuse. In addition to dopamine, a number of other mediator systems are involved in reward and learning mechanisms (e.g., endocannabinoids, γ-Aminobutyric acid, glutamate, serotonin, acetylcholine, endogenous opioids and others) (Koob and Volkow 2010, 2016) (Volkow et al. 2011) (Hyman, Malenka, and Nestler 2006).

Endogenous cannabinoids and endogenous opioids also contribute to the reinforcing effects of addictive drugs through inhibition of negative affective states or modulation of hedonic responses (Mitchell, Berridge, and Mahler 2018). The significant involvement of these non-dopaminergic approaches on the reward processing are not extensively studied in comparison to dopamine. The endogenous cannabinoid system (ECS) modulates other neurotransmitter systems including γ-Aminobutyric acid, glutamate, and dopamine in addiction key areas along the mesolimbic circuitry (Lopez-Moreno et al. 2008; Wang and Lupica 2014). The ECS consists of endogenous cannabinoids (e.g., anandamide and 2-arachidonoylglycerol) and their associated receptors (CB1R and CB2R) (Volkow, Wise, and Baler 2017). The endogenous opioid system modulates the mesolimbic dopaminergic system (Fields and Margolis 2015; Trigo et al. 2010), and is involved in integrating reward-related

information to guide decision-making and execution of goal-directed behaviours (Laurent, Morse, and Balleine 2015). It consists of endogenous opioid peptides including the β-endorphins, enkephalins, and dynorphins peptides, which signal preferentially through mu, delta, and kappa opioid receptors, respectively. Mu receptors are responsible for the rewarding effects of opioids and for analgesia, delta are implicated in analgesia and anxiolysis, while kappa are implicated in the dysphorigenic responses associated with addiction (Le Merrer et al. 2009) and in stress-induced relapse (Graziane et al. 2013). Mu in the VTA and NAc, as well as in the basolateral amygdala, are implicated in opioids' rewarding effects (Figure 1).

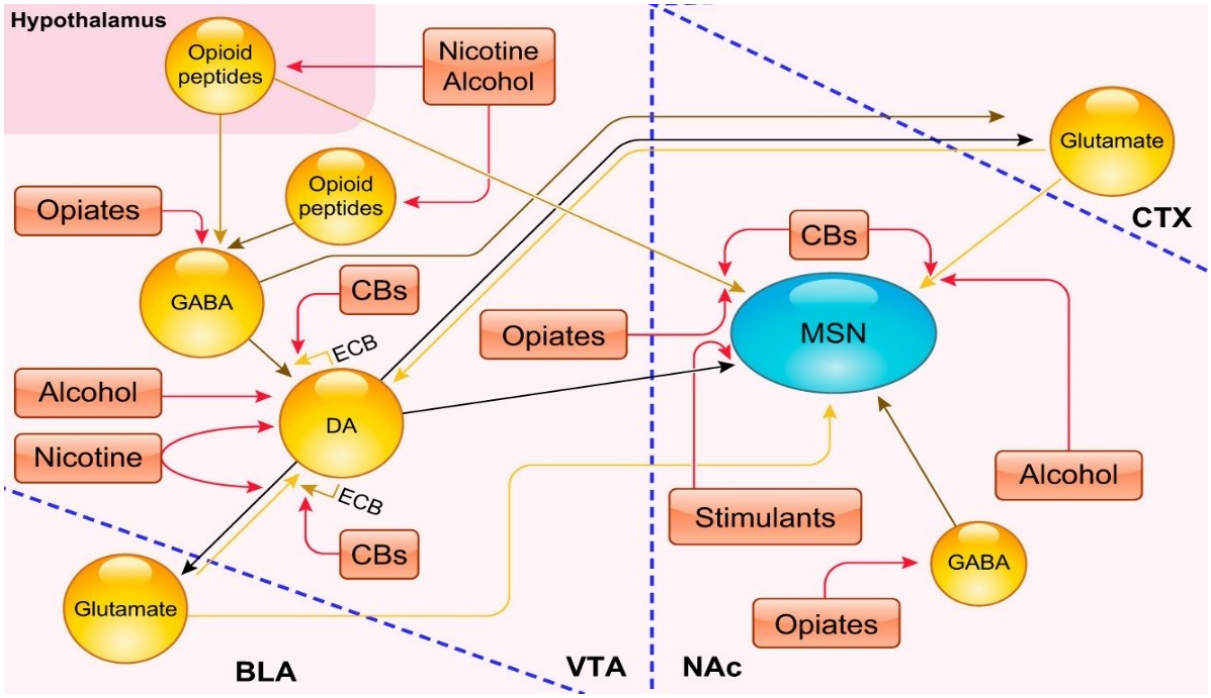

Figure 1 Key target sites for various drugs of abuse across the reward circuity (Volkow, Michaelides, and Baler 2019) (DO-Dopamine, CBs-Cannabinoids, MSN-Medial Septal Nucleus, CTX-Cortex, BLA-Basolateral Amygdala)

2. Cannabinoids

2.1. Current situation – spectrum of use and abuse cannabinoids in the Czech Republic and Europe

Cannabis is the most widely illicit drug available in Europe, where it can be produced domestically but also imported. It is also the most used illicit drug in Europe (EMCDDA 2020). According to the annual reports of the National Monitoring Centre for Drugs and Drug Addiction in the Czech Republic, cannabis is the most frequently used drug after alcohol and tobacco (NMCDA 2019).

Dried female flowers of Cannabis sativa L. or Cannabis indica L. hemp are used for medicinal and recreational purposes with the active ingredients Δ-9-trans-tetrahydrocannabinol (THC) and cannabidiol (CBD). The active substance content can range from 0.3% to 21% for THC and from 0.1% to 19% for CBD. Cannabis is regulated mainly by the Act on Addictive Substances (167/1998 Coll.) and the Decree on the Determination of Conditions for Prescribing, Preparation, Distribution, Dispensing and Use of Individually Prepared Medicinal Products Containing Cannabis for Medical Use (236/2015 Coll.). In the Czech Republic, patients can legally obtain cannabis: a) imported from abroad: the import of cannabis from abroad is permitted by the Ministry of Health of the Czech Republic - Inspectorate of Narcotic and Psychotropic Substances. This cannabis must meet all the requirements in terms of quality and content of active substances, which are based on the applicable legislation and b) Grown in the Czech Republic: this area falls under the State Institute for Drug Control (or the State Agency for Cannabis for Medical Use), which grants licenses for the cultivation of cannabis and provides other related activities (supervision of its cultivation, distribution to pharmacies, etc.) (SUKL 2022).

An estimation of 90.2 million adult population in the European Union (aged 15-64), used cannabis at least once in their lifetime. Around 18.0 million of the younger population (aged 15-34) report using cannabis in the year 2019, with males being typically twice as likely to report use than females (EMCDDA 2020). According to National Survey on Substance Use 2016, the lifetime prevalence in the general population aged 15–64 years reached 26.6% in 2016 (34.6% among males and 19.1% among females), 9.5% of adults reported cannabis use in the last 12 months and 5.5% in the last 30 days (NMCDA 2019).

Problems related to cannabis use are more widespread and frequent in Europe, due to the predominant riskier type of cannabis with a high tetrahydrocannabinol content ("skunk"),

the use of synthetic cannabinoids and the increasing use of cannabinoids by adolescents. Synthetic cannabinoids are a broad group of substances with similar effects to THC, however, they can be much more toxic compared to natural cannabis drugs, some of which are highly toxic. They are most often distributed in the form of a plant mixture or a substance imitating hashish. They are offered under an abbreviation based on the chemical name of the substance or under various commercial names, such as 'Spice Gold', 'Spice Silver', 'Spice Diamond', 'K2', 'Bliss', 'Black Mamba', 'Bombay Blue', 'Blaze'. Synthetic cannabinoids include, for example, JWH-018, APINACA (AKB-48), MDMB-CHMICA, UR-144, AM-2201, ADB-CHMINACA, 4F-MDMB-BINACA (NMCDA 2019).

Social, medical, and legal acceptance of cannabis has grown dramatically in Europe during the past 15 years and the cannabis use for medical and recreational purposes was also increased, yet, the proportion of the public that perceives important harms from cannabis use was decreased (Hasin 2018). In Europe, including Czech Republic, prevails a supply of high-potent/tetrahydrocannabinol strains of cannabis, linked with increased risks of cannabis use disorder (CUD), which includes uncontrolled drug-seeking and withdrawal symptoms, psychotic disorders, dysphoric mood, disturbed sleep, gastrointestinal symptoms, eating disorders etc. It is recently estimated that about 9% of chronic cannabis users display characteristic symptoms of dependence according to WHO/DSM-IV criteria (Zehra et al. 2018). Currently, no specific pharmacotherapies for CUD and dependence are approved, thus cannabinoid addiction treatment remains exclusively symptomatic, unsatisfactory, with low relapse prevention (Kondo et al. 2020). Therefore, new effective treatment strategies are constantly being researched.

2.2. Neurobiological mechanisms of action and risks

The CB1 receptor is entirely responsible for the psychoactive effects of cannabis (Brumback et al. 2016). Cannabis enables/enhances a subjective sense of well-being by stimulating the endocannabinoid system, which plays a key role in modulating the response to stress, reward, and their interactions. Repeated/prolonged activation of the endocannabinoid system by cannabis can trigger neuroadaptations that may impair the sensitivity to stress and reward. In vulnerable individuals, it can lead to addiction and other adverse consequences (Volkow, Wise, and Baler 2017). Endocannabinoid system is characterised by great complexity with central and peripheral effects, several binding sites etc. The most studied endocannabinoids [N-arachidonoylethanolamine/anandamide and 2-arachydonoylglycerol (2-

AG)] are synthetized on demand from the cell phospholipids and through cannabinoid CB1 receptors which retrogradely regulate synaptic neurotransmission [e.g., glutamate, γ-aminobutyric acid (GABA) etc.] overall the brain reward circuitry, controlling both excitatory and inhibitory inputs (Parsons and Hurd 2015; Scherma et al. 2019). The CB1Rs are the most abundant G protein-coupled receptors expressed in the adult brain with particular dense expression in regions involved in reward, addiction, and cognitive functions, including VTA, NAc, substantia nigra etc. (Parsons and Hurd 2015). The CB1Rs are located on various presynaptic axons/inputs in the VTA and the NAc (Parsons and Hurd 2015; Herkenham 1991; Matsuda et al. 1990).

The CB2Rs have been recently found in the midbrain dopamine neuron regions. It seems that the CB1Rs activation produces reinforcing effects, whereas the CB2Rs aversive (Spiller et al. 2019). THC is considered as a partial CB1R and CB2R agonist, shows dose-dependent biphasic/dual effects, with reinforcing low doses (Spiller et al. 2019; Zehra et al. 2018). CBD has variable effects, including indirect agonism (peripheral effects) or antagonism (central effects) of CB1Rs, adenosine-uptake inhibition, allosteric modulation of dopamine D2, GABA-A, glycine, 5-HT1A, μ- and δ- opioid receptors etc. (McPartland et al. 2015; Pertwee 2008).

THC, anandamide, and 2-AG via the mesolimbic CB1Rs increase dopamine concentration in the NACSh followed by further reinforcement, conditioning, and salience alteration processing (Lupica, Riegel, and Hoffman 2004; Panlilio et al. 2013; Parsons and Hurd 2015; Wijayendran, O'Neill, and Bhattacharyya 2018; Zehra et al. 2018). Prolonged/chronic cannabis/THC use leads to downregulation of CB1Rs (but not dopamine D2/D3 receptors), tolerance, amotivational states, dysregulation of stress responsivity, withdrawal symptoms, sensitization of mesocorticolimbic-reward system to cannabis cues and to THC after period of abstinence, increase glutamate signalling during relapses etc. Thus, development of cannabis addiction largely parallels addiction to other drugs of abuse(Hwang and Lupica 2020; Volkow, Hampson, and Baler 2017; Zehra et al. 2018).

2.3. Treatment approaches and limitations in cannabis/cannabinoid addiction

In the last few years, clinical and epidemiological studies have shown that that addiction can be developed in connection with cannabis use (Lang, Engelander, and Brooke 2000). Withdrawal symptoms they are much lighter for cannabis users than for other addicts, so there is no need for pharmaceutical intervention, with a few exceptions (Dvoracek, Cook, and

Klepser 2010). The purpose of detoxification is to prevent drug use and reduce the intensity of withdrawal symptoms syndrome and the risks of use. Treatment is divided into short-term, medium-term and long-term. Short-term treatment lasts usually within three months. Medium-term treatment lasts from three to six months, long-term treatment lasts from half a year to one year, exceptionally up to two years, and is ongoing in the environment of therapeutic communities (Kalina 2008). Other treatment options are therapeutic communities, aftercare programs or self-help activities.

Beside other roles, the endocannabinoid system importantly participates in metabolism, peripheral and central homeostatic as well as non-homeostatic/hedonic food intake, and drug abuse, similarly to the ghrelin system. Particularly, cannabinoid CB1R and ghrelin/GHS-R1A receptors are distributed within overlapping brain regions crucial for feeding (hypothalamus) and reward/reinforcement (ventral tegmental area/VTA, nucleus accumbens/NAc) (Zehra et al. 2018; Maldonado, Valverde, and Berrendero 2006; Manzanares et al. 2018).

The role of ghrelin and its receptor in the cannabinoid pro-addictive effects are currently investigated place in the animal facilities at the Department of Pharmacology of the Third Faculty of Medicine Charles University which for several years is engaged in the experimental research of the ghrelin role and its binding site, the growth's hormone secretagogue receptor type A1 (GHS-R1A) in the pro-addictive effects of selected drugs of abuse, which is part of part of this research thesis (Charalambous et al. 2021; Sustkova-Fiserova et al. 2017). The ghrelin's involvement and the apparent relationship mechanism in the drug addiction is still complicated because of the probable relapse limitation, thus, more rigorous research was appropriate in order to elucidate the cannabis-seeking behaviour. Therefore, to clarify the involved mechanisms and relationships among the cannabis and ghrelin systems within the NAc and/or the VTA, this research thesis focuses on the role of ghrelin in the cannabinoids pro-addictive effects and the significant interaction between ghrelin and endocannabinoids in the cannabis-induced changes in NAc and/or the VTA, as it was never researched before (Charalambous et al. 2021).

3. Opioids

3.1. Current situation – spectrum of abused opioids in the Czech Republic and Europe

In adults, the opioid dependence prevalence differs considerably between the European countries. The use of heroin remains a main concern in several European countries and the use of synthetic opioids is growing alarmingly (EMCDDA 2020). Approximately 20 drug users in the Czech Republic die each year from opioid overdoses (heroin, fentanyl, morphine, codeine, and other opium alkaloids). Preparations used for substitution treatment of opioid dependence escape from standard medical sources and are traded on the illegal market (in the Czech Republic it mainly concerns preparations containing buprenorphine). The likelihood of non-standard acquisition of substitution drugs increases addiction, tighter dispensation control, lower availability of drugs, including affordability and underdosing while in treatment (non-compliance) (NMCDA 2019).

The consumption of opioid analgesics (e.g., morphine, hydromorphone, oxycodone dihydrocodeine, fentanyl) is growing for a long time (from 19 million in 2004 to 52 million daily doses in 2020). Currently, a very widespread alternative source of obtaining psychoactive medicines is the internet, and their cost can be significantly higher compared to a standard source in a pharmacy. The availability of psychoactive medicines on the internet, especially opioids, is higher even on online platforms in the Czech language. Synthetic opioids, such as fentanyl derivatives, are also present as new synthetic drugs. 67 new synthetic opioids are monitored in the EU. Medicines are also available on the illegal market along with other illegal drugs (NMCDA 2019).

3.2. Neurobiological mechanisms of action and risks

Opioids are the most potent analgesics. Their effects but also their most adverse effects are mediated by the specific agonism on opioid receptors. The three major opioid receptors are mu (μ), kappa (κ) and delta (δ). Recently, the morphine-6-glucuronide (M6G) and ORL1 (opioid-receptor-like) were identified as opioid binding sites as well (Schug, Garrett, and Gillespie 2003).

The prototype substance of opioids is morphine, and its effect is due to binding predominantly at the μ receptor. The clinically relevant difference in effects among the opioid analgesics depend on their agonism, partial agonism or antagonism at the various opioid

receptors. Fentanyl is considered as a μ-receptor-selective agonist, 100-fold more potent than morphine, acting on both VTA as well as NAc μ-receptors (Fields and Margolis 2015; Pasternak and Pan 2013; Yoshida et al. 1999).The side effects are usually respiratory depression, which is usually the cause of death in accidental or suicidal overdose (White and Irvine 1999a, 1999b), nausea and vomiting, the most common toxic effect of opioids, serotonin syndrome once there is interaction with monoamine oxidase inhibitors (MAOI) or selective serotonin re-uptake inhibitors (SSRI), muscle rigidity (Bowdle 1998), myoclonus and seizures caused by the excitation of the central nervous system, hallucinations, delirium, and hyperalgesia (Daeninck and Bruera 1999), pruritus during histamine release causing itching, hypotention, bradycardia, myuocardial depression and pulmonary oedema (Bruera and Miller 1989). Opioids also cause constipation, dysuria and urinary retention (Schug, Garrett, and Gillespie 2003), delayed gastric emptying, and several biliary effects (Bowdle 1998).

Physical dependence is typical after a longer period of use (abstinence syndrome in case of abrupt withdrawal or administration of an antidote). Opioids are usually not able to alleviate severe pain completely, their effects on mood, however, they lead to suppression of unpleasant feelings associated with pain. Morphine is available in several dosage forms. For parenteral administration there is no difference between intramuscular, subcutaneous and intravenous administration. The plasma half-life is 2-4 hours and steady-state plasma levels are reached in about 4-5 half-lives (e.g., within 24 hours) (Brunk and Delle 1974).

Another specific group are users of illicit opioids for whom it is common polyvalent use of multiple substances, including opioids, used in substitution therapy, either obtained legally or from an illegal market. They use opioid analgesics because of their intoxicating effects, alleviation of withdrawal symptoms, or in the unavailability of legal substitution treatment. Drugs used in opioid substitution treatment (methadone, buprenorphine), which are leaking from standard medical sources and traded on illegal market. The likelihood of non-standard acquisition of substitution drugs increases addiction, tighter dispensing controls, lower availability of medicines, including affordability and underdosing in treatment (Mravcik et al. 2018).

Extensive knowledge of the complex neurobiological mechanisms of drug effects and their dependence mechanisms is essential for the development of effective treatment strategies, including the discovery of new drugs. Extensive knowledge of the complex neurobiological mechanisms of drug effects and their dependence mechanisms is essential for the development of effective treatment strategies, including the discovery of new drugs. Endocannabinoids, such as anandamide and 2-AG, have been reported to play an important role in the reward/boost

effects of opioids. The CB1 cannabinoid receptor antagonist (SR141716A, Rimonabant) reduced opioid intravenous self-administration (IVSA) (Caille and Parsons 2003; De Vries and Shippenberg 2002; Navarro et al. 2001; Solinas et al. 2003) and opioid-induced site preference (CPP) (Chaperon et al. 1998; Navarro et al. 2001; Singh et al. 2004). A CB1 agonist (9Δ-tetrahydrocannabinol) increased the rewarding effects of heroin during IVSA (Solinas et al. 2005). The rewarding/boosting effects of anandamide have been demonstrated in IVSA in monkeys (Justinova et al. 2005).

3.3. Treatment approaches and limitations in opioid addiction

Early problem identification and early intervention are important for psychoactive drug overuse. Short interventions aimed at gradually reducing the dose to discontinuing the use of opioids are an effective method in indicated patients (opioid tapering). However, in the Czech Republic, the rate of implementation of short interventions is generally relatively low (even for alcohol or tobacco), although, it is mandatory by law for all health professionals. If addiction develops, safe detoxification is performed. Substitution treatment is a specific treatment modality for opioid addicts. It is not only for addicts with illegal opioids but is also indicated for patients with chronic (non-tumor) pain treated with opioid analgesics who have developed addiction (NMCDA 2019).

The offer of treatment and counselling interventions provided via the internet and with the use of new technologies is growing, and their development has been further catalysed by COVID-19 pandemic. One of the most effective interventions when an overdose has occurred is the administration of an opioid antagonist, naloxone, which displaces the acting opioid from the receptors, thereby interrupting the attenuation of the respiratory centre. Naloxone distribution programs provide administration of naloxone to individuals (often other users or family members) who witness an overdose. A pilot naloxone project has just started in the Czech Republic. The implementation of the pilot project to ensure the availability of naloxone to drug users in the Czech Republic was discussed and approved by GCDPC in April and November 2020 (NMCDA 2019).

Naloxone is an opioid antagonist which when administered to an opioid-intoxicated person may avert life-threatening intoxications and fatal outcomes. The project consists of short-term training of drug users (or other people, such as loved ones or field workers) who carry naloxone with them, and of its administration in the event of an overdose. The project in the Czech Republic uses Nyxoid® in the form of a 1.8 mg naloxone nasal spray, which is

registered in the EU market. Naloxone distribution programs, so-called Take-Home Naloxone (THN) programs are available in 11 other EU countries and Norway. The naloxone program is one of the basic interventions to reduce the health risks of drug use. Its administration is simple, it is associated with minimal risks and side effects, and the improvement in opioid overdose is almost immediate. Secondly, training and information on the topic introduces the topic of overdose prevention to the population of drug users and their loved ones and draws attention to the possible risks of drug use (NMCDA 2019).

In a previous study in the animal facilities at the Department of Pharmacology of the Third Faculty of Medicine Charles University, it was documented a significant interaction between ghrelin and endocannabinoids in the morphine-induced changes in the NACSh. (Jerabek et al. 2017). Conclusively, this suggests the significant involvement of central ghrelin system in changes induced by morphine in the mesolimbic dopaminergic system, changes which are associated with processing of neural reward. The ghrelin's involvement and the apparent relationship mechanism in the drug addiction is still complicated because of the probable relapse limitation, thus, more rigorous research was appropriate in order to elucidate the opioid-seeking behaviour. Therefore, to clarify the involved mechanisms and relationships among the opioid and ghrelin systems within the NACSh and/or the VTA, this research thesis focuses on the role of ghrelin in the selective μ opioid receptor pro-addictive effects and the significant interaction between ghrelin and endocannabinoids in the fentanyl-induced changes in NACSh and/or the VTA, as it was never researched before (Sustkova-Fiserova et al. 2017).

4. Ghrelin and GHS-R1A - their role in mechanisms of addiction

4.1. Current knowledge of complex ghrelin/GHA-R1A effects and potential use in addiction therapy

Ghrelin is an endogenous peptide that was discovered in 1999 by Kojima (Kojima et al. 1999). Its discovery was preceded by research into synthetic substances that affect growth hormone secretagogues (GHS). Bowers conducted this research at the 1970s (Bowers et al. 1980). The GHS-specific receptor, GHS-R1A, is a metabotropic type of G protein-coupled receptor. Its activation leads to the release of calcium from the endoplasmic reticulum into the cytoplasm of the cell and subsequently to membrane depolarization (Jensovsky, Lebl, and Christiansen 2000). Due to the existence of such a specific receptor, it was assumed that this receptor would also have some natural endogenous ligand. Extensive research based on monitoring changes in intracellular calcium levels after the addition of individual tissue types has shown that the greatest changes are observed after the addition of extracted tissue from the stomach (Kojima et al. 1999). Ghrelin is a 28 amino acid peptide (Bednarek et al. 2000). Although human and rat ghrelin differ by two amino acids, in both cases the peptide is characterized by an octanoyl ester group on serine at position 3. The acylated form of the peptide is essential for most ghrelin effects (acylated / active ghrelin) (Kojima et al. 1999).

The presence of ghrelin was demonstrated in various central and peripheral structures of human and animal organisms, including rats. Ghrelin secretion is controlled by local and central stimuli, one example of stimuli is glucose solution (Rosicka et al. 2002). Experiments in rats have shown that both peripheral and intracerebral administration of ghrelin leads to stimulation of growth hormone secretion from somatotropic pituitary cells (Date et al. 2000). It has also been shown that both peripheral and central application of ghrelin elicits a marked orexigenic response, thus increasing gastric secretion, motility and motivation to eat. Faster gastric emptying is associated with increased food intake and thus obesity (Masuda et al. 2000). It was further found that that chronic stimulation of the GHS-R1A receptor by ghrelin (Tschop, Smiley, and Heiman 2000) or synthetic growth hormone secretagogue (Lall et al. 2001) led to an increase in adipose tissue in rodents. Ghrelin levels are elevated before meals (Cummings et al. 2001) and have been shown to correlate with feelings of hunger (Cummings et al. 2004). The oregenic effect of ghrelin was observed after applications to different parts of the brain such as the hypothalamus, nucleus tractus solitarius, ventral tegmental area (VTA) and nucleus accumbens (NAc) (Egecioglu et al. 2010). GHS-R1A receptors in addition to brain structures

manage food intake in terms of energy balance. Chronic overeating can lead to similar neuroadaptive changes in the brain as a substance addiction, at least as far as reward mechanisms (Grigson 2002). Behaviour changes also show similar signs of chronic overeating and substance abuse, e.g., loss of control over by their actions. Thus, hypothetically, the principle of ghrelin mechanisms may also apply in the development of substance abuse.

The neurobiology of food intake behaviour seems to share several fundamental features with the neurobiology of drug use and craving (Volkow et al. 2011). According to the gut-brain peptide physiology, the hormone ghrelin plays a central role in the regulation of appetite, energy homeostasis and in extend food intake and reward (Egecioglu et al. 2010). It was also suggested that central ghrelin signalling might play an important role in alcohol reinforcement and reward of other substances of abuse including amphetamines, cocaine, and nicotine (Engel and Jerlhag 2014; Panagopoulos and Ralevski 2014; Sustkova-Fiserova et al. 2022). Ghrelin is synthesized primarily by endocrine cells in the stomach and in smaller amounts by other organs such as the pancreas, salivary glands, and placenta (Kojima et al. 1999). The peripherally produced ghrelin communicates with the central nervous system and affects the brain directly by crossing the blood–brain barrier and indirectly by stimulating the vagus nerve (Cabral et al. 2017).

The growth hormone secretagogue receptors type 1A (GHS-R1a) are expressed in brain-reward areas including nucleus accumbens (NAc), ventral tegmental area (VTA), striatum, prefrontal cortex, hypothalamus, amygdala, and hippocampus (Abizaid et al. 2006; Ferrini et al. 2009; Howard et al. 1996), (Skibicka et al. 2011). The ghrelin administration modulates dopamine transmission in the brain, it releases dopamine in the nucleus accumbens and stimulates behavioural measures of reward processing. GHS-R1a is expressed throughout the mesolimbic reward pathway and forms heterodimers with dopamine D1 and D2 and several other receptors (Abizaid et al. 2006; Guan et al. 1997; Schellekens et al. 2013). These findings suggest that ghrelin may play a central role in reward processing. Also, acyl-ghrelin interacts with the hypothalamic pituitary adrenal axis and modulate stress related behaviours (Bali and Jaggi 2016; Spencer et al. 2015). Ghrelin's involvement in both reward and stress pathways suggests a potential role for the ghrelin signalling in addiction, as drug-seeking behaviour is linked to positive reinforcement (drug reward) and negative reinforcement (stress relief) mechanisms (Koob and Volkow 2016; Panagopoulos and Ralevski 2014).

4.2. Role of ghrelin/GHS-R1A in the cannabinoid addiction

The endogenous cannabinoid system, as well as exogenous cannabinoids such as THC, affects appetite and food intake through the cannabinoid receptors (Kirkham and Williams 2001). Cannabinoids stimulate the mesolimbic/mesocortical endocannabinoid system, which influences the motivation for natural rewards (such as palatable food, social interaction and sexual activity) and also modulates the rewarding effects of addictive drugs. The prolonged activation of the endocannabinoid system by cannabis/cannabinoids can elicit neuroadaptations which may lead to addiction and other adverse consequences (Volkow, Hampson, and Baler 2017; Zehra et al. 2019; Parsons and Hurd 2015; Hwang and Lupica 2020). The endocannabinoid system consists of central and peripheral effects, several binding sites and numerous endogenous ligands (Di Marzo et al. 1998; Mechoulam, Fride, and Di Marzo 1998; Tanda and Goldberg 2003). The most studied endocannabinoids, anandamide and 2-AG are synthetised on demand from the cell phospholipids and through cannabinoid CB1 receptors, retrogradely regulating synaptic neurotransmissions (e.g., glutamate, γ-Aminobutyric within the brain's reward circuitry, controlling both excitatory (glutamate) and inhibitory (GABA) inputs (Zehra et al. 2019; Parsons and Hurd 2015). The endocannabinoid system acts as an important mediator of synaptic plasticity in the mesocorticolimbic/corticostriatal pathways which are involved in motivated behaviour control (Volkow, Hampson, and Baler 2017; Parsons and Hurd 2015; Zlebnik and Cheer 2016). The CB1Rs are the most abundant G protein-coupled receptors expressed in the adult brain with intense expression in regions involved in reward, addiction and cognitive functions including the ventral tegmental area, nucleus accumbens and hippocampus (Parsons and Hurd 2015; Herkenham 1991; Matsuda et al. 1990). The CB2Rs have also been recently found in the midbrain dopamine neuron regions. The CB1Rs activation produces reinforcing effects, whereas the CB2Rs produces aversive effects (Parsons and Hurd 2015; Spiller et al. 2019).

Cannabinoids including the main psychoactive constituent of cannabis, tetrahydrocannabinol as well as the synthetic amino-alkylindol cannabinoid WIN55,212-2, show typical biphasic/dual effects, with reinforcing and hyperactivity-stimulating low doses but aversive and hypoactivity inducing high doses. Tetrahydrocannabinol is considered a partial CB1R/CB2R agonist and WIN55,212-2 a full CB1R/CB2R agonist (Zehra et al. 2019; Spiller et al. 2019; D'Ambra et al. 1992). It was previously described that the CB1 antagonist reduced elevated levels of circulating acyl-ghrelin in rats during food deprivation. The CB1 antagonist administration attenuated the growth hormone secretion (Al Massadi et al. 2017). In

separate experiments investigating the effects of the AMP-activated protein kinase (AMPK), an enzyme that regulates food intake and energy balance, was found that the activity of this enzyme was affected by both ghrelin and cannabinoids. Thus, AMPK could mediate the orexigenic effects of cannabinoids and ghrelin (Kola et al. 2005). Interestingly, administration of a CB1 antagonist in rats inhibited the orexigenic effect of centrally (intracerebroventricularly) ghrelin. Central administration of ghrelin in mice increased the food intake, the AMPK activity, and the presence of endocannabinoids in the hypothalamus. The administration of a cannabinoid antagonist rimonabant reversed these effects (Kola et al. 2005).

The current literature implicating ghrelin in opioid and cannabis use disorders is still limited and inconclusive. Any manipulations that attenuate drug accumbens dopaminergic and behavioural sensitization are essential for understanding the foundation of a future drug addiction treatment (Steketee and Kalivas 2011; Vanderschuren and Kalivas 2000). Therefore, this research thesis focuses on the involved mechanisms and relationships among the cannabis and ghrelin systems within the NACSh and/or the VTA.

4.3. Role of ghrelin/GHS-R1A in the opioid addiction

The opioid system is an important component in regulating of the food reward and reinforcement. Clinical studies showed that administration of an opioid μ receptor antagonist, could reduce pleasure and smell and reduce the preference for sweet and high-energy foods.

Experiments in mice (Engel, Nylander, and Jerlhag 2015) and in rats (Jerabek et al. 2017) demonstrated that a GHS-R1A antagonist (JMV2959) significantly attenuated the ability of acute morphine-induced behavioural stimulation, dopamine efflux in the NAc and the site preference. A heroin self-administration study (Maric et al. 2012) demonstrated that ghrelin was able to increase heroin intake, however, pretreatment with a selective ghrelin antagonist (D-Lys3-GHRP-6) did not influence heroin self-administration. On the other hand, in few studies (Engel, Nylander, and Jerlhag 2015; Sustkova-Fiserova et al. 2014) was demonstrated that the pretreatment with the ghrelin antagonist (JMV2959), the nonpeptidic triazole substance (Moulin et al. 2007), significantly and dose-dependently attenuated acute morphine-induced dopamine release in the NACSh together with concurrent stereotypical behaviour. The ghrelin antagonism was able to inhibit the expression of the repeated morphine-induced behavioural and accumbens dopaminergic sensitization in rats. A prolonged exposure to drugs of abuse lead to drug and behavioural sensitization but also to a differentially sensitized mesolimbic dopaminergic system depending on conditions of the drug manipulations. A higher stimulatory

impact of drug reward combined with drug-conditioned stimuli was observed in the NACSh in comparison to the core dopamine transmission. The mesolimbic dopaminergic neuron hypersensitivity during an extended abstinence may trigger the long-term expression of behavioural sensitization to drugs of abuse as well as the reinstatement of a compulsive drug-seeking behaviour (Robinson and Berridge 1993; Vanderschuren and Kalivas 2000). Behavioural sensitization may be helpful for investigating the motivation of underlying drug-seeking behaviour. The mesolimbic dopaminergic system mediates both sensitizing and motivational properties of drugs of abuse including opioids (De Vries and Shippenberg 2002; Robinson and Berridge 1993; Vanderschuren and Kalivas 2000; Steketee and Kalivas 2011).

The influence of the ghrelin antagonist JMV2959 on sensitized accumbens dopamine release and behavioural sensitization was demonstrated by morphine challenge dose administered on the 12th day of abstinence following subchronic daily morphine treatment for 5 days (Sustkova-Fiserova et al. 2014). Pretreatment with JMV2959 reversed both short-term and long-term morphine-induced increases in anandamide levels in the nucleus accumbens, intensified acute morphine-induced decreases in 2-arachidonoylglycerol (2-AG) levels, and attenuated long-term morphine-induced decreases in 2-AG levels (Sustkova-Fiserova et al. 2016). The opioid-induced conditioned place preference (CPP) is associated with the opioid reinforcing properties that are part of the addiction process (Bardo and Bevins 2000). Pretreatment with systemic JMV2959 also resulted in decreased morphine-induced hyperlocomotion, conditioned place preference, and dopamine release in the nucleus accumbens in mice (Engel, Nylander, and Jerlhag 2015).

Fentanyl and new opioid synthetic derivatives have been recently increasingly abused in the USA, Canada and Europe (Mounteney et al. 2015). Fentanyl, a 4-anilidopiperidin derivate synthetized in 1959 (Janssen et al. 1959), is generally considered as a μ-receptor-selective agonist about 100-fold more potent than morphine (Pasternak and Pan 2013) using active transportation through the blood–brain barrier (Henthorn et al. 1999; Tsuji 2005). The aim of this study was to test whether JMV2959, a growth hormone secretagogue receptor (GHS-R1A) antagonist, can influence the fentanyl-induced effects on anandamide, 2-arachidonoylglycerol (2-AG) and GABA in the NACSh and strength the involvement of GHS-R1A located in the ventral tegmental area (VTA) and nucleus accumbens (NAc). Therefore, this research thesis focuses on the involved mechanisms and relationships among the opioid and ghrelin systems within the NACSh and/or the VTA.

5. Experimental models used in the preclinical addiction research

General scientific methods (in vivo, in vitro, in situ, behavioural etc) as well as specific addiction models are used in order to explore possible addiction mechanisms. In the current experimental research, the involvement of central ghrelin signalling in the mechanisms of cannabis and opioid dependence and GHS-R1A testing antagonism are explored as a potential new promising mechanism for the treatment of these addictions, especially for relapse and drug seeking prevention. A neurobehavioral method (CNS microdialysis in vivo) was used to monitor several neurotransmitter changes in the nucleus accumbens, a brain structure that is essential for rewarding and strengthening effects of addictive substances. Also, a specific addiction behavioural model (LABORAS) was used to monitor behaviour.

5.1. Microdialysis in vivo

The neurobehavioral in vivo microdialysis method allows monitoring neuromediator changes that take place in selected brain structures. A microdialysis cannula is inserted in advance under general anaesthesia into the implanted brain structure nucleus accumbens and/or VTA. The day of the experiment, the cannula is removed, and the perfusion is starting and is flushed at low speed (2 μl / min) with artificial cerebrospinal fluid / treated Ringer's solution (Figure 2). In the current research, the microdialysates were taken in intervals from the NAC structure in free-moving rats monitored changes in extracellular concentrations of dopamine and its metabolites (3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), endocannabinoids (anandamide and 2-AG) and γ-Aminobutyric acid (GABA). The detection of mediators in cooperation with Institute of Chemistry and Technology (ICT) using high - performance liquid chromatography in combination with mass spectrometry (HPLC-MS). During microdialysis, after collecting three basal / unaffected dialysates (basal mediator levels), the administered opioid (fentanyl)/ WIN55,212-2 or vehicle / solvent with JMV2959 premedication or vehicle are collected (Charalambous et al. 2020; Jerabek et al. 2017; Sustkova-Fiserova et al. 2017; Sustkova-Fiserova et al. 2014; Sustkova-Fiserova et al. 2016).

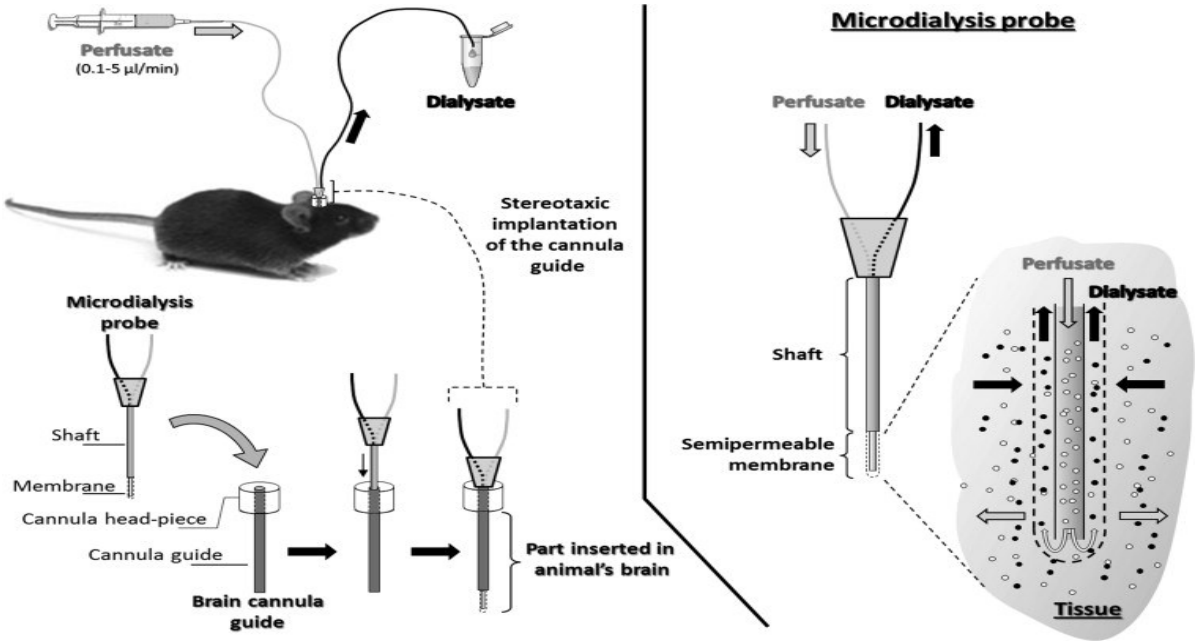

Figure 2. The microdialysis dialysate collection

5.2. LABORAS

The spontaneous exploratory behaviour but also the locomotor and climbing activity of rodents within the known/homecage-like environment can be examined using the Laboratory Animal Behaviour Observation, Registration, and Analysis System (LABORAS, Metris, Hoofddorp, The Netherlands). The behaviour of adult male and female rats can be tested in the LABORAS apparatus (Figure. 3) in a reversed light/dark cycle. The LABORAS is known as a fully automated system for monitoring of the behavioural postures and tracking of small rodents (Schutova et al. 2013). The apparatus consists of a triangular shaped sensor platform connected directly to a computer. A makrolon type III cage (840 cm2) filled with a 2 cm layer of absorbent bedding of natural poplar wood placed on the platform. The platform transforms the mechanical vibrations caused by the movements of the animal into electrical signals. Each movement pattern has its own unique frequency and amplitude pattern and thus separate behavioural categories can be distinguished and classified by the computer. The stored data are processed by the LABORAS software—the recorded signals are compared with the predetermined characteristic patterns and thus are classified into behavioural categories (Borbelyova et al. 2019; Van de Weerd et al. 2001).

Figure 3. The LABORAS system

III. EXPERIMENTAL PART

6. Hypothesis and Aim

The overall outcome from a previous study of the research lab from the Department of Pharmacology of the 3rd Faculty of Medicine Charles University indicated the significant involvement of central ghrelin signalling in the non-selective opioid morphine-induced dopamine as well as endocannabinoid changes in the NACSh in rats. In this study, the systemic GHS-R1A antagonist (JMV2959) administration significantly and dose dependently reduced the morphine-induced dopamine release as well as dopamine sensitization in the NACSh and affected the concentration of by-products associated with dopamine metabolism: 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). The premedication with JMV2959 significantly and dose dependently reversed the morphine-induced anandamide increases in the NACSh in both the acute and chronic models, resulting in a significant anandamide decrease. JMV2959 also significantly intensified acute morphine-induced decreases in accumbens 2-AG levels and attenuated morphine challenge induced 2-AG decreases (Sustkova-Fiserova et al. 2014; Sustkova-Fiserova et al. 2016). The observed morphine-induced anandamide increase and 2-AG decrease in the rat NACSh, was suggested to importantly contribute to the opioid reinforcing effects and addiction (Caille et al. 2007; Vigano et al. 2004). In the Sustkova-Fiserova et al. 2016 study, the co-administered acyl-ghrelin (40 μg/kg i.p.) with the JMV2959 3 mg/kg i.p., abolished completely the monitored effects of this GHS-R1A antagonist on the morphine-induced accumbens neurotransmitter changes. Thus, the participation of ghrelin signalling in the non-selective opioid/morphine-induced anandamide and 2-AG accumbens changes were confirmed. However, the influence of ghrelin antagonism on the accumbens endocannabinoid changes induced within the NAc by the μ-selective opioid agonist and specifically fentanyl (a highly potent opioid), was not known. Furthermore, the participation of the VTA and the NAc brain structures in the observed accumbens effects also remained to be tested.

A study in mice described that the CB1 antagonist reduced the ghrelin-induced locomotor stimulation and dopamine overflow in the NAc (Kalafateli et al. 2018). Collectively with our above-mentioned studies with GHS-R1A antagonist and morphine in rats, these results indicated, that the ghrelin signalling and the CB1 receptors in the NAc might be significantly involved not only in the in the opioid, but also in the cannabinoid reinforcing effects possibly through a dopamine-independent mechanism (Caille et al. 2007; Vigano et al. 2004; Caille and

Parsons 2003). The involvement of ghrelin signalling in the accumbens changes induced by cannabinoids/cannabis have not been previously tested.

Therefore, to clarify the involved mechanisms and relationships among the cannabinoid, opioid, and ghrelin systems within the NACSh and/or the VTA, the following hypotheses must be defined:

1. The systemic pretreatment with the JMV2959 could reduce the intrategmentally (into the VTA) administered synthetic CB1R agonist WIN55,212-2–induced dopamine efflux in the NACSh.
2. The systemic pretreatment with the JMV2959 could reduce the intrategmentally (into the VTA) administered synthetic CB1R agonist WIN55,212-2–induced endocannabinoid and GABA changes in the NACSh.
3. The systemic pretreatment with the GHS-R1A antagonist (JMV2959) could reverse/reduce the anandamide increase and influence the 2-AG decrease in the rat NACSh induced by the selective μ-receptor agonist (fentanyl) systemic administration, similarly to the previously described situation with non-selective opioid (morphine).
4. The central pretreatment with the JMV2959 administered into the VTA and/or the NACSh could influence the fentanyl-induced endocannabinoid (anandamide and 2-AG) changes in the NACSh.
5. The systemic pretreatment with the JMV2959 could also reduce the tetrahydrocannabinol (THC) and/or WIN55,212-2 –induced locomotor stimulation in the LABORAS system.

7. Methods

7.1. Animals

Male adult Wistar rats provided by Velaz, Praha-Lysolaje, Czech Republic, initially aged 8 weeks were used in all the experiments. During the habituation period for at least seven days before the initiation of all experiments and during the experimental days the rats were given free access to food and water and were housed in polycarbonate cages in singles (microdialysis) or threes (LABORAS) per cage with constant humidity (50–60%), room temperature (22–24 C) and reversed 12 h light/dark cycle (6 a.m.–6 p.m.). After the drug administration during the experiments, the food was removed (Charalambous et al. 2021; Sustkova-Fiserova et al. 2017). Procedures involving animals, along with animal care, were conducted in accordance with international laws; protocols complied with the Guidelines of the European Union Council (86/609/EU, 24 November 1986), the EU Directive (2010/63/EU, 22 September 2010) and the instructions of the National Committee for the Care and Use of Laboratory Animals. Experiments were approved by the Expert Committee for Protection of Experimental Animals of the Third Faculty of Medicine, Charles University, Prague, and they were performed in accordance with the Animal Protection Act of the Czech Republic (No. 246/1992 Sb, 15 April 1992).

7.2. Drugs and Chemicals

Fentanyl citrate was provided by Sigma-Aldrich. THC was synthesized in cooperation with the University of Chemistry and Technology Prague (UCT Prague, Czech Republic). The synthetic aminoalkylindole cannabinoid WIN 55,212-2 mesylate salt (WIN55,212-2) was provided by Sigma–Aldrich. The GHS-R1A antagonist, substance JMV2959 (1,2,4-triazole derivate) was provided by Anton Bespalov (AbbVie, Heidelberg, Germany) and also was synthesized at the UCT Prague (Czech Republic). All reagents were of analytical grade.

Both THC and WIN55,212-2 were firstly dissolved in one drop of Polysorbate 80 (Tween 80) and then diluted in saline. Instead of THC/WIN55,212-2 as the vehicle (saline with one drop of Tween 80) and instead of JMV2959/ghrelin pretreatments, saline served as the placebo/control. THC was used in a stimulatory/rewarding 0.1 mg/kg dose in LABORAS and administered intraperitoneally (i.p.) in volumes of 0.1 mL/100 g of body weight. WIN55,212-2 was diluted in saline or Ringer's solution in microdialysis experiments. Saline/Ringers

solution with the drop of Tween 8 (vehicle) and saline were used as a placebo/control. In accordance with the literature (Polissidis et al. 2013; Polissidis et al. 2010), WIN55,212-2 was administered intracerebrally into the posterior ventral tegmental area (VTA) in dose 2.4 mM/0.5 µL within one minute, dissolved in Ringer's solution (pH 7.0); the intra-cerebral cannula stayed in place for another minute and after was retracted (5µL microsyringe; Innovative Labor System, Stutzerbach, Germany). The JMV2959 was administered i.p. at 0.1 mL/100 g of body weight, always 20 min prior to the WIN55,212-2/vehicle administration.

The administration sites were verified following the end of the experiment (Figure 4B). The dialysis probe was used for the administration of JMV2959 into the NAc. During perfusion with Ringer's solution (always 2 µL/min) the inlet tube was switched to tube filled with 8 mM or 40 mM solution of JMV2959 in the Ringer's solution for 15 min, starting 5 min before WIN55,212-2 administration; thereafter, the inlet tube was switched back to Ringer's solution. The position of each dialysis probe was histologically verified after the completion of each microdialysis experiment and only animals with correct probe positions were included into the statistical evaluations (Figure 4A).

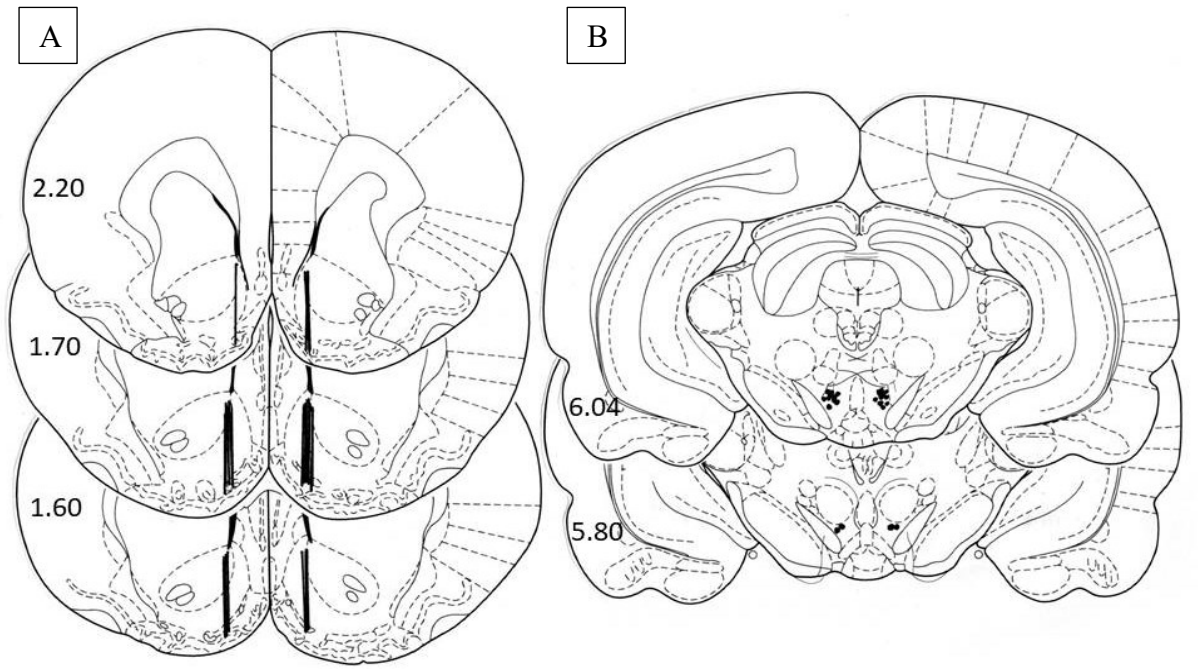

Figure 4. Schematic locations of dialysis probes in WIN55,212-2 rats in the NACSh (A) and sites of infusions into the VTA (B). Schematic locations of probe tips in animals which were involved in analyses of accumbens neurotransmitter concentrations (the bold lines indicate the dialyzing portions (A) and locations of JMV2959/Ringer´s solution administrations into the VTA (B) are illustrated as described in the atlas of Paxinos and Watson (Paxinos and Watson 2007). For each section, the distance from bregma (in mm) is indicated on the left.

Fentanyl was always dissolved in saline and administered subcutaneously (s.c.) 0.1 mL/100 g of body weight; 30 μg/kg was selected following the literature as a reliable analgesic and discriminative dose increasing accumbens dopamine (Di Chiara and Imperato 1988; Zhang et al. 2000; Megens et al. 1998). Saline was used as a placebo. The JMV2959 was dissolved in saline, when administered intraperitoneally (i.p.) 20 min before fentanyl; the selected dose 3 mg/kg JMV2959 s.c. was determined based on previous studies in Wistar rats (Sustkova-Fiserova et al. 2014; Sustkova-Fiserova et al. 2016; Clifford et al. 2012). The intracerebral JMV2959 doses were in accordance with the literature (Hansson et al. 2012; Skibicka et al. 2012). When JMV2959 was administered intra-cerebrally, JMV2959 was dissolved in the Ringer's solution (adjusted to pH = 7.0) and Ringer's solution was used as a placebo. Doses 2 or 10 μg of JMV2959 were administered into the rat VTA at a volume of 0.5 μL for 1 min; the cannula stayed in place for another minute and after was retracted (5 μL microsyringe; Innovative Labor System, Stutzerbach, Germany). The administration sites were verified following the end of the experiment (Figure 5B). The position of each dialysis probe was histologically verified after the completion of each microdialysis experiment and only animals with correct probe positions were included into the statistical evaluations (Figure 5A).

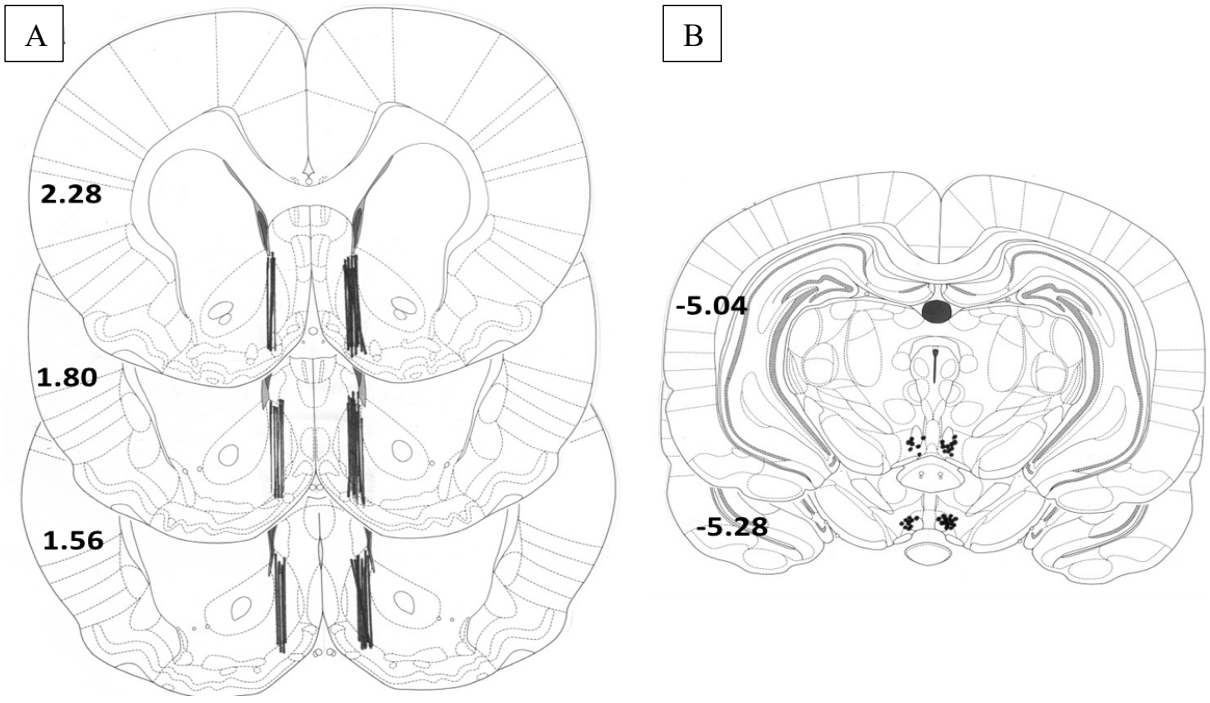

Figure 5. Schematic locations of dialysis probes in fentanyl rats in the NACSh (A) and sites of infusions into the VTA (B). Schematic locations of probe tips in rats which were included in analyses of accumbens neurotransmitter concentrations (the bold lines indicate the dialyzing portions (A) and locations of JMV2959/Ringer's solution administrations into the VTA (B) are illustrated as described in the atlas of Paxinos and Watson (Paxinos and Watson 2007). For each section, the distance from bregma (in mm) is indicated on the left.

7.3. In-vivo microdialysis and chemical analysis assay

The microdialysis surgeries were adapted from the Sustkova-Fiserova previous studies (Sustkova-Fiserova et al. 2014; Sustkova-Fiserova et al. 2016). Under ketamine – xylazine anaesthesia (ketamine 100 mg/kg i.p., Narketan, Vetoquinol; xylazine 10 mg/kg i.p., Xylapan, Vetoquinol), rats were implanted with a disposable dialysis guide cannula (MAB4 probes, Agnthos, Sweden) using a stereotaxic instrument (StoeltingCo) into the NACSh (NACSh: A: +2.0 mm and L: ±1.2 mm from bregma and V: 6.2 mm from occipital bone) (fentanyl experiment systemic) (Paxinos and Watson 2006) and secured to the skull with dental cement and an anchoring screw. The guide was randomly alternated on the left and right side. In experiments, were JMV2959/Ringer's solution was administered into the VTA, two guide cannulas were implanted together on the same site, one into the NACSh (above coordinates) and one into the anterior VTA (VTA: A: -5.3 mm and L: ±0.8 mm from bregma and V: 8.2 mm from the skull) (unilaterally) (fentanyl experiment central). In the further cannabinoid study, in each rat two guide cannulas were randomly and unilaterally implanted, one into the NACSh (NACSh: A: +2.0 mm and L: +/-1.2 mm from bregma and V: 6.2 mm from occipital bone) and another one into the posterior VTA (P: 6.0 mm and L: +/-1.0 mm from bregma and V: 8.0 mm from the skull) (WIN55,212-2 experiment) (Paxinos and Watson 2006). The selected coordinates to target the VTA district that participate in the ghrelin as well as cannabinoid food/drug motivation processes were chosen following literature (Abizaid et al. 2006; Zangen et al. 2006; Skibicka et al. 2011).

Post-surgery, the rats were kept in individual cages. In accordance with Sustkova-Fiserova, 48 h after implantation, the probe (MAB4, 2 mm active cuprophane membrane, Agnthos, Sweden) was inserted into the guide cannula and artificial cerebrospinal fluid (Ringer´s solution; 147 mM NaCl, 2. 2 mM CaCl2 and 4.0 mM KCl; adjusted to pH 7.0) was flushed through the probe at a constant rate of 2.0 µl/min (Univentor 864 Syringe Pump, Agnthos, Sweden) (Sustkova-Fiserova et al. 2014; Sustkova-Fiserova et al. 2016). After minimum 60 min of habituation, 20 µL samples were collected at 20 min intervals in small polyethylene tubes containing 7 µL HCl 0.1 mM to prevent monoamine degradation. The further 20 µL dialysate samples of each 20 min interval were collected in empty small polyethylene tubes for detection of the other neurotransmitters (endocannabinoids and GABA). After three consecutive baseline samples, rats were injected with saline or JMV2959 (i.p.), which was followed (20 min later) by the administration with fentanyl (s.c)/ WIN55,212-2 (VTA) or saline (s.c.). In the fentanyl experiments with JMV2959 administration into the

VTA/NAC four baseline samples and 5 min before fentanyl were collected, JMV2959 was administered into the VTA or NAC perfusion started and samples were further collected starting with fentanyl administration. Samples were collected for 3 h following injection of fentanyl/ WIN55,212-2 or saline. Immediately following collection, the samples were frozen at −70 °C. The amount of anandamide, 2-AG and GABA in the dialysate were quantified using HPLC-MS. The appropriate HPLC-MS determination methods were described in detail earlier (Sustkova-Fiserova et al. 2016; Syslova et al. 2011). The endocannabinoids in the dialysate went under lyophilization in freeze dryer (Labconco Free Zone, USA) to concentrate the substances from the dialysates, and detection using liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) which consisted of a chromatograph Accela 1250 (Thermo Scientific, USA), autosampler Accela (Thermo Scientific, USA) and a TSQ Vantage mass spectrometer (Thermo Scientific, USA). The data were acquired and processed using Xcalibur 2.1.0 software (Thermo Scientific, USA).

7.4. Behavioural Testing in LABORAS

The LABORAS apparatus was used for testing the behavioural changes induced by the WIN55,212-2/vehicle or THC/vehicle with a JMV2959/saline pretreatment from 8 am to 16 pm in reversed light/dark cycle (during the dark period). The day prior to testing, the animals were weighed, and moved to the testing room, where they remained in their home cages for an acclimation period. Immediately after the i.p. injection of saline or JMV2959 (1 or 3 mg/kg), the rats were placed into the LABORAS system for continuous behaviour recognition and tracking and were left there for 20 min of habituation. Thereafter, 0.1 mg/kg of THC/WIN55,212-2 or vehicle was administered i.p. and the rats were left in the apparatus for another 20 min of habituation. Then, the 20 min monitoring period started, within 20–40 min after THC/WIN55,212-2 administration. Following the literature, this interval matched a period when significant 0.1 mg/kg THC/WIN55,212-2-induced behavioural stimulation could be observed (Polissidis et al. 2009). The following parameters were automatically evaluated by LABORAS: time spent in locomotion (s), time spent immobile (s), time spent rearing (s), time spent grooming [s], distance (trajectory length) (m), and average speed (mm/s). The animals were randomly assigned to groups. The vehicle + saline group served as a control to compare the effects of THC and the pretreatment.

7.5. Statistical Analysis

Sigma Plot 13 (Systat Software, Inc., San Jose, CA, USA) was used for the statistical evaluation of the data. The microdialysates raw data for fentanyl endocannabinoids and WIN55,212-2 dopamine and its metabolites, endocannabinoids and GABA expressed as ng/mL were converted to percentage of baseline levels (mean of three 20 min intervals prior to pretreatments). For statistical differences between the treatment groups relative to time-related changes during the in vivo microdialysis experiment, a two-way ANOVA RM/Bonferroni test was used. For statistical analysis of dopamine turnover metabolic ratios (comparison among the groups) a two-way ANOVA/Bonferroni method was used.

The behavioural changes among the rat groups with different treatments observed in the LABORAS cage within 20–40 min after the WIN55,212-2/vehicle administration, were evaluated by one-way ANOVA followed by the Holm–Sidak post–hoc test.

All results are presented as a group mean ± SEM. All statistical tests were evaluated at a significance level of 0.05 (p values of < 0.05, < 0.01, and < 0.001 defined statistical significance).

8. Results

8.1. The JMV2959 effects on the WIN55,212-2-induced increase of dopamine and its metabolites in the NACSh

The extracellular accumbens changes were observed for a total of 200 min (140 min after the cannabinoid application). The two-way ANOVA for repeated measures (RM) followed by Bonferroni's test of accumbens microdialysis measurements of dopamine revealed an overall effect of the treatment ($F_{3,8} = 125.3$, $p < 0.001$), time ($F_{8,168} = 22.1$, $p < 0.001$) and the treatment × time interaction ($F_{8,168} = 15.3$, $p < 0.001$) (Figure 6A). The rapid cannabinoid-induced dopamine increase in NACSh was significantly higher relatively to the vehicle + saline group during the 20–100 min intervals ($p < 0.001$). The maximal WIN55,212-2-induced dopamine increase (130.5% of baseline mean during the 60 min interval) was significantly reduced (by 22.7%) after the GHS-R1A antagonist/JMV2959 pretreatment to the maximum of 107.8% of the baseline mean (during the 40 min interval). However, despite the JMV2959 pretreatment, the cannabinoid-induced increase in accumbens dopamine remained significant relative to the vehicle + saline treatment at the time interval of 40 min ($p < 0.001$) and 60–80 min ($p < 0.05$). Subsequently, the dopamine extracellular concentration moderately dropped below baseline mean levels, oscillating from 95.2 to 91.7% and 85.4% of the baseline mean, respectively; the decrease was transiently significant relatively to the vehicle + saline group at intervals 80 min ($p < 0.01$), 100 min ($p < 0.05$) and 120 min ($p < 0.001$). Within the last 140 min interval, the dopamine levels in the JMV2959 + WIN55,212-2 group were comparable with the saline + WIN55,212-2 and saline + vehicle groups. The effects of WIN55,212-2-induced dopamine metabolism in the NACSh are illustrated in the Figure 6B–D.

The two-way ANOVA RM/Bonferroni analysis of accumbens HVA measurements (Figure 6B) revealed an overall effect of the treatment ($F_{3,8} = 52.4$, $p < 0.001$), time ($F_{8,168} = 61.8$, $p < 0.001$) and treatment × time interaction ($F_{8,168} = 21.8$, $p < 0.001$). The WIN55,212-2 increased accumbens HVA levels were significantly higher relatively to the vehicle + saline group within 40–100 min intervals ($p < 0.001$). Pretreatment with the GHS-R1A antagonist/JMV2959 significantly enhanced the maximal cannabinoid-increased accumbens HVA concentrations (142.2% of the baseline mean at a 60 min interval) by 19.4% to a maximal 161.6% of the baseline mean (at a 40 min interval). The HVA levels were significantly higher relatively to the vehicle + saline group from the first interval after the WIN55,212-2

administration and remained so until the end of the microdialysis trial (20–140 min intervals, $p < 0.001$).

The two-way ANOVA RM/Bonferroni analysis of accumbens 3-MT measurements (Figure 6C) revealed an overall effect of the treatment ($F_{3,8} = 18.0$, $p < 0.001$), time ($F_{8,168} = 12.2$, $p < 0.001$) and the treatment × time reaction ($F_{8,168} = 5.0$, $p < 0.001$). The 3-MT accumbens extracellular levels were increased by the intra-tegmental WIN55,212-2 administration only to a maximum of 113.9% of the baseline mean (60 min interval). The JMV2959 pretreatment seemed to decrease the cannabinoid-induced 3-MT accumbens augmentation, but the effect was not significant with a maximum 111.3% of the baseline at a 60 min interval.

The two-way ANOVA RM/Bonferroni analysis of accumbens DOPAC measurements (Figure 6D) revealed an overall effect of the treatment ($F_{3,8} = 102.3$, $p < 0.001$), time ($F_{8,168} = 53.4$, $p < 0.001$) and the treatment × time reaction ($F_{8,168} = 22.6$, $p < 0.001$). Administration of WIN55,212-2 increased the extracellular accumbens DOPAC to a maximum of 133.5% of the baseline mean (40 min interval) and the DOPAC concentration was significantly higher relative to the vehicle + saline group during 20–60 min intervals ($p < 0.001$) and 80 min interval ($p < 0.05$). Pretreatment with JMV2959 enhanced the cannabinoid-induced DOPAC accumbens' levels by 29% to a maximum of 162.5% of the baseline mean (40 min interval) and prolonged the significantly higher DOPAC levels relative to the vehicle + saline group until the end of the dialysis experiment (20–140 min interval, $p < 0.001$). A single 3 mg/kg intraperitoneal dose of JMV2959 + saline but also the vehicle + saline had no significant effect on dopamine and its metabolites concentrations in the NACSh.

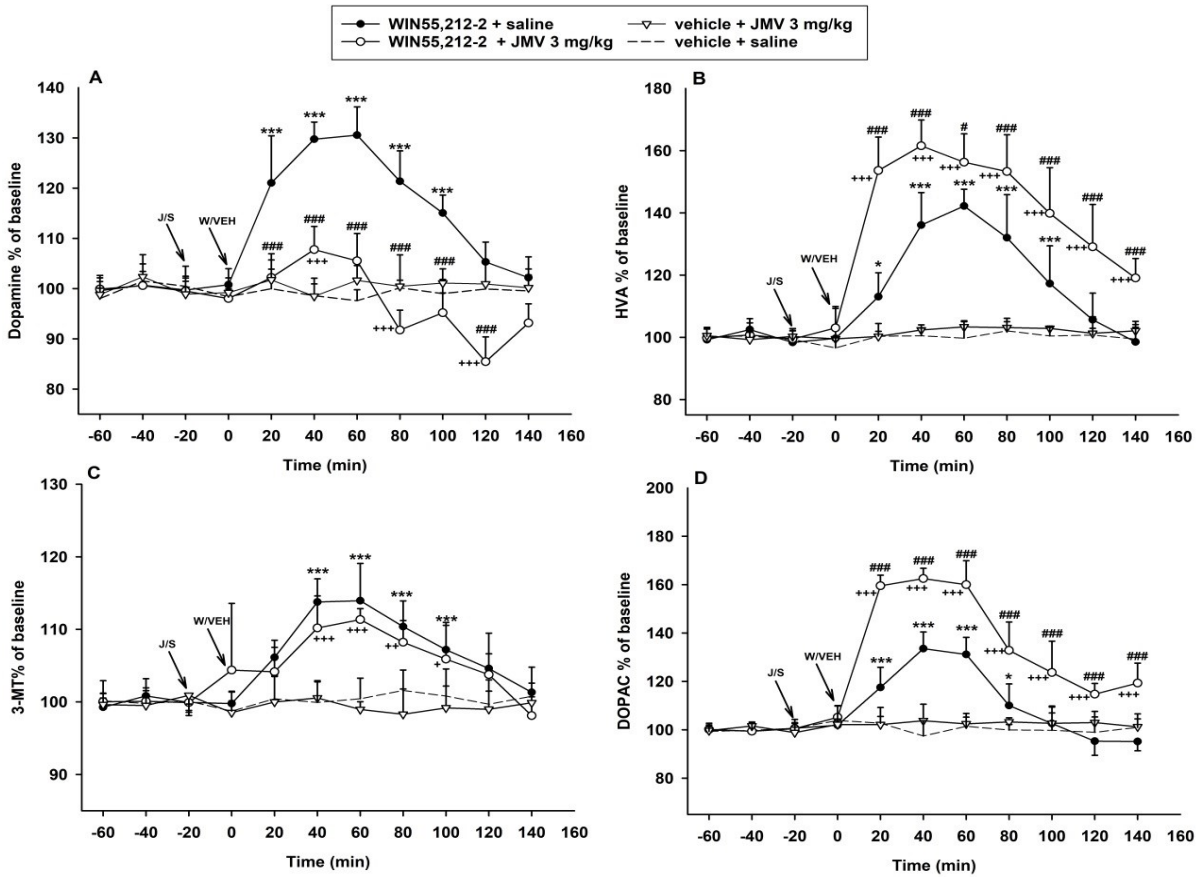

*Figure 6. Effects of growth hormone secretagogoue receptors (GHS-R1A) antagonist (JMV2959) on WIN55,212-2-induced dopamine and its metabolites' extracellular changes in the rat's NACSh. JMV2959 3 mg/kg was given i.p. 20 min before 2.4 mM/0.5 µL WIN55,212-2 or vehicle 0.5 µL was administered into the posterior ventral tegmental area (VTA) injection (n = 6; means ± SEM). Changes in accumbens dopamine are illustrated in graph (A), changes in HVA, 3-MT, and DOPAC are illustrated in the graphs (B–D), respectively. The effects are illustrated as follows: saline + WIN55,212-2 (filled circle; n = 7), 3 mg/kg JMV2959 + WIN55,212-2 (open circle; n = 6), 3 mg/kg JMV2959 + vehicle (open triangle; n = 4), saline + vehicle (dotting; n = 7). Differences between saline + WIN55,212-2 and saline + vehicle groups are expressed as *** $p < 0.001$, * $p < 0.05$. Differences JMV2959 + WIN55,212-2 and saline + vehicle groups are expressed as +++ $p < 0.001$, ++ $p < 0.01$, + $p < 0.05$. Differences between groups saline + WIN55,212-2 and 3 mg/kg JMV2959 + WIN55,212-2 are expressed as ### $p < 0.001$, # $p < 0.05$.*

8.2. The JMV2959 effects on the WIN55,212-2-induced extracellular turnover of dopamine in the NACSh

The dopamine turnover metabolic ratios of baseline levels (three 20 min intervals before pretreatments) with two intervals with the highest observed dopamine/metabolite changes induced by WIN55,212-2 administration, the 40 and 60 min intervals are illustrated Figure 7. The groups WIN55,212-2 + saline, WIN55,212-2 + JMV2959 and vehicle + saline

were chosen for comparison of the JMV2959 pretreatment effect. For analysis of dopamine turnover, the values of detected concentrations (pg or ng/mL) were used within the appropriate intervals.

Comparison of the dopamine turnover metabolic HVA/DA ratios, illustrated in Figure 7A, using two-way ANOVA/Bonferroni revealed significant difference among groups ($F_{1,2} = 121.0$; $p < 0.001$), treatment effect ($F_{2,99} = 95.2$; $p < 0.001$) and group × treatment interaction ($F_{2,99} = 78.4$; $p < 0.001$). These results indicate that total accumbens dopamine metabolism was significantly elevated after the WIN55,212-2 intracerebral administration, since its final metabolite (HVA) was increasingly produced; hence, the HVA/DA ratio was enhanced in comparison to the baseline as well as to the vehicle + saline group ($p < 0.05$). The JMV2959 pretreatment significantly increased the general accumbens dopamine turnover in comparison to saline pretreatment before WIN55,212-2, because, despite the observed accumbens dopamine decrease, the HVA concentration increased after the JMV2959 pretreatment together with the HVA/DA ratios ($p < 0.001$).

Comparison of the dopamine turnover metabolic 3-MT/DA ratios, illustrated in Figure 7B, using two-way ANOVA/Bonferroni revealed significant difference among groups ($F_{1,2} = 4.3$; $p < 0.05$), treatment ($F_{2,99} = 18.5$; $p < 0.001$) and group × treatment interaction ($F_{2,99} = 12.5$; $p < 0.001$). The dopamine efflux induced by WIN55,212-2 intracerebral administration in the NACSh was higher in comparison to the 3-MT levels production; thus, the 3-MT/DA ratios decreased relative to the baseline as well as to the vehicle + saline group ($p < 0.001$). The JMV2959 pretreatment almost prevented the WIN55,212-2-induced accumbens dopamine increase, yet the accumbens 3-MT production did not significantly change; thus, the 3-MT/DA ratios increased relative to the WIN55,212-2 + saline group ($p < 0.001$).

Comparison of the dopamine turnover metabolic DOPAC/DA ratios, illustrated in Figure 7C, using two-way ANOVA/Bonferroni revealed significant difference among groups ($F_{1,2} = 153.0$; $p < 0.001$), treatment effect ($F_{2,99} = 171,2$; $p < 0.001$) and group × treatment interaction ($F_{2,99} = 135.1$; $p < 0.001$). Production of DOPAC in the NACSh after the cannabinoid administration was significantly increased only in comparison to the vehicle + saline group ($p < 0.001$). The JMV2959 pretreatment before WIN55,212-2 significantly elevated the accumbens DOPAC levels while reducing dopamine, so the DOPAC/DA ratios significantly increased relative to the WIN55,212-2 + saline group ($p < 0.001$).

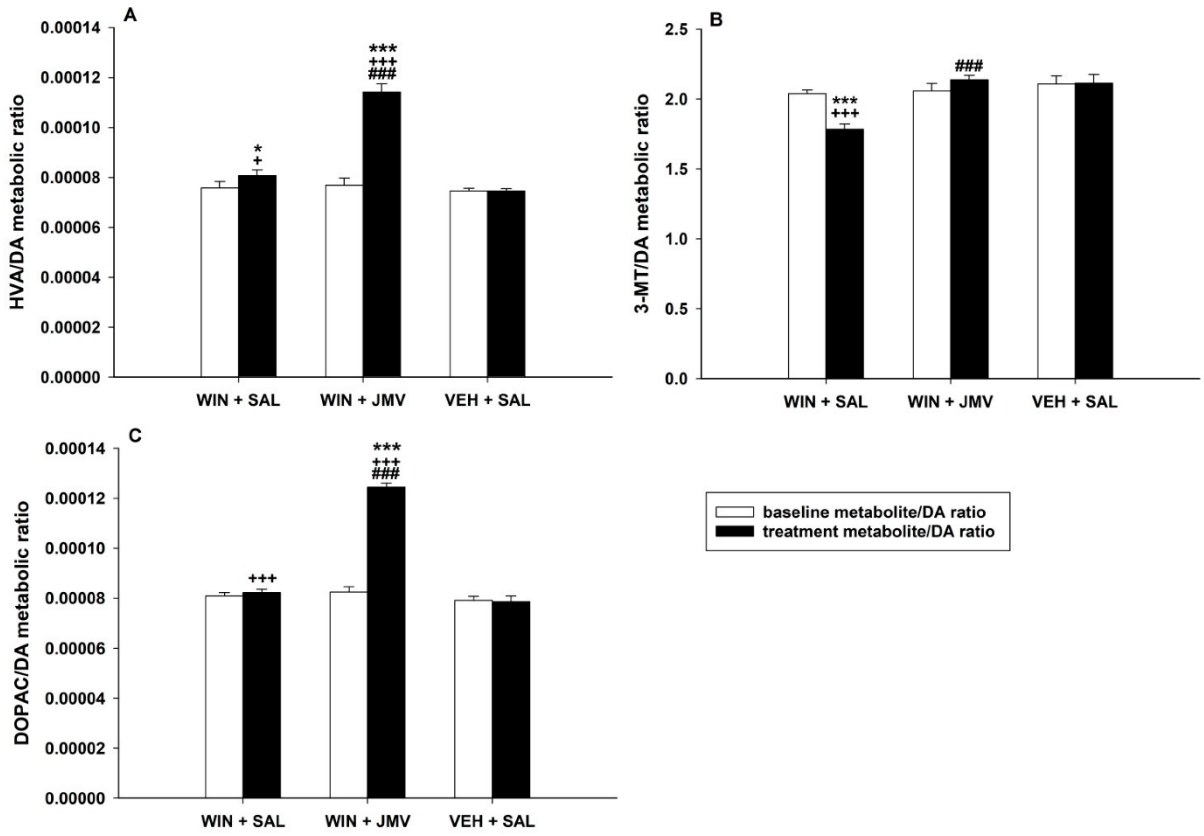

*Figure 7. Extracellular DA metabolic turnover in the NACSh. The graphs show the metabolite/dopamine ratios, the means ± SEMs (n = 6) of concentration values of the metabolite divided by the corresponding values of dopamine concentrations (metabolite/DA). The HVA/DA ratio are illustrated in the graph A, the 3-MT/DA ratio in the graph B and the DOPAC/DA ratio in the graph C. Three baseline interval concentration values before pretreatments (baseline metabolite/DA ratio–white bars) and two intervals with maximal WIN55,212-2 effects (40 min and 60 min intervals; treatment metabolite/DA ratio–black bars) were used for statistical comparison within WIN55,212-2 + saline versus WIN55,212-2 + JMV2959 versus vehicle + saline groups. The effect of JMV2959 pretreatment, thus differences between WIN55,212-2 + saline and WIN55,212-2 + JMV2959, are illustrated expressed as ### $p < 0.001$. Differences between groups relative to the vehicle + saline group are illustrated expressed as + $p < 0.05$ and +++ $p < 0.001$. Differences between treatment and baseline are illustrated expressed as * $p < 0.05$ and *** $p < 0.001$.*

8.3. The JMV2959 effects on the WIN55,212-2-induced changes of anandamide, 2-AG and GABA in the NACSh

The two-way ANOVA RM/Bonferroni analysis of accumbens anandamide measurements (Figure 8A) revealed an overall effect of the treatment ($F_{3,8} = 660.3$; $p < 0.001$), time ($F_{8,168} = 274.4$; $p < 0.001$) and the treatment × time interaction ($F_{8,168} = 97.5$; $p < 0.001$). WIN55,212-2 administration into the VTA induced significant anandamide increase in the NACSh to a maximum of 164.1% of the baseline mean (80 min interval), and the

anandamide accumbens levels were significantly higher relatively to the vehicle + saline group at 20 min interval and remained so until the end of experiment (140 min interval) ($p < 0.001$). Pretreatment with JMV2959 transiently significantly reduced the cannabinoid-induced anandamide increase within a 20 min interval ($p < 0.05$) and 40–100 min intervals ($p < 0.001$) to a maximum of 151.4% of the baseline mean; thus, no more than by 12.7%. Despite the JMV2959 pretreatment, the cannabinoid-induced anandamide increase remained significantly relative to the vehicle + saline group from the 40 min interval until the end of the experiment (140 min interval).

The two-way ANOVA RM/Bonferroni analysis of accumbens 2-AG measurements (Figure 8B) revealed an overall effect of the treatment ($F_{3,8} = 182.5$; $p < 0.001$), time ($F_{8,168} = 79.8$; $p < 0.001$) and the treatment × time interaction ($F_{8,168} = 30.7$; $p < 0.001$) (two-way ANOVA RM/Bonferroni). The accumbens 2-AG extracellular concentration was significantly increased by WIN55,212-2 administration from 20 min interval until the end of microdialysis (140 min interval) relatively to the vehicle + saline group ($p < 0.001$) with a maximum 120.1% of the baseline mean. The JMV2959 pretreatment transiently significantly reduced the cannabinoid-induced 2-AG elevation within 20 min and 60–80 min intervals ($p < 0.001$) and 40 min and 120 min intervals ($p < 0.05$) by 5.5% to a maximum of 114.5% of the baseline. Despite JMV2959 pretreatment, the WIN55,212-2-induced accumbens 2-AG elevation remained significant relative to the vehicle + saline group within 40–140 min intervals.

The two-way ANOVA RM/Bonferroni analysis of accumbens GABA measurements (Figure 8C) revealed an overall effect of the treatment ($F_{3,8} = 52.4$; $p < 0.001$), time ($F_{8,168} = 8.5$; $p < 0.001$) and the treatment × time interaction ($F_{8,168} = 24.7$; $p < 0.001$). The WIN55,212-2 administration into the VTA induced a significant decrease of accumbens GABA extracellular levels within 20–120 min intervals ($p < 0.001$) with a maximum of 78.9% of the baseline mean at an 80 min interval. When JMV2959 was injected i.p. 20 min before the cannabinoid, we observed an initial moderate diminution of GABA in the NACSh significant relatively to vehicle + saline group within 20 min and 40 min intervals ($p < 0.01$ and $p < 0.001$, respectively) with a maximum of 92.6% of the baseline. However, the drop in accumbens GABA was transformed into a mild but significant increase at the 60 min interval ($p < 0.05$) to a maximum of 106.6% of the baseline at the 80 min interval, and then the GABA levels decreased again back to baseline mean levels (non-significant relative to the vehicle + saline group). Thus, the WIN55,212-2 + saline induced 2-AG decrease in the NACSh was significantly changed/reversed by JMV2959 pretreatment within 60–100 min intervals ($p <$

0.001). A single dose of JMV2959 3 mg/kg i.p. had no effect on accumbens anandamide, 2-AG, or GABA and neither for saline i.p.

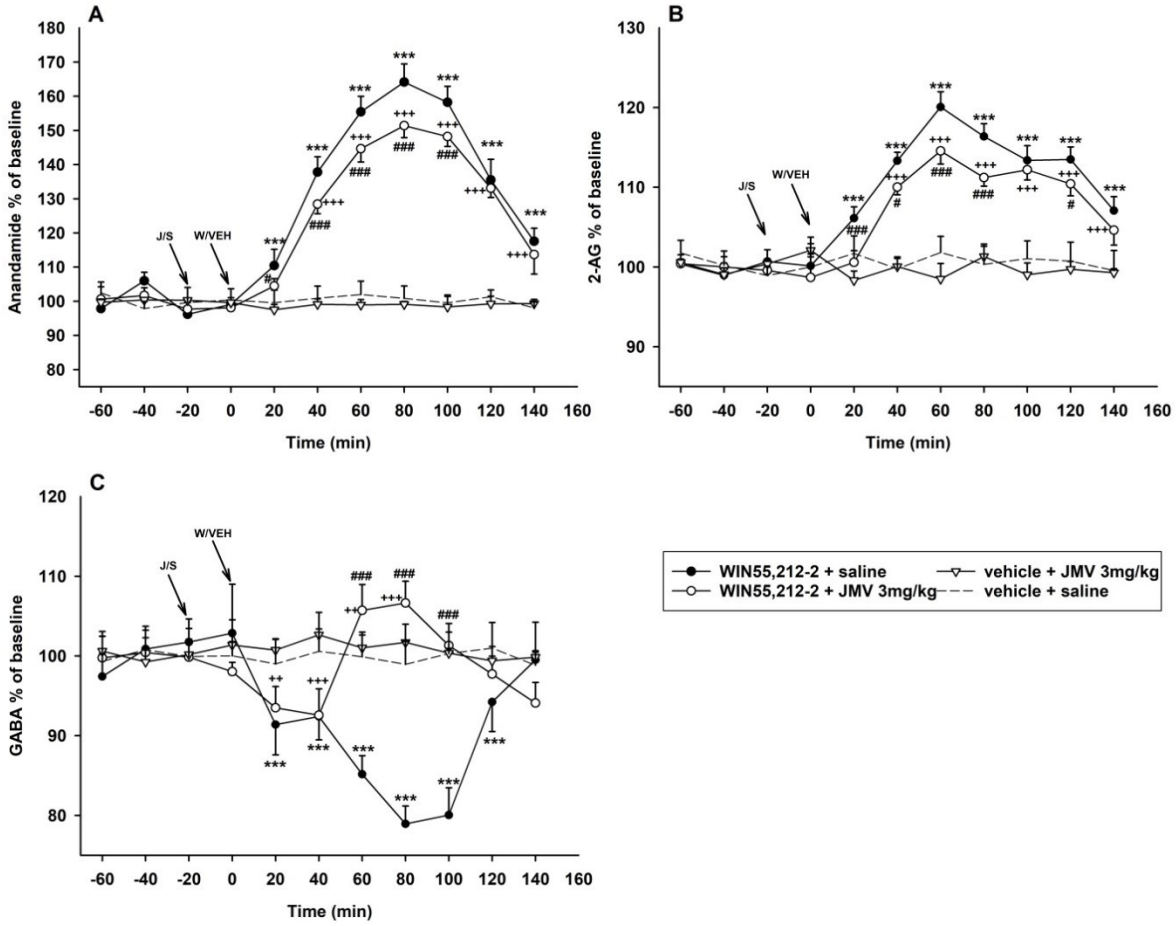

*Figure 8. Effects of GHS-R1A antagonist (JMV2959) on WIN55,212-2-induced endocannabinoid and GABA extracellular changes in the rat NACSh. The JMV2959 3 mg/kg was injected i.p. 20 min before 2.4 mM/0.5 µl WIN55,212-2 or vehicle 0.5 µl administered into the posterior VTA injection (n = 6; means ± SEM). Changes in accumbens anandamide levels are illustrated in graph A, changes in 2-AG levels in the graph B and GABA changes are shown in graph C. The effects are illustrated as follows: saline + WIN55,212-2 (filled circle; n = 7), 3 mg/kg JMV2959 + WIN55,212-2 (open circle; n = 6), 3 mg/kg JMV2959 + vehicle (open triangle; n = 4) and saline + vehicle (dotting; n = 7). Differences between saline + WIN55,212-2 and saline + vehicle groups are expressed as ***p < 0.001. Differences between JMV2959 + WIN55,212-2 and saline + vehicle groups are expressed as +++p < 0.001, ++p < 0.01. Differences between groups saline + WIN55,212-2 and 3 mg/kg JMV2959 + WIN55,212-2 are expressed as ###p < 0.001, #p < 0.05.*

8.4. The JMV2959 effects on the μ-selective opioid fentanyl-induced changes of anandamide, 2-AG and GABA in the NACSh

The influence of intraperitoneally administered ghrelin antagonist on fentanyl-induced extracellular anandamide increase and 2-AG increase in the NACSh are illustrated in Figure 9. The acute systemic fentanyl (30 μg/kg s.c.) administration evoked a statistically significant efflux of anandamide in the NACSh. The two-way ANOVA for repeated measures (RM) followed by Bonferroni's multiple comparisons procedure has shown a significant group effect: saline + fentanyl 30 μg/kg vs. saline + saline group ($F_{1,10} = 813.8$, $p < 0.001$) and time effect ($F_{10,100} = 81.9$, $p < 0.001$); time course of anandamide changes in the NACSh after saline/fentanyl injection differed significantly between the two groups of rats (time × group interaction, $F_{10,100} = 86.8$, $p < 0.001$). The fentanyl-induced anandamide increase reached the maximum effect 220% of baseline mean level 60 min after fentanyl administration ($p < 0.001$). The acute systemic fentanyl administration also induced a statistically significant decrease of NACSh 2-AG with maximum drop 81% of baseline level 1 h after fentanyl administration: saline + fentanyl 30 μg/kg vs. saline + saline: effect of group $F_{1,10} = 197.6$, $p < 0.001$; effect of time $F_{10,100} = 18.4$, $p < 0.001$; time × group interaction $F_{10,100} = 16.0$, $p < 0.001$.

Pre-treatment with the GHS-R1A antagonist JMV2959 administered intraperitoneally (i.p.) turned the fentanyl-induced accumbens anandamide increase and induced a significant decrease with the maximum drop 50% of baseline mean level. Thus, the JMV2959 pre-treatment effect was highly statistically significant: JMV2959 3 min/kg + fentanyl 30 μg/kg vs. saline + fentanyl 30 μg/kg: effect of group $F_{1,10} = 217.3$, $p < 0.001$; effect of time $F_{10,100} = 7.9$, $p < 0.001$; time × group interaction $F_{10,100} = 78.0$, $p < 0.001$. Observed changes within the JMV2959 pre-treatment group in comparison to baseline were also significant ($p < 0.001$) (JMV2959 3 mg/kg + saline vs. saline + saline: effect of group $F_{1,10} = 18.7$, $p < 0.01$; effect of time $F_{10,100} = 14.9$, $p < 0.001$; time × group interaction $F_{10,100} = 13.7$, $p < 0.001$). The JMV2959 pre-treatment induced decrease/reversal of accumbens anandamide was observed within about 20–150 min after fentanyl administration, then the anandamide levels crossed the baseline levels and reached a significant anandamide increase with maximum 117% of baseline.

Pre-treatment with JMV2959 intensified the fentanyl-induced accumbens 2-AG decrease. The 3 mg/kg i.p. JMV2959 pre-treatment significantly deepened the fentanyl-induced 2-AG drop into maximum 59% of baseline level: JMV2959 3 mg/kg + fentanyl 30 μg/kg vs. saline + fentanyl 30 μg/kg: effect of group $F_{1,10} = 566.1$, $p < 0.001$; effect of time

F10,100 = 246.0, p < 0.001; time × group interaction F10,100 = 33.1, p < 0.001. (JMV2959 3 mg/kg + fentanyl 30 µg/kg vs. saline + saline: effect of group F1,10 = 391.4, p < 0.001; effect of time F10,100 = 321.5, p < 0.001; time × group interaction F10,100 = 290.7, p < 0.001). A single dose of JMV2959 3 mg/kg i.p. had no effect on accumbens anandamide/2-AG and neither for saline i.p.

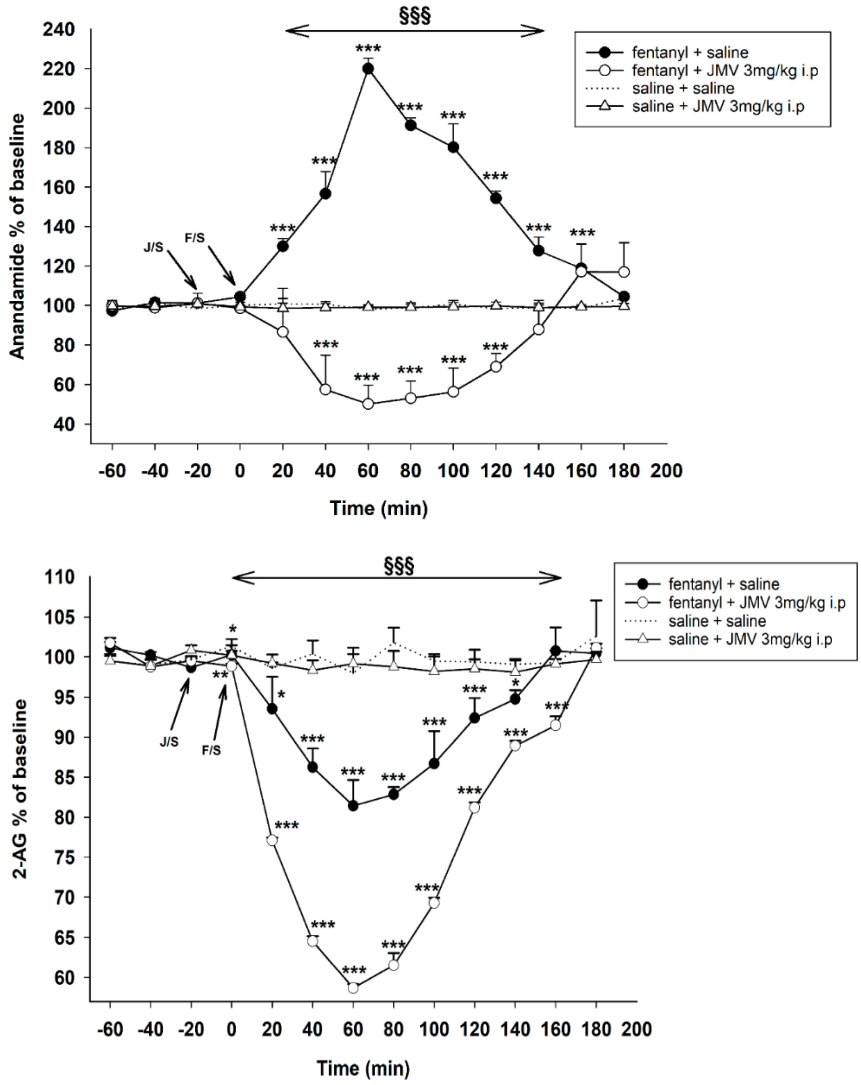

*Figure 9. Effects of the ghrelin receptor antagonist JMV2959 3 mg/kg administered intraperitoneally (i.p.) on the fentanyl-induced accumbens anandamide and 2-AG levels. JMV2959 was administered following three 20 min baselines and 20 min before fentanyl/saline (intervals: baseline = −60 to −20 min; JMV2959 pre-treatment = 0 min; fentanyl = 20–180 min) (means ± SEM). The effects are illustrated as follows: saline + fentanyl (filled circle), 3 mg/kg JMV2959 + fentanyl (open circle), 3 mg/kg JMV2959 + saline (open triangle), saline + saline (dotting). Differences to saline + saline group are expressed as *** p < 0.001, ** p < 0.01, * p < 0.05. Differences between fentanyl + saline and 3 mg/kg JMV2959 + fentanyl effects are expressed as §§§ p < 0.001. The horizontal arrow shows intervals with appropriate significant changes (§§§); the oblique arrows show the time of administration of J/S = JMV2959/saline and F/S = fentanyl/saline*

The influence of intraperitoneally administered ghrelin antagonist on fentanyl-induced extracellular GABA increase in the NACSh are illustrated in Figure 10. The acute systemic fentanyl (30 µg/kg s.c.) administration induced a statistically significant increase of GABA in the NACSh (saline + fentanyl 30 µg/kg vs. saline + saline: effect of group $F_{1,10} = 105.0$, $p < 0.001$; effect of time $F_{10,100} = 57.8$, $p < 0.001$; time × group interaction $F_{10,100} = 56.4$, $p < 0.001$; maximum increase 192% of baseline level). The GHS-R1A antagonist, JMV2959 i.p. administration 20 min before fentanyl prevented the fentanyl-induced accumbens GABA increase maintaining its concentration almost on the baseline level: JMV2959 3 min/kg + fentanyl 30 µg/kg vs. saline + fentanyl 30 µg/kg: effect of group $F_{1,10} = 158.1$, $p < 0.001$; effect of time $F_{10,100} = 65.2$, $p < 0.001$; time × group interaction $F_{10,100} = 55.9$, $p < 0.001$. Accumbens GABA levels within the JMV2959 + fentanyl group were significantly above the saline levels during the first four intervals after fentanyl (JMV2959 3 mg/kg + saline vs. saline + saline: effect of group $F_{1,10} = 50.1$, $p < 0.05$; effect of time $F_{10,100} = 4.6$, $p < 0.001$; time × group interaction $F_{10,100} = 4.9$, $p < 0.001$; maximum increase 112% of baseline). A single dose of JMV2959 3 mg/kg i.p. had no effect on accumbens GABA and neither for saline i.p.

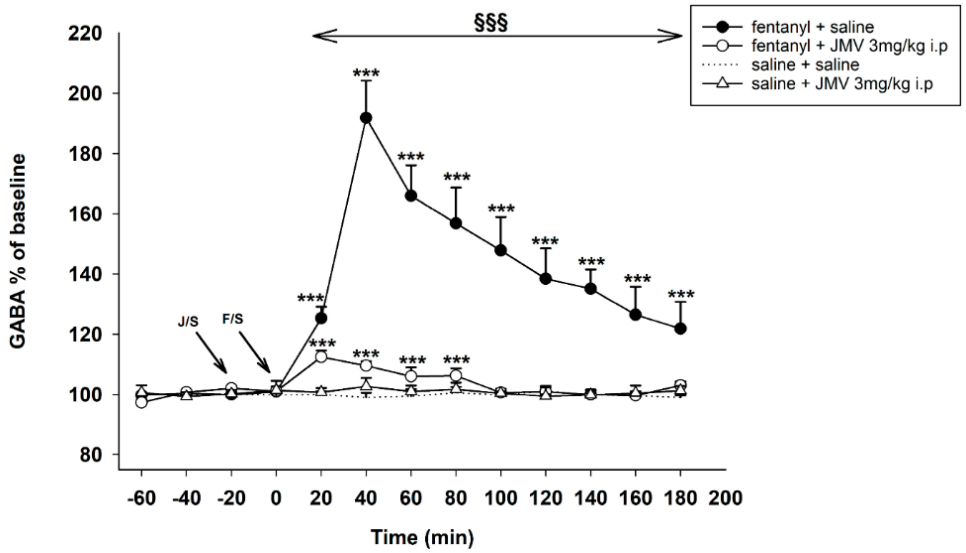

*Figure 10. Effects of GHS-R1A antagonist JMV2959 3 mg/kg i.p. on the fentanyl-induced accumbens γ-aminobutyric (GABA) concentration. JMV2959 was administered following three 20 min baselines and 20 min before fentanyl/saline (means ± SEM). The effects are illustrated as follows: saline + fentanyl (filled circle), 3 mg/kg JMV2959 + fentanyl (open circle), 3 mg/kg JMV2959 + saline (open triangle), saline + saline (dotting). Differences to saline + saline group are expressed as *** $p < 0.001$. Differences between fentanyl + saline and 3 mg/kg JMV2959 + fentanyl effects are expressed as §§§ $p < 0.001$. The horizontal arrow shows intervals with appropriate significant changes (§§§); the oblique arrows show the time of administration of J/S= JMV2959/saline and F/S = fentanyl/saline.*

8.5. The JMV2959 effects on the μ-selective opioid fentanyl-induced changes of accumbens anandamide and 2-AG when administered into the VTA or NACSh

Pre-Treatment with JMV2959 administered into the VTA

The observed influence of ghrelin antagonist, administered into the VTA, on changes in accumbens anandamide and 2-AG induced by 30 μg/kg s.c. fentanyl can be read in detail at Sustkova-Fiserova et al., 2017 (Sustkova-Fiserova et al. 2017).

The 30 μg/kg dose of fentanyl together with intra-VTA Ringer's solution induced practically the same anandamide increase as described above with fentanyl and systemic saline. The lower dose (2 μg) pre-treatment caused a drop of anandamide accumbens levels to the baseline concentration and the higher dose (10 μg) induced significant anandamide decrease. The effects of JMV2959 2 μg/VTA with fentanyl 30 μg/kg on the accumbens anandamide did not significantly differ from Ringer's solution/VTA with saline. A single dose of JMV2959 2 μg as well as 10 μg/VTA, similarly to Ringer's solution/VTA had no significant effect on accumbens anandamide.

The 30 μg/kg dose of fentanyl together with intra-VTA Ringer's solution induced practically the same 2-AG decrease as described above with fentanyl and systemic saline. Pre-treatment with JMV2959 influenced the fentanyl-induced accumbens 2-AG decrease differently depending on the given dose. The 2 μg JMV2959/VTA dose slightly but significantly attenuated and simultaneously also prolonged the fentanyl-induced 2-AG decrease. The 10 μg JMV2959/VTA dose significantly deepened the accumbens fentanyl-induced 2-AG decrease. In comparison to the control group the lower 2 μg/kg JMV2959 dose with fentanyl significantly decreased the accumbens 2-AG. The higher 10 μg JMV2959 dose with fentanyl, also induced significant 2-AG decrease in the NACSh. A single dose of JMV2959 2 μg as well as 10 μg/VTA did not significantly influence the accumbens 2-AG levels, the same as Ringer's solution/VTA with saline s.c.

Pre-Treatment with JMV2959 Administered into the NAc

The observed influence of ghrelin antagonist administered into the NACSh on accumbens fentanyl-induced anandamide and 2-AG changes can be read in detail at Sustkova-Fiserova et al., 2017 (Sustkova-Fiserova et al. 2017).

The 30 μg/kg fentanyl effects on accumbens anandamide were practically the same with both pre-treatment with saline i.p. and Ringers's solution into the VTA. Thus, for ethical

reasons, we did not create a new group with fentanyl 30 μg/kg without any pre-treatment, but we have used the group with systemic saline pre-treatment instead for testing the JMV2959 effects when administered into the NACSh.

We have used the dialysis probe for administration of JMV2959 into the NAC. Pre-treatment with both JMV2959 doses into the NACSh dose dependently reversed the fentanyl-induced accumbens anandamide increase. The JMV2959/NAc pre-treatment induced decrease/reversal of accumbens anandamide after fentanyl administration were observed only during first intervals, the anandamide levels returned to baseline levels at about 90 min (lower JMV2959 dose) and 120 min (higher JMV2959 dose) after fentanyl administration. Administration of single lower 8 mM JMV2959 dose into the NAC and saline i.p. did not significantly influence the accumbens anandamide. Administration of single higher 40 mM JMV2959 dose induced slight but significant anandamide decrease.

The 30 μg/kg fentanyl effects on accumbens 2-AG were practically the same with both, pre-treatment with saline i.p. as well as Ringers's solution into the VTA. Thus, again, we have used the group with systemic saline pre-treatment of fentanyl for testing the JMV2959 effects when administered into the NAc. Pre-treatment with both JMV2959 doses into the NAC significantly and dose dependently deepened the fentanyl-induced accumbens 2-AG extracellular concentrations. Both JMV2959 pre-treatments with fentanyl induced significant 2-AG decrease in comparison to saline + saline. Single lower JMV2959 dose administered into the NAC and saline i.p. did not significantly influence the accumbens 2-AG. Administration of single higher JMV2959 dose 40 mM/15 min/NAC induced slight but significant 2-AG decrease.

8.6. JMV2959 effects on THC/WIN55,212-2 -induced behavioural stimulation

In comparison to the vehicle + saline group, the 0.1 mg/kg i.p. THC dose produced significant behavioural stimulation in the habituated rats monitored using the LABORAS apparatus within 20–40 min after the THC administration (Figure 11), which is in accordance with the literature (Bardo and Bevins 2000). Following THC administration, the locomotion, rear, distance travelled, and average speed were significantly increased, while immobility was significantly decreased in comparison to the saline + vehicle group. The 1 or 3 mg/kg JMV2959 administered 20 min before THC, significantly and dose-dependently reduced the THC-induced changes in all monitored parameters using one-way ANOVA followed by Holm–Sidak test, specifically: locomotion duration ($F_{5,38} = 4.66$, $p = 0.002$) (see Figure 11A), rear duration

($F_{5,38} = 5.37$, $p < 0.001$) (Figure 11B), immobility duration ($F_{5,38} = 5.19$, $p < 0.001$) (Figure 11C), distance travelled ($F_{5,38} = 5.78$, $p < 0.001$) (Figure 11D), and average speed overall ($F_{5,38} = 6.60$, $p < 0.001$) (Figure 11E). Both doses of JMV2959 administered alone/with the vehicle did not cause significant changes in rat behaviour during the monitored period in comparison to the vehicle + saline group.

Within 20–40 min after administration, the 0.1 mg/kg i.p. WIN55,212-2 dose produced significant behavioural stimulation in the habituated rats monitored using the fully automated LABORAS apparatus, in comparison to the vehicle + saline group (Figure 12). This is in accordance with the literature (Polissidis et al. 2009; Polissidis et al. 2013). Following WIN55,212-2 administration, the locomotion, rear, distance travelled, and average speed significantly increased, while immobility significantly decreased in comparison to the saline + vehicle group. The 1 or 3 mg/kg JMV2959 administered 20 min before WIN55,212-2, dose-dependently reduced the WIN55,212-2-induced changes in all monitored parameters using one-way ANOVA followed by the Holm–Shidak test; however, only pretreatments with the higher dose, 3 mg/kg JMV2959, reached significance in their effects; specifically: locomotion duration ($F_{5,37} = 3.65$, $p = 0.009$) (Figure 12A), rear duration ($F_{5,37} = 5.88$, $p < 0.001$) (Figure 12B), immobility duration ($F_{5,37} = 5.72$, $p < 0.001$) (Figure 12C), distance travelled ($F_{5,37} = 4.22$, $p = 0.004$) (Figure 12D) and average speed overall ($F_{5,37} = 5.59$, $p < 0.001$) (Figure 12E). Both doses of JMV2959 administered alone/with vehicle did not cause significant changes in rat behaviour during the monitored period in comparison to the vehicle + saline group.

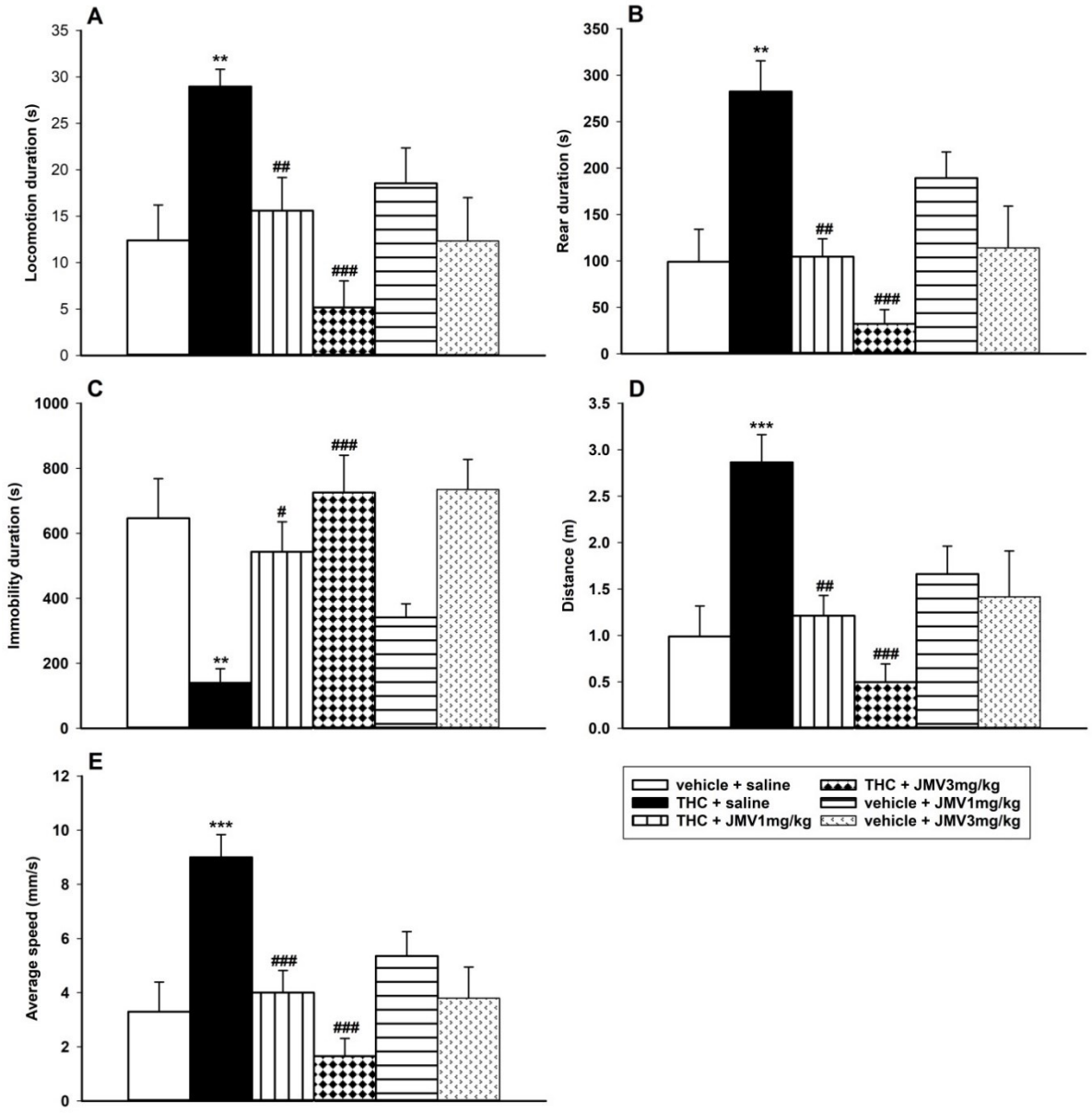

*Figure 11. Effects of JMV2959 on the THC-induced behavioural changes in rats in the LABORAS apparatus. JMV2959 (0, 1, 3 mg/kg i.p.) was administered immediately before placing the rats into the apparatus and after 20 min of habituation, a stimulatory THC dose 0.1 mg/kg or saline was administered intraperitoneally. After another 20 min of habituation, behaviour monitoring started and lasted for 20 min (20–40 min after THC administration). Changes in locomotion duration (A), rear duration (B), immobility duration (C), distance traveled (D), and average speed overall (E) are illustrated as follows: saline + vehicle (open bar) (n = 9), saline + THC (filled bar) (n = 7),1 mg/kg + THC (vertically striped bar) (n = 8), JMV2959 3 mg/kg + THC (diamond bar) (n = 8), JMV2959 1mg/kg + vehicle (horizontally striped bar) (n = 4), JMV2959 3 mg/kg + vehicle (little arrows bar) (n = 8). The JMV2959 pretreatment effects in comparison to saline + THC group are expressed as # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$. Differences between groups in comparison to vehicle + saline group are expressed as ** $p < 0.01$, *** $p < 0.001$. The results are presented as group means with 95% confidence intervals.*

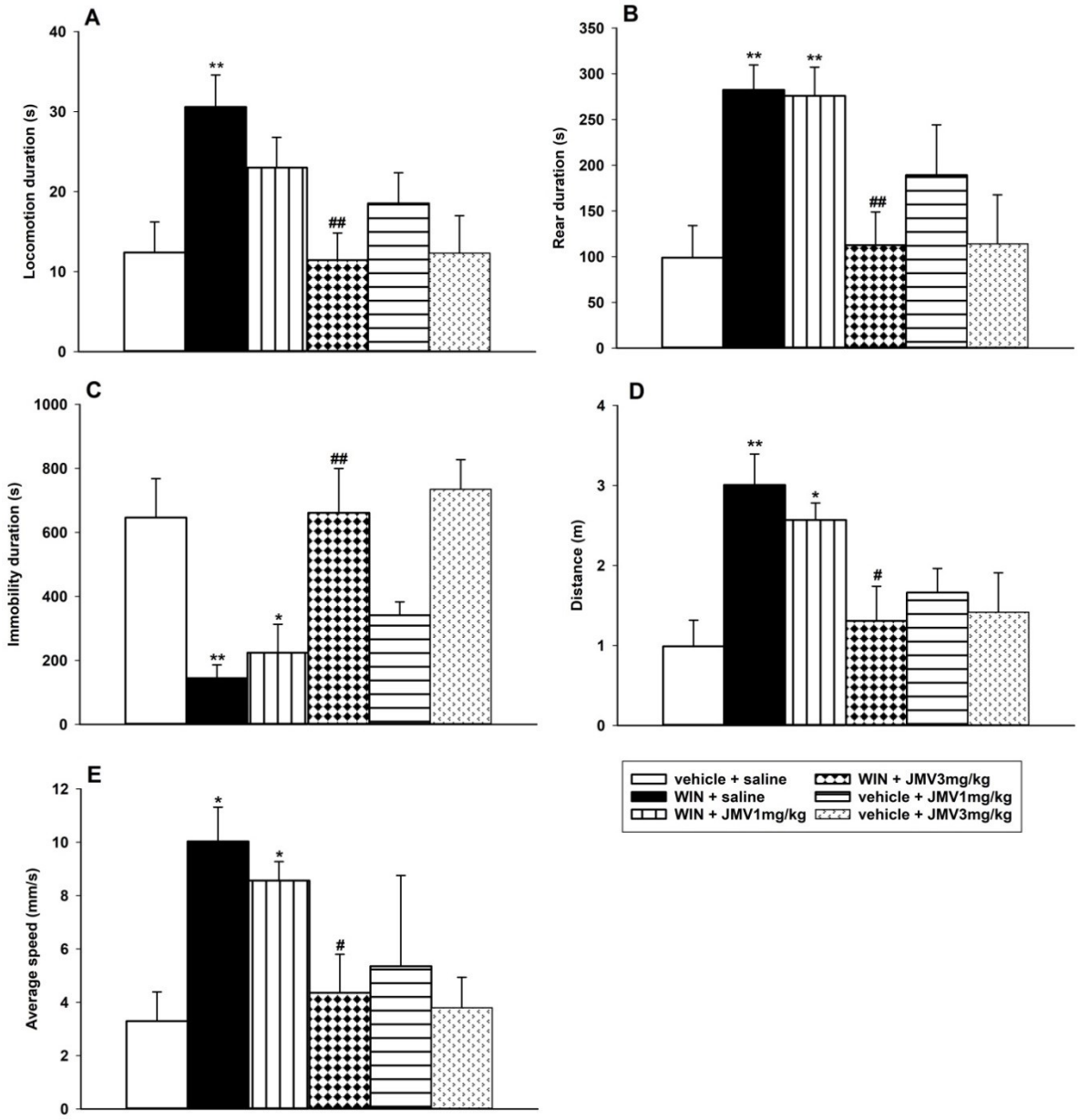

*Figure 12. Effects of JMV2959 on the WIN55,212-2 -induced behavioural changes in rats in the LABORAS apparatus. JMV2959 (0, 1 and 3 mg/kg i.p.) was injected immediately before placing the rat into the cage, and after 20 min of habituation a stimulatory WIN55,212-2 dose 0.1 mg/kg or vehicle was administered intraperitoneally. After another 20 min of habituation, behaviour monitoring started and lasted for 20 min (20–40 min after WIN55,212-2 administration). Changes in locomotion duration (A), rear duration (B), immobility duration (C), distance travelled (D) and average speed overall (E) are illustrated as follows: saline + vehicle (open bar; n = 9), saline + WIN55,212-2 (filled bar; n = 7), JMV2959 1 mg/kg + WIN55,212-2 (vertically striped bar; n = 7), JMV2959 3 mg/kg + WIN55,212-2 (diamond bar; n = 8), JMV2959 1 mg/kg + vehicle (horizontally striped bar; n = 4), JMV2959 3 mg/kg + vehicle (little arrows bar; n = 8). The JMV2959 pretreatment effects in comparison to saline + WIN55,212-2 group are expressed as # $p < 0.05$, ## $p < 0.01$. Differences among groups in comparison to vehicle + saline group are expressed as * $p < 0.05$, ** $p < 0.01$.*

9. Discussion

The suggested hypotheses and the presented results indicate interactions among the GHS-R1A, the CB1R, and the μ-opioid receptors within the brain mesolimbic system. The GHS-R1A, CB1 and μ-opioid receptors are expressed within the NACSh as well as the VTA, thus, interaction among the appropriate signalling systems within these brain structures could be considered (Ferrini et al. 2009; Befort 2015; Fattore et al. 2005; Maldonado, Valverde, and Berrendero 2006; Pickel et al. 2004; Gomes et al. 2013). Midbrain GHS-R1As are co-localized with dopaminergic and cholinergic receptors (Ferrini et al. 2009; Guan et al. 1997) and presumably interact in amplification of the dopaminergic signalling in the VTA neurons and stimulate accumbens dopamine efflux (Jerlhag et al. 2011; Jerlhag et al. 2006). The ability of ghrelin antagonism to decrease morphine-induced NACSh dopamine efflux was documented previously (Engel, Nylander, and Jerlhag 2015; Sustkova-Fiserova et al. 2014). The brain endocannabinoid system is important for the regulation of the dopamine signalling during reinforcement processes (Solinas, Goldberg, and Piomelli 2008; Lupica and Riegel 2005). It was also suggested that possible interactions between endocannabinoids and central ghrelin signalling and the functional cooperation of CB1/endocannabinoids and GHS-R1A/ghrelin within hypothalamus may contribute to ghrelin's orexigenic effects (Cani et al. 2004; Kola et al. 2008; Folgueira, Seoane, and Casanueva 2014; Al Massadi et al. 2017; Tucci et al. 2004).

Cannabinoids including the main cannabis constituent tetrahydrocannabinol (THC) and synthetic cannabinoids (e.g., WIN55,212-2) most likely mediate their pleasurable, anxiolytic, and rewarding/reinforcing effects through the CB1Rs located within the central brain reward circuits, particularly the VTA and the NAc (Parsons and Hurd 2015; Herkenham 1991; Matsuda et al. 1990). In our in vivo microdialysis study, the local administration of the low 2.4 mM/ 0.5 μL dose of WIN55,212-2 into the posterior VTA induced significant dopamine efflux in the NACSh of Wistar rats in accordance with the literature (Volkow, Hampson, and Baler 2017; Zehra et al. 2018; Parsons and Hurd 2015; Bloomfield et al. 2016; Zangen et al. 2006; Polissidis et al. 2009). The intracerebral 2.4 mM dose of WIN55,212-2 induced dopamine release with a maximum of 131% of the baseline mean, which approximately corresponds with published microdialysis experiments when WIN55,212-2 was administered systemically (i.p., i.v.) and the dopamine release was prevented by CB1 antagonist (rimonabant) co-administration (Tanda, Pontieri, and Di Chiara 1997; Polissidis et al. 2009). The intra-VTA administration of WIN55,212-2 also increased accumbens extracellular concentrations of dopamine degradation metabolites: specifically, the 3-methoxytyramine/3-MT (a maximum of

114% of the baseline mean), 3,4-dihydroxyphenylacetic acid/DOPAC (133%) and homovanillic acid/HVA (142% of the baseline mean). The maximal microdialysate concentration values (40 and 60 min intervals after WIN55,212-2 administration) were used for calculation of the extracellular dopamine metabolic turnover ratios. The cannabinoid application significantly increased the extracellular dopamine metabolic turnover HVA/DA ratio ($p < 0.05$) in comparison with the control/vehicle + saline group. The 3-MT/DA ratio significantly decreased ($p < 0.001$) and the DOPAC/DA ratio increased ($p < 0.001$). Increases in dopamine metabolism, measured with the DOPAC/DA ratio, have been reported in most but not all rodent studies with cannabinoids (Bloomfield et al. 2016; Polissidis et al. 2010). Some resulting inconsistencies within the early ex-vivo studies could be explained due to the technical limitations in detecting the rapid changes in extracellular dopamine/metabolite concentration, detectable by microdialysis techniques used more recently (Bloomfield et al. 2016).

Pretreatment with the GHS-R1A antagonist/JMV2959 3 mg/kg i.p. 20 min before WIN55,212-2 significantly reduced the cannabinoid-induced dopamine efflux in the NACSh. The same 3 mg/kg i.p. JMV2959 dose "per se" did not influence accumbens dopamine, as it has already been described (Jerlhag et al. 2009; Engel, Nylander, and Jerlhag 2015; Sustkova-Fiserova et al. 2019). Also, the lower JMV2959 dose 1 mg/kg alone did not induce significant changes in accumbens dopamine/metabolites. The observed GHS-R1A antagonist effect is in accordance with previous studies in mice/rats experimental models with alcohol (Jerlhag et al. 2009), stimulants/cocaine, amphetamine (Jerlhag et al. 2010), nicotine (Jerlhag and Engel 2011) and opioids/morphine and fentanyl (Engel, Nylander, and Jerlhag 2015; Sustkova-Fiserova et al. 2014; Sustkova-Fiserova et al. 2019). Contrary to alcohol and stimulant models, the JMV2959 pretreatment did not completely abolish the cannabinoid-induced accumbens dopamine increase. The dopamine extracellular concentrations remained transiently significantly increased (108% of the baseline mean) in comparison with baseline and the vehicle + saline group, similar to the studies with opioids, when the JMV2959 pretreatment effects were weaker (Engel, Nylander, and Jerlhag 2015; Sustkova-Fiserova et al. 2014; Sustkova-Fiserova et al. 2019). However, in the present study, the JMV2959 + WIN55,212-2-induced transient dopamine increase (40–80 min intervals) was changed to transient moderate but significant decrease (in the 120 min interval; maximum of 86% of the baseline mean, $p < 0.001$), which was not observed in previous studies. The observed gradually intensified JMV2959 pretreatment effect could indicate some modulatory mechanisms of the GHS-R1A antagonism specifically in combination with the cannabinoid. However, in contrast

to previous studies, when the drugs were administered systemically (s.c. or i.v.), the cannabinoid was injected directly into the VTA, which also may impact the effect proportions. Nevertheless, the GHS-R1A antagonism significantly reduced the cannabinoid/CB1R-induced accumbens dopamine efflux, the trigger of reinforcement effects, which indicates the important involvement of the ghrelin/GHS-R1A system in the cannabinoid reinforcement effects, hence, addiction processing. Concerning the GHS-R1As, it was described that intra-VTA administered ghrelin induced accumbens dopamine release and the intra-VTA GHS-R1A antagonist supressed this effect (Jerlhag et al. 2006; Jerlhag et al. 2007). The GHS-R1A is expressed on dopaminergic neurons (Tucci et al. 2004), and it has been suggested that GHS-R1A regulates the activity of VTA dopamine neurons via heterodimerization of the GHS-R1A to the D1R (especially with stimulants), as well as by the strong constitutive activity of the GHS-R1A (Holst et al. 2003; Jiang, Betancourt, and Smith 2006). Further, the midbrain GHS-R1As, co-localized with dopaminergic and cholinergic receptors (Ferrini et al. 2009; Guan et al. 1997), activate the cholinergic– dopaminergic reward link and trigger the VTA dopaminergic signalling, which can be blocked by intra-VTA administration of nicotinic cholinergic and glutamatergic receptor antagonists (Jerlhag et al. 2006; Jerlhag et al. 2011; Jerlhag et al. 2012). Also, GHS-R1A is known to modulate synaptic plasticity in the brain (Serrenho, Santos, and Carvalho 2019; Castaneda et al. 2010). The GHS-R1As are also expressed on the GABA VTA neurones (Serrenho, Santos, and Carvalho 2019), however, the ghrelin-induced activation of VTA dopamine neurons was not blocked in presence of GABA-A antagonist (Abizaid et al. 2006). In the hypothalamus, the GHS-R1A is present at GABAergic presynaptic terminals, and it attenuates GABA release in the hypothalamic neurons (Lopez Soto et al. 2015). CB1-dependent long-term depression (LTD) in the VTA GABA neurons induced by cannabinoid/THC has been described (Friend et al. 2017).

The accumbens extracellular dopamine metabolic turnover increase, induced by intracerebral WIN55,212-2 during its maximal effect (40 and 60 min intervals) in the present study, was significantly augmented after JMV2959 pretreatment in comparison to the WIN55,212-2 + saline group, measured with metabolic ratios HVA/DA, DOPAC/DA and 3-MT/DA ($p < 0.001$). The substantial increase in final metabolite HVA formation (HVA/DA ratio) was produced by enhanced DOPAC production (DOPAC/DA ratio). This is in accordance with our previous opioid microdialysis studies when a significantly increased formation of DOPAC and HVA in the NACSh after JMV2959 pretreatment before morphine and fentanyl was observed (Sustkova-Fiserova et al. 2014; Sustkova-Fiserova et al. 2019). Thus, a significant impact of JMV2959 pretreatment was observed on cannabinoid- and opioid-

provoked accumbens dopamine metabolism due to the monoamine oxidase (MAO) (the significant DOPAC and DOPAC/DA increase). Moreover, the relatively high 6 mg/kg i.p. JMV2959 dose, when administered alone, moderately but significantly increased DOPAC and HVA accumbens levels in rats (Sustkova-Fiserova et al. 2014); we did not find any significant influence of single JMV2959 3 mg/kg on the accumbens dopamine/metabolite concentrations (Sustkova-Fiserova et al. 2014; Sustkova-Fiserova et al. 2019). Altogether, it seems that GHS-R1A antagonism might be associated with increased metabolism of dopamine by MAO.

In our study, the WIN55,212-2 intra-VTA administration also induced a significant decrease in the γ-aminobutyric acid/GABA extracellular concentrations in the NACSh (maximum of 79% of the baseline in an 80 min interval), which was normalised within the last 140 min interval. The accumbens GABA decrease was presumably a consequence of the endocannabinoid presynaptic modulations (Depolarization-induced suppression of inhibition - DSI and Long-term depression LTD) within the NAc, where CB1Rs are located on both the medium spiny neurons (MSN) collaterals and on intrinsic GABA neuron terminals, just as in the VTA (Lupica, Riegel, and Hoffman 2004; Friend et al. 2017). The CB1R agonists (CP55940, THC) were found to decrease the accumbens GABA release (Manzoni and Bockaert 2001). It was suggested that the (endo)cannabinoid-induced inhibition of GABA release onto the MSNs in the NAc might increase/cause the disinhibition effect of the GABAergic output to the VTA and thus further participate on the cannabinoid reinforcement effects (Lupica and Riegel 2005). The JMV2959 pretreatment reversed the cannabinoid-induced accumbens GABA decrease to transiently significant increase (maximum of 107% of the baseline mean in the 80 min interval), which again was normalised within the last 140 min interval. The single 3 mg/kg JMV2959 dose did not significantly influence the accumbens GABA levels as in previous experiments (Sustkova-Fiserova et al. 2014); however, accumbens GABA levels are influenced by opioids and cannabinoids in different ways (Aono et al. 2008; Saigusa et al. 2008). The GHS-R1As are expressed on GABA neurons in the VTA, the NAc and elsewhere in the brain (Serrenho, Santos, and Carvalho 2019). Important interactions between ghrelin and GABA systems have been implicated within the CeA (Cruz et al. 2013). The GHS-R1As are located on the hypothalamic GABAergic presynaptic terminals; the GHS-R1A constitutive activity itself and administration of the GHS-R1A agonist attenuated GABA release. Presumably, the GHS-R1A antagonist and/or inverse agonist would reduce/abolish this effect. Similar interaction can be presumed within the NAc/VTA (Serrenho, Santos, and Carvalho 2019). It seems that ghrelin and endocannabinoids show similar synaptic plasticity mechanisms, so they might cooperate on GABAergic neurons, where they are both located on

the presynaptic axons; moreover, the CB1/GHS-R1A dimers were indicated (Wellman and Abizaid 2015).

In the fentanyl experiments, pretreatment with JMV2959 in all doses and types of administration (i.p., into the VTA or NAc) significantly changed the fentanyl-induced accumbens anandamide increase and the 2-AG decrease. The fentanyl evoked anandamide increase was reversed by the pretreatment with the intraperitoneal 3 mg/kg dose of JMV2959, inducing a significant decrease. The fentanyl-induced 2-AG decrease was significantly intensified by the 3 mg/kg i.p. JMV2959. This is fully in accordance with the Sustkova-Fiserova et al. 2016 study with morphine and the intraperitoneal JMV2959 pretreatment (Sustkova-Fiserova et al. 2016). The fentanyl-induced accumbens anandamide increase was reversed when fentanyl s.c. was administered together with JMV2959 into the NAc (perfusion with 8 or 40 mM for 15 min); however, the observed significant dose-dependent anandamide decrease had a sharper drop and shorter duration in comparison with the intraperitoneal JMV2959 effect, which was possibly due to the local (NAc) type of JMV2959 administration. The intra-VTA pretreatment with JMV2959 (2 or 10 μg) prevented the fentanyl-induced accumbens anandamide increase, but only the higher dose reversed the anandamide levels to partly significant decrease. It is difficult to compare effects of differently administered various doses, but considering the effects of the pretreatment JMV2959 doses, which alone did not significantly influence the accumbens anandamide, it can be suggested that GHS-R1A receptors of both VTA and NAc brain structures participate in the significant JMV2959 reversal effects on the fentanyl/opioid-induced anandamide increase in the NASh, with major involvement of the NAc structure. The GHS-R1A antagonism significantly affected the selective μ-opioid fentanyl-induced accumbens anandamide changes which are believed to contribute to opioid reinforcement. Also, all pre-treated JMV2959 doses and types of administration affected the fentanyl-induced anandamide increase in a similar manner without showing significant influence on the anandamide levels. The JMV2959 3 mg/kg i.p. dose and the doses administered into the VTA and lower dose into the NAc did not induce significant changes in accumbens anandamide, although the higher 40 nM/NAc dose produced slight but significant anandamide decrease (94% of baseline mean). This corresponds with the Sustkova-Fiserova et al. 2016 study, when 6 mg/kg i.p. JMV2959 dose induced significant anandamide drop to 92% of baseline and 3 mg/kg i.p. dose did not have a significant influence (Sustkova-Fiserova et al. 2016). This indicates a presumable complexity of possibly several ghrelin involving neural pathways participating in the observed antagonism of fentanyl-induced

accumbens anandamide increase. Possible cooperation of two or more neural systems and/or indirect effects might be considered.

The role of ghrelin in the accumbens dopamine-involving changes in the rewarding/reinforcing processing was previously described (Jerlhag et al. 2012). Also, it was documented, that ghrelin's orexigenic effects are dependent on several central networks, such as dopamine, cannabinoid, opioid and serotonine systems (Al Massadi et al. 2017). In addition, the high constitutive activity of the GHS-R1A might possibly play some role in the ghrelin´s effects (Holst et al. 2003). Blocking of the GHS-R1A by JMV2959 pre-treatments reduced several observed fentanyl-induced effects, which suggests that the ghrelin/GHS-R1A at least partly contribute in these massive pre-treatment effects, although the JMV2959 doses were not effective per se. As mentioned earlier, the opioid-induced anandamide increase in the NACSh possibly contribute to the opioid reinforcement through CB1 receptor-mediated process independent of dopamine (Caille and Parsons 2003, 2006; Caille et al. 2007). Our presented results sugget, that the GHS-R1A receptors within the NACSh as well as VTA both participate significantly in the opioid-induced accumbens anandamide increase, with possibly emphasized impact of the NACSh ghrelin signalling. Similarly, to the systemic 3 mg/kg JMV2959 i.p. administered effects, the intra-accumbens administration of JMV2959 (the NAc perfusion with 8 or 40 mM for 15 min) also intensified significantly and dose-dependently the fentanyl-induced 2-AG accumbens decrease. When JMV2959 was administered into the VTA (2 or 10 μg), only the higher JMV2959 dose significantly intensified/deepened the fentanyl-induced 2-AG decrease. The lower JMV2959/VTA dose attenuated but prolonged the fentanyl-induced accumbens 2-AG decrease.

The opioid-induced GABA efflux in the NACSh contributes to the opioid reinforcing properties (Aono et al. 2008; Xi and Stein 2000). The simultaneous activation of μ and GABA-A receptors which are co-expressed on GABAergic interneurons in the NAc, markedly suppresses GABA release onto dopamine nerve endings, thus disinhibiting/enhancing dopamine efflux (Aono et al. 2008). Alternatively, dopamine release may be evoked indirectly through activation of GABA-A and μ receptors on GABAergic medium spiny projection neurons in the VTA (Creed, Ntamati, and Tan 2014; Fields and Margolis 2015). GABA elevated concentrations potentiate opioid-induced accumbens dopamine release. In the present research, the pre-treatment with ghrelin antagonist in all given doses and types of administration prevented the opioid/fentanyl-induced accumbens GABA efflux in a very similar way. These findings suggest that ghrelin antagonism may inhibit opioid-induced dopamine release, which was also described previously in other studies (Engel, Nylander, and

Jerlhag 2015; Sustkova-Fiserova et al. 2016), by attenuating opioid-evoked GABA release in the NACSh. The activation of opioid receptors in the NAc decreases GABA release in their major projection area the ventral pallidum, generating dopamine-independent biological effects. It is documented that CB1, μ as well as GHS-R1As are present on GABA presynaptic terminals in various brain structures (De Vries and Shippenberg 2002; Maldonado, Valverde, and Berrendero 2006; Ting and van der Kooy 2012; Lopez Soto et al. 2015). CB1 receptors are located on inhibitory inputs to GABAergic medium spiny projection neurons in the NAc and it is suggested that endocannabinoids release evoked by depolarization in the NAc and from the VTA dopaminergic neurons may act as retrograde messengers on reachable receptors of GABAergic afferents (Lupica, Riegel, and Hoffman 2004). VTA GABA-A/GABAergic system importantly contributes to the dopamine-independent opioid reinforcement (Ting and van der Kooy 2012; Laviolette and van der Kooy 2001). Interactions between central ghrelin and GABAergic systems have been implicated within the CeA (Cruz et al. 2013) and hypothalamus (Lopez Soto et al. 2015) hereby the observed significant ghrelin and accumbens GABA interaction.

The cannabinoids/CB1R agonists, including WIN55,212-2, are known for their biphasic/ dual effects, with low doses inducing the locomotor hyperactivity and with higher doses the hypoactivity. Behavioural stimulation, frequently observed in drugs of abuse, is considered as a sign of dopaminergic nigrostriatal pathway activation and constitutes to part of the addiction process associated with drugs' reinforcing effects, hence, contributing to drug dependence (Wise 1987; Polissidis et al. 2009; Polissidis et al. 2013; Koob and Volkow 2016). The low cannabinoid/WIN55,212-2 dose (0.1 mg/kg i.p.) increased the horizontal and vertical activity in habituated rats (Polissidis et al. 2009; Polissidis et al. 2013).

In the LABORAS experiment, a significant increase in locomotion, rearing, distance travelled, and the overall average speed of behaviour was observed in comparison to the vehicle treated group, within 20–40 min after the WIN55,212-2 as well as the THC administration. The JMV2959 (1 and 3 mg/kg) administration 20 min before the cannabinoid significantly and dose-dependently reduced the monitored cannabinoid-stimulated behavioural parameters; however, in case of WIN55,212-2 the effect was significant only when the higher dose 3 mg/kg JMV2959 was used ($p < 0.001$). These results are in accordance with previous studies, when JMV2959 in mice or rats reduced locomotor hyperactivity induced by alcohol (Jerlhag et al. 2009), nicotine (Jerlhag et al. 2011), cocaine and amphetamine (Jerlhag et al. 2010), morphine (Engel, Nylander, and Jerlhag 2015; Sustkova-Fiserova et al. 2014) and fentanyl (Sustkova-Fiserova et al. 2017). When JMV2959 was administered alone (in the same doses), no

significant influence on rat behaviour was observed within the relevant period in LABORAS, which corresponds well with the Jerabek et al. 2017 study in activity cage/open field (Jerabek et al. 2017). To summarize, the behavioural stimulation observed within 20–40 min after the 0.1 mg/kg THC as well as WIN55,212-2 intraperitoneal administration was significantly reduced by the GHS-R1A antagonist (JMV2959) pretreatment.

IV. CONCLUSION

Substance use disorders are associated with extensive substantial burdens, including the increased risk for neuropsychological deficits, suicidality, infectious disease, and diminished quality of life (Grant et al. 2015). Addiction is a chronic relapse disease with a multidimensional aetiology and natural history. In order to develop better preventative and therapeutic interventions, which currently remain inadequate, crucial biological mechanisms involved in the pathophysiology of drug addiction should be thoroughly researched. Research must search for new, more effective mechanisms to prevent frequent addiction relapses.

In recent years, evidence from some of the peripheral hormones including ghrelin, affect food intake on mesolimbic dopaminergic system in the brain. These hormones in addition to the known homeostatic energy control food intake which are located in the hypothalamus, its structures are linked with the brain reward system (Narayanan, Guarnieri, and DiLeone 2010). These hormones participate in the energy-homeostasis but also in the hedonic principle of food intake and overeating and are explored in the last few years in the context of their possible involvement in substance abuse.

Our experimental research with the GHS-R1A antagonist JMV2959 in rats demonstrated for the first time the important role of GHS-R1A in the mechanisms of both cannabis and μ-selective opioid fentanyl dependence and significantly contributed to understanding the role of ghrelin / GHS-R1A in the mechanisms of these dependencies. We further corroborated a significant interaction between ghrelin / GHS-R1A systems, endocannabinoids (anandamide/2-AG), GABA and opioid /μ -receptor in the NAc / VTA region (Sustkova-Fiserova et al. 2017; Charalambous et al. 2021; Sustkova-Fiserova et al. 2022). Collectively, all presented results (i) demonstrated that the GHS-R1 antagonism (JMV2959) significantly reduced the cannabinoid/WIN55,212-2 – induced dopamine release in the NACSh; also, (ii) it reduced the WIN55,212-2- and also the fentanyl-induced endocannabinoid (anandamide, 2-AG) and GABA changes in the NACSh. Additionally, (iii) the GHS-R1 antagonism (JMV2959) significantly reduced the CB1R agonist/WIN55,212-2 as well as the THC-induced behavioural stimulation.

These results signify a strong participation of accumbens endocannabinoids, particularly anandamide, but also GABA in the neural opioid/fentanyl reinforcing processes and suggest that ghrelin antagonism may play an important role in the NACSh endocannabinoid/anandamide and GABA changes possibly related to opioid/μ-receptor agonist reinforcement. Although GHS-R1A receptors within both NACSh as well as VTA have

been found to contribute significantly to these effects, administration of ghrelin antagonist into the NACSh seemed to have stronger impact on the accumbens endocannabinoid opioid-induced changes. Our findings further suggest substantial involvement of ghrelin/GHS-R1A central signalling in the cannabinoid rewarding/ reinforcement pro-addictive effects, which encourages further investigation of the GHS-R1A antagonism as a potential approach to cannabinoid addiction treatment. Further investigation to assess if substances affecting GABA or endocannabinoid concentrations and action, such as GHS-R1A antagonists, can be used to prevent cannabis/opioid -seeking behaviour and as a potential new therapeutic direction in these addictions.

LIST OF FIGURES

Figure 1 Key target sites for various drugs of abuse across the reward circuity 17
Figure 2. The microdialysis dialysate collection 32
Figure 3. The LABORAS system 33
Figure 4. Schematic locations of dialysis probes in WIN55,212-2 rats in the NACSh (A) and sites of infusions into the VTA (B).....37
Figure 5**.** Schematic locations of dialysis probes in fentanyl rats in the NACSh (A) and sites of infusions into the VTA (B) 38
Figure 6**.** Effects of growth hormone secretagogoue receptors (GHS-R1A) antagonist (JMV2959) on WIN55,212-2-induced dopamine and its metabolites' extracellular changes in the rat's NACSh. 44
Figure 7. Extracellular DA metabolic turnover in the NACSh.....46
Figure 8**.** Effects of GHS-R1A antagonist (JMV2959) on WIN55,212-2-induced endocannabinoid and GABA extracellular changes in the rat NACSh 48
Figure 9**.** Effects of the ghrelin receptor antagonist JMV2959 3 mg/kg administered intraperitoneally (i.p.) on the fentanyl-induced accumbens anandamide and 2-AG levels 50
Figure 10**.** Effects of GHS-R1A antagonist JMV2959 3 mg/kg i.p. on the fentanyl-induced accumbens γ-aminobutyric (GABA) concentration.....51
Figure 11. Effects of JMV2959 on the THC-induced behavioural changes in rats in the LABORAS apparatus.....55
Figure 12. Effects of JMV2959 on the WIN55,212-2 -induced behavioural changes in rats in the LABORAS apparatus.....56

LIST OF PUBLISHED ARTICLES

IF publications, which are the basis and related to the dissertation

1. Charalambous, Ch.; Lapka, M.; Havlíčková, T.; Syslova K.; Sustkova-Fiserova, M.: Alterations in Rat Accumbens Dopamine, Endocannabinoids and GABA Content During WIN55,212-2 Treatment: The Role of Ghrelin. International Journal of Molecular Sciences, 2021, 22 (1): Article 210. IF: 5,923/2021
2. Šustková-Fišerová, M.; Charalambous, C.; Havlíčková, T.; Lapka, M.; Jeřábek, P.; Puškina, N.; Syslová, K.: Alterations in Rat Accumbens Endocannabinoid and GABA Content during Fentanyl Treatment: The Role of Ghrelin. International Journal of Molecular Sciences, 2017, 18(11): Article 2486. IF: 3,687/2017; Q2/2017; Aktuální IF časopisu: 4,183/2018; Q2/2018
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Alterations in Rat Accumbens Dopamine, Endocannabinoids and GABA Content During WIN55,212-2 Treatment: The Role of Ghrelin

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Abstract: The endocannabinoid/CB1R system as well as the central ghrelin signalling with its growth hormone secretagogoue receptors (GHS-R1A) are importantly involved in food intake and reward/reinforcement processing and show distinct overlaps in distribution within the relevant brain regions including the hypothalamus (food intake), the ventral tegmental area (VTA) and the nucleus accumbens (NAC) (reward/reinforcement). The significant mutual interaction between these systems in food intake has been documented; however, the possible role of ghrelin/GHS-R1A in the cannabinoid reinforcement effects and addiction remain unclear. Therefore, the principal aim of the present study was to investigate whether pretreatment with GHS-R1A antagonist/JMV2959 could reduce the CB1R agonist/WIN55,212-2–induced dopamine efflux in the nucleus accumbens shell (NACSh), which is considered a crucial trigger impulse of the addiction process. The synthetic aminoalklylindol cannabinoid WIN55,212-2 administration into the posterior VTA induced significant accumbens dopamine release, which was significantly reduced by the 3 mg/kg i.p. JMV2959 pretreatment. Simultaneously, the cannabinoid-increased accumbens dopamine metabolic turnover was significantly augmented by the JMV2959 pretreament. The intracerebral WIN55,212-2 administration also increased the endocannabinoid arachidonoylethanolamide/anandamide and the 2-arachidonoylglycerol/2-AG extracellular levels in the NACSh, which was moderately but significantly attenuated by the JMV2959 pretreatment. Moreover, the cannabinoid-induced decrease in accumbens γ-aminobutyric acid/gamma-aminobutyric acid levels was reversed by the JMV2959 pretreatment. The behavioural study in the LABORAS cage showed that 3 mg/kg JMV2959 pretreatment also significantly reduced the systemic WIN55,212-2-induced behavioural stimulation. Our results demonstrate that the ghrelin/GHS-R1A system significantly participates in the rewarding/reinforcing effects of the cannabinoid/CB1 agonist that are involved in cannabinoid addiction processing.

Keywords: synthetic cannabinoid WIN55,212-2; endocannabinoids; anandamide/AEA; 2-arachidonoylglycerol/2-AG; dopamine; dopamine metabolism; GABA; ghrelin/GHS-R1A; addiction; nucleus accumbens shell microdialysis

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1. Introduction

Cannabinoids enhance the subjective sense of well-being by stimulating the mesolimbic/mesocortical endocannabinoid system, which influences the motivation for natural rewards (such as palatable food, social interaction and sexual activity) and modulates the rewarding effects of addictive drugs. Prolonged activation of the endocannabinoid

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Alterations in Rat Accumbens Endocannabinoid and GABA Content during Fentanyl Treatment: The Role of Ghrelin

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Abstract: The opioid-induced rise of extracellular dopamine, endocannabinoid anandamide and γ-aminobutyric acid (GABA) concentrations triggered by opioids in the nucleus accumbens shell (NACSh) most likely participate in opioid reward. We have previously demonstrated that systemic administration of ghrelin antagonist (JMV2959) significantly decreased morphine-induced dopamine and anandamide (*N*-arachidonoylethanolamine, AEA) increase in the NACSh. Fentanyl is considered as a μ-receptor-selective agonist. The aim of this study was to test whether JMV2959, a growth hormone secretagogue receptor (GHS-R1A) antagonist, can influence the fentanyl-induced effects on anandamide, 2-arachidonoylglycerol (2-AG) and GABA in the NACSh and specify the involvement of GHS-R1A located in the ventral tegmental area (VTA) and nucleus accumbens (NAC). Using in vivo microdialysis in rats, we have found that pre-treatment with JMV2959 reversed dose dependently fentanyl-induced anandamide increases in the NACSh, resulting in a significant AEA decrease and intensified fentanyl-induced decreases in accumbens 2-AG levels, with both JMV2959 effects more expressed when administered into the NACSh in comparison to the VTA. JMV2959 pre-treatment significantly decreased the fentanyl-evoked accumbens GABA efflux and reduced concurrently monitored fentanyl-induced behavioural stimulation. Our current data encourage further investigation to assess if substances affecting GABA or endocannabinoid concentrations and action, such as GHS-R1A antagonists, can be used to prevent opioid-seeking behaviour.

Keywords: fentanyl; ghrelin; endocannabinoids; anandamide; 2-arachidonoylglycerol; GABA; neural reward system; nucleus accumbens shell; ventral tegmental area; microdialysis

1. Introduction

Gut-brain orexigenic peptide ghrelin [1], a natural ligand of the growth hormone secretagogue receptor (GHS-R1A), has been recently shown to play a critical role in food reward [2] as well as reward, motivation and intake of alcohol and reward of several stimulants (for review, see [3,4]). In addition to hypothalamus, the central GHS-R1As are expressed in important reward related areas including striatum, nucleus accumbens (NAC), amygdala, prefrontal cortex, hippocampus, and ventral tegmental area (VTA) [5–12]. Available literature involving ghrelin in opioid abuse and addiction is still limited and inconclusive [13–17]. The self-administration study of Maric et al. [13] showed that ghrelin

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