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Research on ghrelin mechanisms for the prevention of relapse in cannabinoid addiction

Výzkum ghrelinových mechanizmů pro prevenci relapsu u závislosti na kanabinoidech

Dissertation thesis

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Supervisor

Prague, 2023

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ABSTRACT

Background: Cannabis and cannabinoids are frequently used for recreational and therapeutic purposes, but people tend to overlook the associated risks that comes with them. Cannabinoid-associated use disorders and dependence are alarmingly increasing, and an effective treatment is currently lacking. Recently, the growth hormone secretagogue receptor (GHSR1A) antagonism was proposed as a promising mechanism for drug addiction therapy. However, the role of GHS-R1A and its endogenous ligand ghrelin in cannabinoid abuse remains unclear.

Aim: The principal aim of this research thesis was to further investigate whether the GHS-R1A antagonist JMV2959 could reduce the WIN55,212-2 intravenous self-administration (IVSA) and the tendency to relapse, but also reduce the tetrahydrocannabinol (THC)-induced conditioned place preference (CPP).

Methods: In a rat model, the intravenous self-administration directly measured the rat's response to the reinforcement effects of WIN55,212-2 as spontaneous drug-seeking and consumption with pretreatments of GHS-R1A antagonist/JMV2959 or saline. Further, the behavioral changes in rats were observed on the conditioned place preference apparatus which monitored the influence of JMV2959 on the THC effects.

Findings: Following the ongoing WIN55,212-2 self-administration, JMV2959 3 mg/kg was administered intraperitoneally 20 min before for three daily consequent 120-min IVSA sessions, which significantly reduced the number of the active lever-pressing, the number of infusions, and in extent, the cannabinoid intake. Pretreatment with JMV2959 also suggested the reduction of the WIN55,212-2-seeking/relapse-like behavior tested in rats on the 12 day of the forced abstinence period. Conversely, the pretreatment with ghrelin, significantly increased the cannabinoid IVSA as well as enhanced the relapse-like behavior. Co-administration of ghrelin with JMV2959 abolished/reduced the significant efficacy of the GHS-R1A antagonist in the cannabinoid IVSA. Furthermore, the pretreatment with JMV2959 significantly and dose-dependently reduced the manifestation of THC-induced CPP. The THC-CPP development was also reduced after the simultaneous administration of JMV2959 with THC during conditioning.

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

Key words

tetrahydrocannabinol (THC); synthetic cannabinoid; WIN55,212-2; ghrelin; GHS-R1A; JMV2959; intravenous self-administration (IVSA); conditioned place preference (CPP)

ABSTRAKT

Úvod: Konopí a kanabinoidy jsou často užívány k rekreačním a léčebným účelům, ale rizika, která jsou s nimi spojena, bývají přehlížena. Poruchy a závislost spojené s užíváním kanabinoidů znepokojivě přibývají a účinná léčba v současné době chybí. Nedávno byl jako slibný mechanismus pro léčbu drogové závislosti navržen antagonismus receptoru růstového hormonu (GHSR1A). Úloha GHS-R1A a jeho endogenního ligandu ghrelinu ve zneužívání kanabinoidů však zůstává nejasná.

Cíl: Hlavním cílem této práce bylo prozkoumat, zda antagonista GHS-R1A, látka JMV 2959, může snížit intravenózní autoaplikaci (IVSA) WIN55,212-2 a tendenci k relapsu, a také snížit tetrahydrokanabinolem (THC) indukovanou podmíněnou preferenci místa (CPP).

Metody: Pomocí intravenózní autoaplikaci (IVSA) u potkanů byla měřena reakce na posilující účinky WIN55,212-2 jako spontánní vyhledávání a konzumace drogy po premedikaci GHS-R1A antagonistou/JMV2959 nebo fyziologickým roztokem. Další změny chování potkanů byly pozorovány v modelu podmíněné preference místa (CPP), který hodnotil vliv JMV2959 na účinky THC.

Výsledky: Po samostatné autoaplikaci WIN55,212-2 u potkanů byla látka JMV2959 v dávce 3 mg/kg podána intraperitoneálně 20 minut před třemi po sobě jdoucími denními 120minutovými sezeními, což významně snížilo počet stisknutí aktivní páky, počet infuzí a rozsah příjmu kanabinoidů. Premedikace látkou JMV2959 vedla také ke snížení vyhledávání WIN55,212-2/relapsu-podobného chování testovaného ve dvanáctý den období nucené abstinence. Naopak, premedikace ghrelinem významně zvýšila užívání kanabinoidu v modelu IVSA a zvýšila jeho vyhledávání. Současné podávání ghrelinu a JMV2959 zrušilo/snížilo významnou účinnost antagonisty GHS-R1A v modelu kanabinoidní IVSA . Dále, premedikace JMV2959 významně a v závislosti na dávce snížila projevy THC-indukovaného CPP. Rozvoj THC-navozeného CPP byl snížen při současném podávání JMV2959 s THC během podmiňování.

Závěry: Výsledky tohoto výzkumu zdokumentovaly významný podíl ghrelinu/GHS-R1A na pro-adiktivních účincích kanabinoidů a podpořily další výzkum ghrelinového antagonismu jako potenciálního terapeutického směru u těchto závislostí.

Klíčová slova

konopí - WIN55,212-2 - THC- ghrelin – GHS-R1A - JMV2959 – IVSA - CPP

I. INTRODUCTION

Addiction refers to persistent and complex conditions, both psychological and physiological in nature, where an individual experiences a diminished ability to exert control over a particular pattern of behavior. This relapsing disease or disorder has negative complex effects on the individual and on society to some extent. Substance dependence refers to the persistent urge or desire to repeatedly use the substance or drug (constantly or intermittently) in order to achieve expected psychological effect(s) (excessive well-being/satisfaction/reward) or to prevent the occurrence of unpleasant conditions that arise in the absence of the substance/drug in the body (withdrawal symptoms); substance or drug use occur even though there are clear evidence of their harmful consequences (NIDA 2018).

Dopamine plays a crucial role in the reward system associated with drug use (Di Chiara and Imperato 1988, Koob and Bloom 1988). The acute consumption of any substance(s) that known to cause addiction leads to an elevation in the extracellular levels of dopamine in the nucleus accumbens (NAc) (Weiss, Paulus et al. 1992). In the nucleus accumbens shell (NACSh), all addictive drugs significantly activate dopaminergic transmission, which is considered an important initial impulse of the addiction processes, which are linked with reward, reinforcement, and disruption of salience attribution (Nestler 2005, Hyman, Malenka et al. 2006, Koob and Volkow 2010). Addictive drugs and the release of dopamine in the NAc caused by them, initiate consequent conditioning processes in the brain that form associations of drug reward with particular cues/conditions and reinforce the drug-seeking behavior (Adinoff 2004).

In Europe, the most utilized illegal substances are cannabinoids. Abused cannabinoids, except the natural constituents of Cannabis sativa, also include several synthetic cannabinoids used in several ways, such as "spice" in herbal mixtures, infused papers, or adulterating cannabis with synthetic cannabinoids. Between 2002 to 2019, various chemical structures of more than 180 synthetic cannabinoids, such as aminoalkylindoles, were detected on the illegal drug market by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (EMCDDA 2020). Legal, medical, and social acceptance of cannabis is grown rapidly during the last 15 years not only in Europe but also in North America. The recreational and medical use of cannabis is increased, but a public proportion that perceives the important harms from the cannabis use is decreased (Hasin 2018, EMCDDA 2020). Over the past few years, in Czech Republic, but also in Europe in general, there has been a presence of high-potency tetrahydrocannabinol (THC) strains supply, linked with increased risks of cannabis use

disorder, which includes uncontrollable drug-seeking and symptoms of withdrawal, psychotic disorders, dysphoria, sleep, and eating disorders (Zehra, Burns et al. 2018, EMCDDA 2020). It was estimated that about 9% of chronic cannabis users show characteristic symptoms and signs of dependence according to the Diagnostic and Statistical Manual of Mental Health of the World Health Organization (WHO/DSM-IV) criteria (Zehra, Burns et al. 2018). Similar potential risks were associated with many new synthetic cannabinoids, such as aminoalkylindole derivatives, which were broadly abused in Europe and elsewhere during the last 15 – 20 years (EMCDDA 2020).

The significant findings of our rigorous research of the ghrelin involvement in the cannabinoids (WIN55,212-2 and THC) pro-addictive effects are summarized in this dissertation thesis. Particularly, we tested whether ghrelin GHS-R1A antagonism could reduce the cannabinoid reinforcement effects. However, I participated also in other research projects. The achieved cannabinoid-linked innovative results were published together with findings from further drug addiction models (opioid, methamphetamine), in total 8 articles in international journals with high IF with average IF=4,759/2021. Nevertheless, this dissertation thesis is focused only on the cannabinoid experimental results. The theoretical part of this dissertation thesis addresses the issues around the consumption of addictive substances, focusing on the cannabinoids, and suggests how it affects the brain in a molecular and physiological point of view. The experimental part of this dissertation thesis documents the significant experimental findings of the ghrelin/GHS-R1A and cannabinoid addiction relationships, which were obtained during our rigorous investigation. The obtained and published results encourage additional research of the GHS-R1A antagonism as a potential novel approach to treating cannabinoid addiction, favourably, to decrease the cannabinoid craving and in extent, relapse. Specific pharmacotherapies are currently not approved for cannabis use disorder and dependence, therefore, cannabis addiction treatment remains unsatisfactory, symptomatic, and with a low relapse prevention; seeking new effective therapeutical approaches are currently needed (Kondo, Morasco et al. 2020).

II. THEORITICAL PART

1. Introduction to the problematic issue of cannabinoids and addiction

1.1. Cannabinoid use prevalence and trends

Europe is a significant market for drugs that come from both local production and trafficking from other parts of the world. Illicit drugs including cannabis and synthetic cannabinoids entering Europe often come from South America, West Asia, and North Africa, while China is a primary source for new psychoactive substances, drug precursors, and associated chemicals. Some drugs also pass through Europe on their way to other continents. Cannabis and synthetic drugs are produced in Europe, primarily for consumption within Europe, but synthetic drugs are also manufactured for export to other regions. The utilization of drugs in Europe covers a broad spectrum of substances. Cannabis is the most widely used drug, with usage rates about five times higher than other substances. Males tend to have higher drug use rates than females, and this gender difference is more pronounced for more frequent or persistent patterns of drug use. It is estimated that approximately 83 million adults in the European Union, which accounts for 28.9% of those aged 15-64, have used illicit drugs at least once in their lives. More men (50.6 million) tend to report having used drugs compared to women (32.8 million) (EMCDDA 2021).

The cannabis resin that is sold in Europe currently has a higher potency than in the past, with an average THC content of between 20% and 28%, which is nearly double that of herbal cannabis. In addition to traditional cannabis products, new forms of cannabis and high-THC content products are available on the illicit market, while commercially sold products contain cannabis extracts with low THC levels. These changes in the market are occurring alongside a rise in the number of individuals seeking treatment for cannabis use for the first time. Cannabis is the most used drug with 47.6 million males and 30.9 million females. According to surveys of the general population, approximately 1.8% of adults aged 15-64 in the European Union use cannabis daily or almost daily, meaning they have used it 20 or more days within the last month. Of these individuals, the majority (61%) are under the age of 35. In 2019, more than half (51%) of those seeking treatment for cannabis use for the first time reported using the drug daily within the last month (EMCDDA 2021).

1.2. Fundamental principles of cannabinoid addiction mechanisms

In general, addiction refers to a chronic mental illness and physical condition characterized by the individual's inability to control a particular behavior. Any substance addiction, including cannabinoids, is classified by the World Health Organization (WHO) within the International Classification of Diseases (ICD/ICD-10), as F10-F19 - Mental and behavioral disorders caused by psychoactive substances. If an individual exhibits three or more of the following symptoms for at least one month, or if at least three of the following symptoms are observed repeatedly during one year, their condition can be classified as addictive (Hasin, O'Brien et al. 2013). The following symptoms are commonly monitored to determine a diagnosis:

- Strong desire or compulsive need to take an addictive substance
- Impaired ability to control behavior associated with substance use
- Somatic withdrawal syndrome
- Demonstrable development of tolerance to the effects of the addictive substance
- Gradual neglect and abandonment of other interests and pleasures in favour of substance use
- Continued use of the substance despite demonstrably harmful consequences

All known addictive substances, including cannabinoiuds, lead to an increase in extracellular dopamine concentration in the NAc upon acute administration. The mesolimbic dopaminergic pathways between NAc and the ventral tegmental area (VTA), further connections with the medial prefrontal cortex (mPFC), and eventually other structures, are part of the brain reward system. All the mention structures are essential for reward and satisfaction feeling and play a role in addiction but not only in addictive substances. The release of dopamine in the NAc is the main indicator of the importance of the given change/reward; initiating trigger for subsequent conditioning, comparative, and control processes (Volkow, Wang et al. 2011). The brain reward system is a crucial neural circuit for the preservation of the species and gender. Life sustaining behaviors (consumption of energetically rich food, sexual behavior, etc.) lead to the release of dopamine in the NAc and simultaneously induce pleasant feelings in the individual. The individual then has a greater tendency to repeat and seek out this behavior and situations because they are associated with "reward". Other mediator systems, except dopamine, participate in learning and reward mechanism(s) (e.g.,

endocannabinoids, GABA, glutamate, and others) (see Figure 1) (Hyman, Malenka et al. 2006, Koob and Volkow 2010, Volkow, Wang et al. 2011, Koob and Volkow 2016).

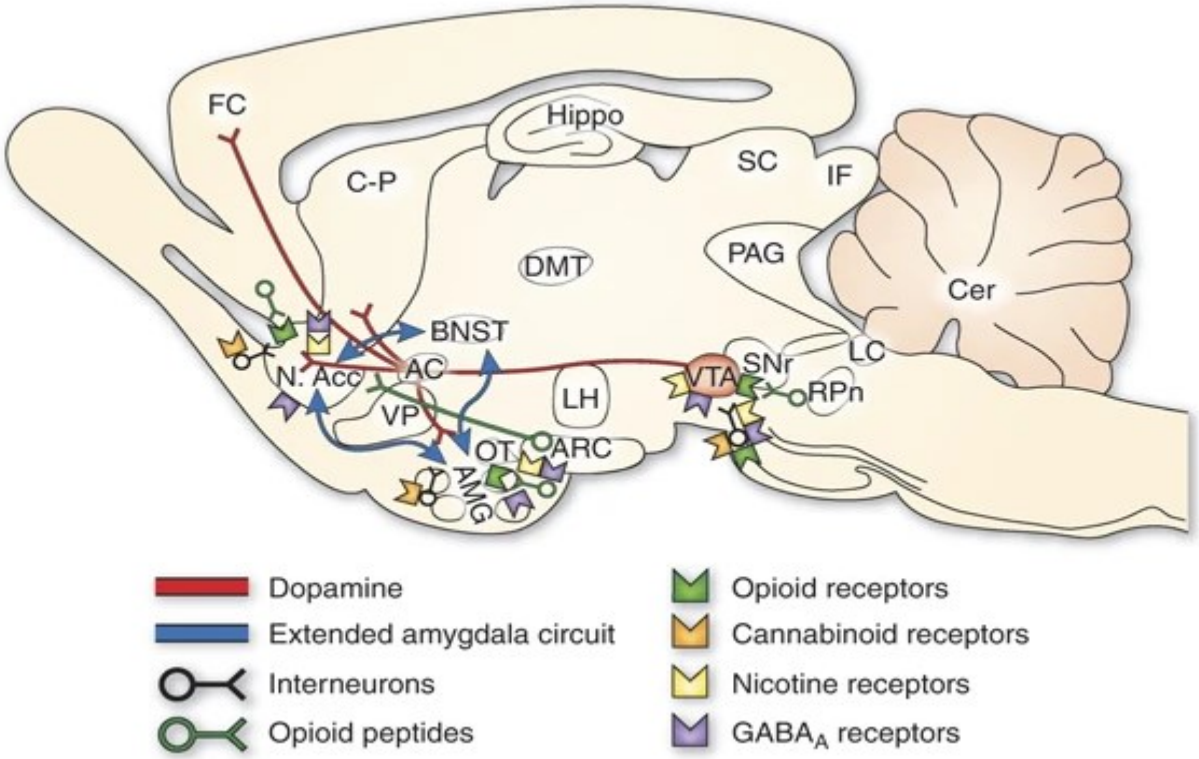

Figure 1 Schematic representation of the neurochemical circuits involved in the development of addiction to addictive substances, a diagram of a rat brain section.
anterior commissure (AC); amygdala (AMG); arcuate nucleus (ARC); bed nucleus of the stria terminalis (BNST); cerebellum (Cer); caudate-putamen (C-P); dorsomedial thalamus (DMT); frontal cortex (FC); hippocampus (Hippo); inferior colliculus (IF); locus coeruleus (LC); lateral hypothalamus (LH); nucleus accumbens (N Acc); olfactory tract (OT); periaqueductal gray (PAG); reticular pontine nucleus (RPn); superior colliculus (SC); substantia nigra pars reticulata (SNr); ventral pallidum (VP); ventral tegmental area (VTA) (Koob and Volkow 2010).

Sagittal section through a representative rodent brain illustrates the pathways and receptor systems implicated in the acute reinforcing actions of drugs of abuse (see Figure 1). Cannabinoids activate cannabinoid CB1 receptors in the VTA, nucleus accumbens, and amygdala. They facilitate the release of dopamine in the NAc through an unknown mechanism either in the VTA or the NAc. The blue arrows represent the interactions within the extended amygdala system hypothesized to have a key function in drug reinforcement. The medial

forebrain bundle represents ascending and descending projections between the ventral forebrain (nucleus accumbens, olfactory tubercle, septal area) and the ventral midbrain (Koob and Volkow 2010).

2. Cannabinoids

2.1. Current situation - range of cannabinoid use and abuse in the Czech Republic and Europe.

Cannabis is the illegal substance most commonly used in all countries of the European Union, where it can be imported but also domestically produced (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 commonly used drug following tobacco and alcohol (NMCDA 2019).

The dried female flowers of Cannabis indica L. or Cannabis sativa L., commonly known as hemp, are utilized for both medicinal and recreational purposes with the active ingredients cannabidiol (CBD) and Δ-9-trans-tetrahydrocannabinol (THC). The active substance content can range from 0.1% to 19% for CBD and from 0.3% to 21% for THC. 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.). Legal cannabis for medical purposes can be obtained by patients in the Czech Republic permitted by the Ministry of Health of the Czech Republic - Inspectorate of Narcotic and Psychotropic Substances.

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

Cannabis use related problems are more prevalent and are more frequently occur in Europe compared to other regions, because of the prevailing presence of a more hazardous

variety of cannabis containing elevated levels of THC ("skunk"), the increasing use of cannabinoids by adolescents and the use of synthetic cannabinoids. Synthetic cannabinoids represent a diverse group of various substances with similar effects to THC, however, they pose significantly greater danger compared to natural cannabis drugs, some are highly toxic and more addictive. They are distributed as plant mixtures or as substances imitating hashish. Synthetic cannabinoids are often sold and referred to using abbreviations based on the chemical names of the specific substances or under various commercial names, such as 'Spice Diamond', 'Spice Gold', 'Bliss', 'Spice Silver', 'Black Mamba', 'Bombay Blue', 'K2', or 'Blaze'. Common abbreviations used for synthetic cannabinoids include JWH-018, MDMB-CHMICA, APINACA (AKB-48), AM-2201, UR-144, ADB-CHMINACA, 4F-MDMB-BINAC. The synthetic cannabinoids are frequently used as spray form on the natural cannabis products to increase their effectiveness (EMCDDA 2019).

In Europe, legal, medical, and social cannabis acceptance is significantly grown during the last 15 year. The use of cannabis for recreational and medical purposes is furthermore increased, however, the proportion of the public that perceives important harms from cannabis use was decreased (Hasin 2018). In Czech Republic but also in Europe in general, subsists 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, gastrointestinal symptoms, dysphoric mood, psychotic disorders, disturbed sleep, and eating disorders. According to the World Health Organization (WHO) and the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria, it is estimated that approximately 9% of chronic cannabis users display characteristic symptoms of dependence (Zehra, Burns et al. 2018). At present, there are no approved pharmacological treatments specifically designed for CUD and dependence, thus cannabinoid addiction treatment remains exclusively symptomatic, unsatisfactory, and with a low relapse prevention (Kondo, Morasco et al. 2020). Hence, ongoing research strategies are focused on discovering novel and efficient treatment approaches.

2.2. Neurobiological mechanisms of cannabinoids action and risks

The psychoactive effects of cannabis are commonly attributed to the CB1 receptor, as it is widely believed to be solely responsible for these effects (Brumback, Castro et al. 2016). Cannabis induces or amplifies the feeling of well-being by stimulating the endocannabinoid system. It plays a crucial role in modulating the response to stress and reward and their

interactions. A repeated and the prolonged activation of the endocannabinoid system can trigger neuroadaptations that may impair the sensitivity to stress and reward. In susceptible individuals, cannabis use can lead to addiction and various negative consequences (Volkow, Wise et al. 2017). The endocannabinoid system exhibits significant complexity, encompassing both central and peripheral effects, and involving multiple binding sites and mechanisms of action. The extensively researched endocannabinoids including N-arachidonoylethanolamine/anandamide and 2-arachydonoylglycerol (2-AG) are synthetized on demand from cell phospholipids. They primarily act via the cannabinoid CB1 receptors, that play a retrograde role in regulating synaptic neurotransmission, overall the brain reward circuitry, controlling both excitatory and inhibitory inputs (Parsons and Hurd 2015, Scherma, Masia et al. 2019). The CB1Rs are the most abundant with particular dense expression in regions involved in addiction, reward, and cognitive functions, including NAc, VTA, substantia nigra and others. (Parsons and Hurd 2015). The CB1Rs are located on various presynaptic inputs/axons in the NAc and the VTA (Matsuda, Lolait et al. 1990, Herkenham 1991, Parsons and Hurd 2015).

Recent research revealed the presence of CB2 receptors (CB2Rs) in midbrain dopamine neuron regions. Interestingly, the activation of CB1 receptors (CB1Rs) has been associated with reinforcing effects, while the activation of CB2Rs appears to elicit aversive responses (Spiller, Bi et al. 2019). THC is classified as a partial agonist of both CB1 receptors and CB2 receptors. Its effects are known to exhibit a dose-dependent biphasic or dual pattern. At lower doses, THC has reinforcing properties (Zehra, Burns et al. 2018, Spiller, Bi et al. 2019). CBD has variable effects, including antagonism (central effects) of CB1Rs, adenosine-uptake inhibition or indirect agonism (peripheral effects), GABA-A, allosteric modulation of dopamine D2, glycine, 5-HT1A, μ- and δ- opioid receptors etc. (Pertwee 2008, McPartland, Duncan et al. 2015).

THC, anandamide, and 2-AG act on the mesolimbic CB1Rs to increase dopamine levels in the NACSh. This leads to subsequent reinforcement, conditioning, and alterations in the processing of salience (Lupica, Riegel et al. 2004, Panlilio, Zanettini et al. 2013, Parsons and Hurd 2015, Wijayendran, O'Neill et al. 2018, Zehra, Burns et al. 2018). Extended or chronic use of cannabis or THC results in the downregulation of CB1Rs in the brain, although dopamine D2/D3 receptors remain unaffected. This downregulation contributes to the development of tolerance, dysregulation of stress responses, amotivational states, withdrawal symptoms, and sensitization of the mesocorticolimbic reward system to cannabis cues and THC after a period of abstinence. Additionally, during relapses, there is an increase in glutamate signalling. Therefore, the progression of cannabis addiction closely resembles the addiction patterns

observed with other substances of abuse (Volkow, Hampson et al. 2017, Zehra, Burns et al. 2018, Hwang and Lupica 2020).

2.3. Treatment approaches and limitations in cannabis/cannabinoid addiction

Recent epidemiological and clinical studies have provided evidence that addiction can indeed develop in relation to cannabis use (Lang, Engelander et al. 2000). The primary goal of detoxification is to prevent drug use and minimize the severity of withdrawal symptoms and associated risks. Treatment for substance use disorders is typically categorized into short-term, medium-term, and long-term interventions. Short-term treatment typically spans a duration of up to three months, while medium-term treatment extends from three to six months. Long-term treatment generally lasts from six months to a year, occasionally lasting up to two years, especially when it involves therapeutic communities as a supportive environment for ongoing care (Kalina 2008). Additional treatment options for cannabis use disorder include therapeutic communities, aftercare programs, and self-help activities. It is important to note that cannabis intoxication is typically mild and often resolves spontaneously without the requirement for medication. In cases where more severe symptoms such as anxiety, panic attacks, or psychosis are present, medications such as benzodiazepines or atypical antipsychotic drugs may be utilized to manage these symptoms. These medications can help alleviate the distressing effects associated with cannabis-induced psychiatric symptoms (Danovitch and Gorelick 2012). There is currently no medication that has been approved specifically for the treatment of symptoms related to cannabis use.

The involvement of ghrelin and the ghrelin receptor in the pro-addictive effects of cannabinoids is currently being investigated in animal research conducted at the Department of Pharmacology of the Third Faculty of Medicine, Charles University. The research aims to better understand the ghrelin role and its receptor in the mechanisms underlying cannabinoid addiction. Our department 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 this research thesis (Sustkova-Fiserova, Charalambous et al. 2017, Charalambous, Lapka et al. 2021).

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

3.1. The complex effects of ghrelin and the potential use of ghrelin mechanisms in addiction therapy

The endogenous peptide ghrelin was firstly discovered by Kojima in 1999 (Kojima, Hosoda et al. 1999). In the 1970s, Bowers conducted a research on synthetic substances that impact growth hormone secretagogues (GHS), which preceded the discovery of ghrelin (Bowers, Momany et al. 1980). The GHS-specific receptor, known as GHS-R1A, is classified as a metabotropic type of G protein-coupled receptor. When activated, it triggers the release of calcium from the endoplasmic reticulum into the cytoplasm of the cell, resulting in membrane depolarization (Jensovsky, Lebl et al. 2000). The presence of this receptor led to the assumption that it would have a natural endogenous ligand. Through extensive research focused on monitoring alterations in intracellular calcium levels following the introduction of different tissue types, it has been observed that the most significant changes occur when tissue extracted from the stomach is added (Kojima, Hosoda et al. 1999). Ghrelin is a peptide composed of 28 amino acids (Bednarek, Feighner et al. 2000). While human and rat ghrelin exhibit a difference of two amino acids, they are 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, Hosoda et al. 1999).

Ghrelin has been shown to be present in various central and peripheral structures of both human and animal organisms, including rats. The secretion of ghrelin is regulated by both local and central stimuli; a glucose solution is an example of stimuli (Rosicka, Krsek et al. 2002). Experiments on rats demonstrated that both peripheral and intracerebral administration of ghrelin result in stimulation of growth hormone secretion from somatotropic pituitary cells (Date, Kojima et al. 2000). Furthermore, both central and peripheral application of ghrelin induce a significant orexigenic response, leading to an increase in gastric secretion, motility, and overall motivation to eat. Faster gastric emptying is associated with increased food intake and thus obesity (Masuda, Tanaka et al. 2000). The chronic stimulation of the GHS-R1A receptor by ghrelin (Tschop, Smiley et al. 2000) or synthetic growth hormone secretagogue (Lall, Tung et al. 2001) led to an increase in adipose tissue in rodents. Ghrelin levels are elevated prior meals (Cummings, Purnell et al. 2001) and correlate with hunger feeling (Cummings, Frayo et al. 2004). The orexigenic effects of ghrelin were observed after the administration to different parts of the brain including hypothalamus, ventral tegmental area, nucleus tractus solitarius, and nucleus accumbens (Egecioglu, Jerlhag et al. 2010). In addition to brain structures, GHS-R1A receptors also play a role in regulating food intake and energy balance.

Chronic excessive consumption of food can result in comparable neuroadaptive alterations in the brain, akin to those observed in substance addiction, in relation to reward mechanisms (Grigson 2002). Behavioral changes showed similar signs of substance abuse and chronic overeating. Hypothetically, it is plausible that the principle of ghrelin mechanisms may also have implications in the development of substance abuse.

The neurobiology underlying food intake behavior exhibits several fundamental similarities to the neurobiology of drug use and craving (Volkow, Wang et al. 2011). Based on the physiology of gut-brain peptide, the ghrelin hormone plays a central role in the regulation of energy homeostasis, appetite, and in extend food intake and reward (Egecioglu, Jerlhag et al. 2010). The central ghrelin signalling may have a significant impact on reinforcing and rewarding effects not only in alcohol but also in other substances of abuse, such as amphetamines, nicotine, and cocaine (Engel and Jerlhag 2014, Panagopoulos and Ralevski 2014, Sustkova-Fiserova, Charalambous et al. 2022). Ghrelin is primarily synthesized in the stomach by endocrine cells and in smaller amounts by other organs including the salivary glands, pancreas, and placenta (Kojima, Hosoda 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, Lopez Soto et al. 2017).

The growth hormone secretagogue receptors type 1A (GHS-R1a) are expressed in brain-reward areas including amygdala, hypothalamus, hippocampus, prefrontal cortex, striatum, nucleus accumbens and ventral tegmental area (Howard, Feighner et al. 1996, Abizaid, Liu et al. 2006, Ferrini, Salio et al. 2009), (Skibicka, Hansson et al. 2011). Administration of ghrelin showed to modulate dopamine transmission in the brain. It induces the release of dopamine in the NAc and stimulates behavioral measures of reward processing. GHS-R1a is expressed throughout the mesolimbic reward pathway and has the ability to form heterodimers with dopamine D1 and D2 receptors, as well as several other receptors (Guan, Yu et al. 1997, Abizaid, Liu et al. 2006, Schellekens, van Oeffelen et al. 2013). Based on these findings, it is suggested that ghrelin may have a central role in reward processing. Acyl-ghrelin interacts with the hypothalamic-pituitary-adrenal axis and can modulate stress-related behaviors (Spencer, Emmerzaal et al. 2015, Bali and Jaggi 2016). The involvement of ghrelin in both reward and stress pathways indicates a potential role for ghrelin signalling in addiction-related processes. Drug-seeking behavior is associated with both positive reinforcement (drug reward) and negative reinforcement (stress relief) mechanisms (Panagopoulos and Ralevski 2014, Koob and Volkow 2016).

3.2. Summary of currently obtained results at the Department of Pharmacology/3rd. Faculty of Medicine/Charles University.

The Department of Pharmacology (3rd. Faculty of Medicine Charles University) has been intensively studying the involvement of ghrelin/GHS-R1A in dependence mechanisms for several years, and I was actively participating in this research throughout my studies. The department focused primarily on the involvement of GHS-R1A and ghrelin in opioid dependence mechanisms, as well as in methamphetamine dependence mechanisms and, more recently, in cannabinoid dependence mechanisms. In neurobehavioral studies with rats, we found that the GHS-R1A antagonist JMV 2959 (a triazole derivative) plays a significant role in the rewarding and reinforcing effects of morphine (a non-specific opioid agonist) and fentanyl (a more specific μ-opioid agonist). For the first time, we demonstrated in rats that ghrelin antagonism reduced intravenous self-administration (IVSA), or spontaneous intake of opioid (fentanyl), and reduced the seeking of fentanyl after a period of enforced abstinence, thus reducing the tendency to relapse. The GHS-R1A antagonist JMV2959 reduced the development/induction and expression of morphine- and fentanyl-induced place preference (CPP). GHS-R1A antagonism reduced opioids (morphine, fentanyl) induced dopamine release and dopamine sensitization in the NAc, indicating a mechanism preceding the observed effects. For the first time, we also found a significant interaction between the ghrelin/GHS-R1A system, endocannabinoids (anandamide, 2-arachidonoyl glycerol), gamma-aminobutyric acid (GABA), opioids (μ-receptor), and dopamine in important reward-related brain structures, the NAc and ventral tegmental area. Thus, ghrelin/GHS-R1A is expected to significantly contribute to dopamine and dopamine-independent mechanisms of opioid reward. JMV2959 premedication also significantly attenuated the effects of opioids on behavioral stimulation and stereotypic behavior, and reduced morphine-induced behavioral sensitization. Further, pretreatment with JMV2959 significantly reduced the methamphetamine self-administration as well as seeking, expression, and development of the CPP in rat models. Altogether, our results support further research on ghrelin antagonism as a potential new therapeutic direction for opioid as well as methamphetamine dependence (Jerabek, Havlickova et al. 2017, Sustkova-Fiserova, Charalambous et al. 2017, Sustkova-Fiserova, Puskina et al. 2020, Sustkova-Fiserova, Charalambous et al. 2022).

3.3. The role of ghrelin and its receptor GHS-R1A in the cannabinoid addiction

The endogenous cannabinoid system, along with the exogenous cannabinoids like THC, impact appetite and food intake through the cannabinoid receptors (Kirkham and Williams 2001). Cannabinoids stimulate the endocannabinoid system in the mesolimbic/mesocortical pathways, which plays a role in influencing the motivation for natural rewards, including include palatable food, sexual activity, and social interaction, and modulates the rewarding effects of addictive drugs. Continuous activation of the endocannabinoid system through the prolonged use of cannabis or cannabinoids can induce neuroadaptations that have the potential to result in addiction and various adverse consequences (Parsons and Hurd 2015, Volkow, Hampson et al. 2017, Zehra, Burns et al. 2019, Hwang and Lupica 2020). The endocannabinoid system encompasses both central and peripheral effects, multiple binding sites, and a variety of endogenous ligands. (Di Marzo, Melck et al. 1998, Mechoulam, Fride et al. 1998, Tanda and Goldberg 2003). The extensively researched endocannabinoids, anandamide and 2-AG, are synthetised on demand from the cell phospholipids and act through cannabinoid CB1 receptors, retrogradely regulating synaptic neurotransmissions (e.g., glutamate and γ-Aminobutyric acid) regulating both excitatory (glutamate) and inhibitory (GABA) processes (Parsons and Hurd 2015, Zehra, Burns et al. 2019). The endocannabinoid system plays a crucial role as a mediator of synaptic plasticity in the mesocorticolimbic/corticostriatal pathways, which are involved in the regulation of motivated behavior control (Parsons and Hurd 2015, Zlebnik and Cheer 2016, Volkow, Hampson et al. 2017). The CB1Rs are the most prevalent G protein-coupled receptors found in the adult brain, exhibiting high levels of expression in regions associated with reward, addiction, and cognitive functions. This include the nucleus accumbens, ventral tegmental area, and hippocampus (Matsuda, Lolait et al. 1990, Herkenham 1991, Parsons and Hurd 2015). Recent discoveries have revealed the presence of the CB2Rs in the midbrain dopamine neuron regions as well. Activation of CB1Rs is associated with the production of reinforcing effects, while the activation of CB2Rs is associated to the production of aversive effects (Parsons and Hurd 2015, Spiller, Bi et al. 2019).

Tetrahydrocannabinol as well as the synthetic amino-alkylindol cannabinoid WIN55,212-2, exhibit a characteristic biphasic/dual effects, with reinforcing and hyperactivity-stimulating low doses but aversive and hypoactivity-inducing high doses. Tetrahydrocannabinol is classified as a partial CB1R/CB2R agonist and WIN55,212-2 as a full CB1R/CB2R agonist (D'Ambra, Estep et al. 1992, Spiller, Bi et al. 2019, Zehra, Burns et al. 2019). Previously it was described that the CB1 antagonist decreased elevated levels of circulating acyl-ghrelin in rats experiencing food deprivation. The CB1 antagonist administration was found to reduce/attenuate the secretion of growth hormone (Al Massadi, Lopez et al. 2017). In

independent experiments examining the impact of AMP-activated protein kinase (AMPK), an enzyme that regulates food intake and energy balance, was discovered that the activity of the AMPK was influenced by both cannabinoids and ghrelin. Thus, AMPK could mediate the orexigenic effects of cannabinoids and ghrelin (Kola, Hubina et al. 2005). Interestingly, the administration of a CB1 antagonist in rats inhibited the orexigenic effect of centrally administered (intrącerebroventricularly) ghrelin. Central administration of ghrelin in mice led to an increase in food intake, the AMPK activity, and the presence of endocannabinoids in the hypothalamus. The administration of the cannabinoid antagonist rimonabant reversed those effects (Kola, Hubina et al. 2005).

The ghrelin involvement in cannabis use disorders is currently limited and inconclusive according to the existing literature. Manipulations that reduce dopaminergic activity in the nucleus accumbens and behavioral sensitization is crucial for gaining insights into the fundamental aspects of future drug addiction treatment strategies (Vanderschuren and Kalivas 2000, Steketee and Kalivas 2011).

4. Experimental models used in this preclinical addiction research

General scientific methods including in vivo, in vitro, in situ, and behavioral approaches as well as specific addiction models are used to explore possible addiction mechanisms. The current experimental research explores the role of central ghrelin signalling in the mechanisms of cannabis dependence. Additionally, the testing of GHS-R1A antagonism is investigated as a potential promising mechanism for the treatment of cannabis addiction, with a particular focus on preventing relapse and drug-seeking behaviors. This research aims to contribute to the development of novel strategies for the management and prevention of cannabis addiction. Thus, the chosen methods are based on the direct influence of a substance/drug on the organism and primarily study specific dependency manifestations, such as active seeking of the substance/drug by animals and spontaneous consumption of the substance, preference for the place associated with substance application etc. Among the most useful addiction models are self-administration of the substance and drug-conditioned place preference (Panlilio and Goldberg 2007) (see below for more details). Further experimental methods used at our workplace include e.g. behavioral techniques monitoring behavior in the “open field”, “plus maze”, Laboras cage, the neurobiological method CNS microdialysis in vivo and others; however, these techniques are not included in this thesis. The self-administration method can involve the oral, intragastric, intramuscular, intracerebroventricular, intracranial, pulmonary, or

intravenous route of administration. Currently, the most commonly used self-administration methods are oral (e.g., for alcohol) and intravenous (for injectable substances/drugs such as methamphetamine, amphetamine, opioids, cocaine)(Panlilio and Goldberg 2007). Although cannabinoids are frequently smoked or inhaled, we chose intravenous/intramuscular administrations, because our experiments were to test for the first time whether a ghrelin GHS-R1A antagonist can affect cannabinoid reinforcement effects, thus we needed a reliable rewarding cannabinoid effect, which requires precise dosing (also due to the dual effect of cannabinoids).

4.1. Intravenous self-administration

Intravenous self-administration (IVSA) in animals, and particularly in rats, is a widely used sophisticated technique to study the neurobiological and behavioral mechanisms of drug addiction and other behaviors related to reward and motivation (Sustkova-Fiserova, Charalambous et al. 2022). Intravenous self-administration closely mimics the human experience of drug addiction, providing a high degree of face validity to the research findings, it allows precise dosing of drugs or other substances, which is important for studying the dose-response relationships and pharmacokinetics of drugs. Furthermore, it allows us to control the amount and duration of drug exposure, which is important for studying the effects of acute and chronic drug exposure on behavior and brain function. It is a widely accepted method in preclinical research for studying the mechanisms of drug addiction and can provide valuable insights into the neural mechanisms underlying drug addiction and related behaviors, helping to identify potential targets for therapeutic interventions (Havlickova, Charalambous et al. 2018, Sustkova-Fiserova, Puskina et al. 2020, Charalambous, Havlickova et al. 2021).

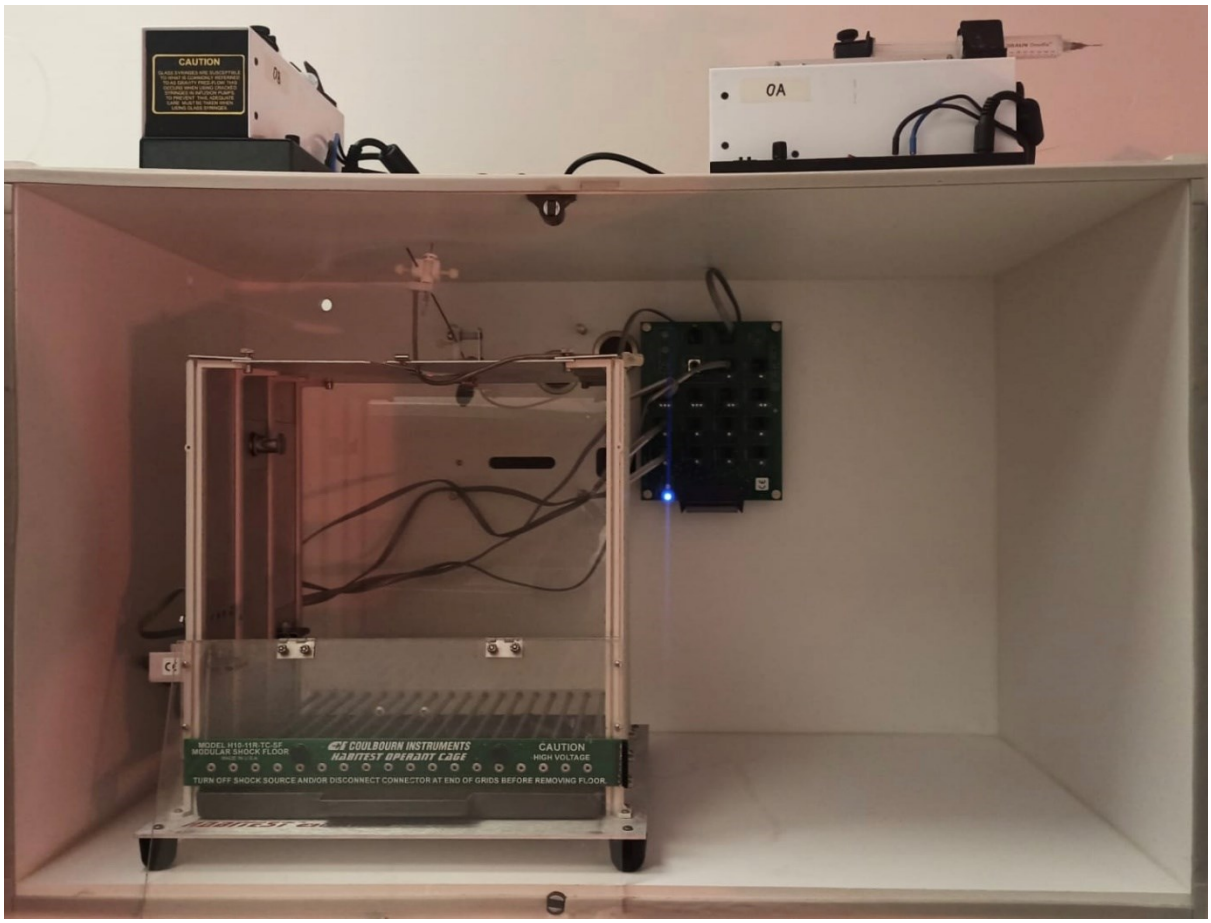

Figure 2. Scheme of the experimental apparatus for intravenous self-administration.

4.2. Conditioned place preference

Conditioned place preference (CPP) is an animal model widely used in drug addiction research. It is based on the principle of conditioning the drug effect with a specific environment. A set of neutral environmental cues is associated/conditioned with drug administration, and the animal forms a conditioned association between the pleasurable effects of the drug and the originally neutral environmental cues in one of the compartments, where the addictive drug is repeatedly for several days administered by the experimenter. If the administered substance has a positive reinforcing effect, the animal actively seeks out the environment (defined by the set of clearly recognizable originally neutral characters) where the substance was administered (Desousa, Wunderlich et al. 1998, Sanchis-Segura and Spanagel 2006).

Figure 3. Scheme of the experimental apparatus for conditioned place preference.

The experimental apparatus consists of two equally sized compartments/boxes connected by a smaller transition cell. Both compartments/boxes differ in their floor surface (coarse/fine mesh) and wall appearance (horizontal/vertical black and white stripes). The middle transition box has a neutral appearance and has entrances to both compartments/boxes. These entrances can be closed when necessary. The entire apparatus is illuminated during the experiment with a light intensity of 45 lux.

III. EXPERIMENTAL PART

5. Hypothesis and Aim

The overall outcome from our previous neurobiological research in the Department of Pharmacology of the 3rd Faculty of Medicine Charles University indicated a significant involvement of the central ghrelin signalling in the cannabinoid-induced dopamine as well as the endocannabinoid (anandamide and 2-AG) and GABA changes observed in the NACSh in rats. 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 to determine the WIN55,212-2 effects on anandamide, 2-AG and GABA accumbens content. 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 comprehensive findings of this research highlight the substantial involvement of ghrelin and its receptor GHS-R1A in the pro-addictive effects of cannabinoids. These findings also provide support for additional research exploring ghrelin antagonism as a potential novel therapeutic approach in the treatment of cannabinoid addiction. This research helps potential interventions targeting the ghrelin system to mitigate the addictive effects of cannabinoids (Charalambous et al 2021)

For this recent research thesis, the intravenous self-administration paradigm was used to provide valuable information about the addictive potential of cannabinoids and the neural mechanisms involved in reward and motivation. Furthermore, the conditioned place preference paradigm was also used to study the rewarding effects of cannabinoids, environmental stimuli, and other manipulations on rats. The CPP paradigm is based on the principle of Pavlovian conditioning, where the rat learns to associate a particular environmental context with a rewarding stimulus. The CPP paradigm is a versatile and widely accepted tool for studying reward processing and addiction, and it contributed significantly to our understanding of the

neurobiological and behavioral mechanisms underlying these processes. Therefore, to further clarify the involved mechanisms and relationships among the cannabinoid and ghrelin systems, the following hypotheses were defined:

1. The systemic pretreatment with the GHS-R1A antagonist JMV2959 could reduce the WIN55,212-2 intravenous self-administration and the tendency to relapse.
2. The systemic pretreatment with acyl-ghrelin could enhance the WIN55,212-2 intravenous self-administration and the tendency to relapse.
3. The co-administration of JMV2959 together with acyl-ghrelin will reduce the GHS-R1A antagonist effectiveness (confirmation of the GHS-R1A involvement in the observed changes).
4. The chosen intravenous WIN55,212-2 self-administration arrangement will provide a reliable model of cannabinoid dependence (method validation using parallel groups of rats self-administering saline or WIN55,212-2).
5. Pretreatments with the GHS-R1A antagonist JMV2959 during IVSA will not significantly affect the rat body weight of the rats.
6. The GHS-R1A antagonist JMV2959 could reduce the THC-induced conditioned place preference in both arrangements, JMV2959 could reduce the CPP expression as well as development.

6. Methods

6.1. Animals

Male adult Wistar rats (Velaz, Prague, Czech Republic) were utilized in the experiments, initially aged around 8 weeks. Prior to the commencement of the experiments (at least 7 days), the rats were provided unrestricted access to food and water. They were housed in polycarbonate cages under consistent environmental conditions, including a constant humidity level of 50-60% and room temperature maintained at 22-24 °C. The conditions were also the same between the experimental procedures, except for the rats throughout the IVSA conditioning and tests, where they received a 20 g/d standard chow food and ad libitum water. In our studies, the food was always removed (if it was not consumed) following any drug administration while running the daily experiments. The rats in the IVSA experiments were housed in a reverse 12-h light/dark cycle and the rats in the CPP experiment were housed in a normal 12-h light/dark cycle (6 a.m.–6 p.m.). In the intravenous self-administration (IVSA) study, the rats were individually housed, while in the conditioned place preference (CPP) study, three rats were accommodated per cage. To ensure the rats in the IVSA study were accustomed to the experimental procedures and experienced reduced stress, they were handled daily before the experiments. All procedures involving the animals, as well as their care, were conducted in compliance with international laws and guidelines regarding the ethical treatment of animals in research. The protocols complied with the Guidelines of the European Union Council (86/609/EU, 24 November 1986) and the EU Directive (2010/63/EU, 22 September 2010), and followed the instructions of the National Committee for the Care and Use of Laboratory Animals. All experiments were under the Expert Committee for Protection of Experimental Animals of the Third Faculty of Medicine, Charles University in Prague, and they were performed in accordance with the Animal Protection Act of the Czech Republic (No. 246/1992 Sb, 15 April 1992).

6.2. Drugs and Chemicals

THC was synthesized in cooperation with the University of Chemistry and Technology Prague (UCT Prague, Czech Republic) and WIN 55,212-2 mesylate salt (synthetic aminoalkylindole cannabinoid) was provided by Sigma–Aldrich (Prague, Czech Republic). Ghrelin was purchased from Essence Line (Prague, Czech Republic). The GHS-R1A

antagonist, the substance JMV2959 (1,2,4-triazole derivate) (Moulin, Brunel et al. 2013), was synthetized by the UCT Prague (Czech Republic). 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 rewarding 0.3 mg/kg dose in CPP, in accordance with the literature (Sanudo-Pena, Romero et al. 2000, Katsidoni, Kastellakis et al. 2013), and it was administered intraperitoneally (i.p.) in volumes of 0.1 mL/100 g of body weight. It was described that, in comparison to THC, WIN55,212-2 is reliably self-administered in rodents/rats (Fattore, Cossu et al. 2001, Amchova, Kucerova et al. 2014, Lefever, Marusich et al. 2014); therefore WIN55,212-2 was used for intravenous self-administration in 12.5 μg/kg/infusion in volumes of 0.1 mL per infusion/active lever press. The selected doses of JMV2959 (1 or 3 mg/kg) were determined based on our previous studies in Wistar rats (Havlickova, Charalambous et al. 2018, Sustkova-Fiserova, Puskina et al. 2020) and had no significant effect on the rat locomotor behavior (Jerabek, Havlickova et al. 2017). JMV2959 was administered 20 min prior the IVSA and CPP sessions or together with THC during the conditioning process during the second CPP experimental arrangement. Ghrelin was administered in dose 40 μg/kg i.p. 20 min prior to the IVSA sessions.

6.3. WIN55,212-2 Intravenous Self-Administration

This study involved a total of forty-four male rats with no prior exposure to the experimental conditions. For the main intravenous self-administration (IVSA) study using WIN55,212-2, statistical analyses were conducted on groups consisting of 10 rats (JMV2959), 9 rats (saline group), and 8 rats (ghrelin group). Additionally, in an additional IVSA experiment, four rats self-administered the vehicle, while another four rats self-administered WIN55,212-2. Seven rats were excluded from the analysis because they did not achieve the minimum daily cannabinoid intake requirement of at least 14 infusions, and two rats were excluded due to catheter obstruction causing catheter leakage. The rats underwent surgery under ketamine-xylazine anaesthesia, which involved the administration of ketamine at a dosage of 100 mg/kg intraperitoneally (i.p.) (Narketan, Vetoquinol, France) and xylazine at a dosage of 10 mg/kg i.p. (Xylapan, Vetoquinol, France). A permanent intracardiac silastic catheter was surgically implanted into the rats through the external jugular vein, with the catheter extending to the right atrium. The external part of the catheter protruded through the skin in the midscapular region and was securely attached to the needleless input (SAI Infusion Technologies, Lake Villa, IL,

USA). Following the surgical implantation of the catheters, they were flushed with heparin (heparin sodium/Heparin Leciva, Zentiva) to prevent clotting and maintain catheter patency. Additionally, antibiotics (cefazoline/Cefazolin, Sandoz, Austria) were administered to prevent infection, and analgesics (meloxicam, Metacam, Boehringer Ingelheim/Rhein, Germany) were given to provide pain relief for the rats. These measures were taken to ensure the well-being of the animals and minimize potential post-surgical complications. The self-administration sessions commenced on the sixth day following the catheter implantation surgery. Before and after each self-administration session, the catheters were flushed with a mixture of 0.3 mL of saline and heparin solution (5 IU). This flushing procedure served two purposes: to ensure the patency of the catheters and to prevent any blockages from occurring. By flushing the catheters with the saline-heparin solution, we could assess and maintain the functionality of the catheters throughout the self-administration sessions. Throughout the study, daily records were maintained to monitor various aspects of the rats' well-being. These records included observations of general behavior, assessment of catheter patency, measurements of body weight, and documentation of food intake for each individual animal. These daily recordings were crucial for tracking any changes or deviations in these parameters, allowing for the evaluation of the animals' overall health and the potential impact of the experimental procedures on their behavior, physiological status, and nutritional intake. The experimental cages used in the study were equipped with two levers positioned on one side of the cage. The levers and associated behavioral tasks were programmed using Graphic State Notation 3.0.3. Software (Coulbourn Instruments, Whitehall, PA, USA). The intravenous self-administration (IVSA) sessions were conducted following a fixed ratio schedule of reinforcement. This schedule required the rats to perform a specific number of lever presses to receive a reinforcement or reward (FR1; each correct response reinforced). An active lever-pressing (combined with a cue light) led to the activation of the infusion pump and administration of a single infusion of WIN55,212-2 (dose 12.5 μg/kg/infusion/0.1 mL) followed by a 15-s time-out, while an inactive lever-pressing was recorded but not rewarded. The cue light was flashing during dose infusion and off during the time-out. The house light was also flashing during each infusion. The sessions lasted for 120 min and were performed twice daily (once daily for each animal) from Monday to Friday. In the main IVSA study, we wanted to test the potential antagonistic effects of the GHS-R1A antagonist/JMV2959 in the reliable WIN55,212-2 self-administration model; thus, we chose adequate exclusion criterion which would guarantee a convincing level of self-administration. After a stabile drug consumption for at least seven sessions (above 70% preference of the active lever, minimum 14 infusions during a session) and after two consequent

sessions with a maximal deviation of 15%, rats were pretreated with JMV2959 (3 mg/kg i.p.) or ghrelin (40 μg/kg i.p.) or saline (0.1 mL/100 g body weight i.p.) 20 min before the IVSA session for three consecutive days. The next day, the 11-day abstinence period started. During the abstinence period, animals were housed individually in their cages. On the twelfth day of abstinence, the rats were placed again into their IVSA cages for one session, yet disconnected from the infusion pump, in order test the cannabinoid-seeking/relapse-like behavior (the lever-pressings were monitored). Twenty minutes before this drug-seeking test session, the rats were again pretreated with JMV2959 (3 mg/kg) or ghrelin (40 μg/kg i.p.) or saline (0.1 mg/100 g). The experimental schedule of the main IVSA study is illustrated below in Figure 4A. The numbers of active and inactive lever-pressing, number of infusions, and WIN55,212-2 consumption (μg/kg) were statistically analysed. The last three sessions/days with a stabilized WIN55,212-2 IVSA intake before pretreatment (5.–7. baseline), three consequent JMV2959/saline pretreatment sessions and "relapse-test" sessions were finally used in the statistical analysis. In the additional IVSA study, we wanted to document the WIN55,212-2 reinforcement effects in comparison to the vehicle IVSA and to test the pretreatment (JMV2959 and ghrelin) effects per se in the control vehicle IVSA conditions. Thus, instead of the cannabinoid, the vehicle was self-administered by four rats and WIN55,212-2 was self-administered by another four rats. After 14 days of IVSA (with no exclusive criterion), these rats were pretreated equally with JMV2959 (3 mg/kg i.p.) 20 min before the two consequent IVSA sessions; then, they were pretreated with JMV2959 (3 mg/kg i.p.) together with ghrelin (40 μg/kg i.p.) before the third pretreatment session and then, they were pretreated again with only the ghrelin (40 μg/kg i.p. 20 min before IVSA) for another two consequent sessions. In the main IVSA study, we observed slightly intensified pretreatment effects during the second pretreatment session; thus, we wanted to observe the effect of repeated JMV2959/ghrelin administration per se in the vehicle IVSA. The combination of the GHS-R1A antagonist/JMV2959 with GHS-R1A agonist/ghrelin should show the co-administration effect on the vehicle IVSA and try to prove the involvement of the GHS-R1A in the JMV2959 effects. Specifically, we wanted to test if co-administration with ghrelin would attenuate the JMV2959-induced reduction of the WIN55,212-2 IVSA. The co-administration was used as an interface between the single JMV2959 and ghrelin pretreatments. The experimental schedule of the additional IVSA study is illustrated below in Figure 4B. During the whole IVSA experiment, the body mass of all rats was monitored daily, and the difference between groups and possible impact of JMV2959 treatment on the body mass was statistically evaluated in the main IVSA study, within the last seven days before pretreatment, during the three days of pretreatment, the

tested relapse-like behavior day, and during all evaluated periods (7 baselines + 3 pretreatment days + relapse-like behavior day = 11 days).

WIN55,212-2 intravenous self-administration (IVSA) timeline
Scheme A (main study)

catheter implantation / recovery	self-administration aquisition / baseline measurments	pretreatment with JMV2959 3mg/kg or ghrelin 40μg/kg or saline 20 minutes before each session	abstinence period	relapse test / preatreatment with JMV2959 3mg/kg or ghrelin 40μg/kg or saline 20 minutes before the session
Day 1-5	minimum 14 days	3 days	11 days	1 day

Scheme B (additional study)

catheter implantation / recovery	self-administration aquisition / baseline measurments	pretreatment with JMV2959 3mg/kg 20 minutes before each session	co-administration of JMV2959 3mg/kg + ghrelin 40μg/kg 20 minutes before the session	preatreatment with ghrelin 40μg/kg 20 minutes before the session
Day 1-5	minimum 14 days	2 days	1 day	2 days

Figure 4. Timeline schedules of the IVSA experiments within the main IVSA study (A) and the additional IVSA study (B).

6.4. THC-Conditioned Place Preference

The biased conditioned place preference method was based on our previous experiences and the literature (Sanchis-Segura and Spanagel 2006, Jerlhag, Egecioglu et al. 2010, Jerabek, Havlickova et al. 2017, Havlickova, Charalambous et al. 2018, Sustkova-Fiserova, Puskina et al. 2020). The study utilized a three-compartment conditioned place preference (CPP) apparatus. The apparatus consisted of three compartments with distinctive visual and tactile cues in the outer compartments. The compartments were separated by gates that could be opened or closed as needed. The entire apparatus was illuminated with a light intensity of 45 lux, providing a consistent level of illumination throughout the experiment. The experiment consisted of pre-conditioning (day 1), conditioning (days 2–9), and post-conditioning (day 10). On day 1 (pre-conditioning), each rat received an intraperitoneal (i.p.) injection of saline 20 minutes before the testing session. The rat was then placed in the central compartment of the CPP apparatus with both gates open. During this 20-minute period, the initial or spontaneous place preference of the rat was determined, observing its natural preference for one particular compartment over the others. This pre-conditioning phase provided a baseline assessment of the rat's innate place preference prior to any conditioning or drug-related associations. During the conditioning phase, a repetitive procedure was employed to establish an association between THC (0.3 mg/kg i.p.) and the compartment that was initially the least preferred by the rat. This

involved pairing the administration of THC with the least preferred compartment in a systematic manner. Through repeated pairings of THC with the least preferred compartment, the aim was to condition the rat to associate the effects of THC with that specific compartment, potentially leading to a shift in the rat's place preference over time. In the first experimental arrangement, during the 8-day conditioning period, each rat received a total of two i.p. injections per day in a balanced design; THC was administered in the morning and saline in the afternoon and vice versa. After each drug injection, the rat was placed in the appropriate outer compartment (for 30 min, with the gate closed). On day 10 (post-conditioning test session), the rats were placed in the central compartment (with the gates open) and were given free access to both compartments for 20 min. To evaluate the effects of the GHS-R1A antagonist on the expression of THC-CPP, each rat was acutely injected with JMV2959 (1 or 3 mg/kg i.p.) or saline (i.p.) 20 min prior to the test session (number of rats in the groups n = 8–11). In the second experimental arrangement, the effects of GHS-R1A antagonism on the development of THC CPP were tested in a separate experiment, when JMV2959 (1 or 3 mg/kg i.p.) or saline (i.p.) were administered repeatedly during the 8-day conditioning phase, always together with THC in separate injections into different sites on the rat (n = 9–10). The calculation of conditioned place preference (CPP) involved comparing the percentage of total time spent in the THC-paired compartment (i.e., least spontaneously preferred) during the post-conditioning and pre-conditioning sessions. Previous studies have demonstrated that the administration of the vehicle/saline as well as JMV2959 per se does not induce any CPP conditioning (Jerlhag, Egecioglu et al. 2009); therefore, these experiments were not included.

A) JMV2959 effect on THC-induced craving

DAY	1	2	3	4	5	Weekend	6	7	8	9	10
Spontaneous Preference -assigned into groups of 2		**Conditioning** -saline application in the morning in the preferred compartment -THC application in the afternoon in the non-preferred compartment (0.3 mg/kg)				-no experimental procedures	**Conditioning** -THC application in the morning in the non-preferred compartment (0.3 mg/kg) -saline application in the afternoon in the preferred compartment				**Testing of condition place preference** - JMV2959 (0, 1, 3 mg/kg i.p.) pretreatment 20 min before testing

B) JMV2959 effects on the THC-induced reward

DAY	1	2	3	4	5	Weekend	6	7	8	9	10
Spontaneous Preference -assigned into groups of 2		**Conditioning** -saline application in the morning in the preferred compartment - JMV2959 (0, 1, 3 mg/kg i.p.) was administered repeatedly together with THC (0.3 mg/kg i.p.) in the non-preferred compartment in the afternoon				-no experimental procedures	**Conditioning** - JMV2959 (0, 1, 3 mg/kg i.p.) was administered repeatedly together with THC (0.3 mg/kg i.p.) in the non-preferred compartment in the morning --saline application in the afternoon in the preferred compartment				**Testing of condition place preference** - saline pretreatment 20 min before tetsing

Figure 5. Timeline schedules of the CPP experiments.

6.5. Statistical Analysis

For evaluation of the IVSA study data, the R program (Lucent Technologies, Vienna, Austria; R Core Team 2013) was used and for the statistical evaluation of the data from CPP, Sigma Plot 13 (Systat Software, Inc., San Jose, CA, USA) was used. The data from the IVSA study were subjected to Lilliefors test of normality (Kolmogorov–Smirnov test for not fully specified normal distribution) and the data from the CPP study were subjected to the Shapiro–Wilk normality test. Homogeneity of variance for analysis of variance (ANOVA) was tested using Levene´s test.

The obtained IVSA evaluated data, meaning the numbers of active and inactive lever presses and number of infusion, failed the equal variance tests. Furthermore, when the Lilliefors test of normality was applied, the acceptable use of normal distribution was rejected for all rat groups in IVSA. Due to the suggestion that the lognormal distribution would be appropriate, the data was subjected to a logarithmic transformation (LN) in order to achieve normality. For

the statistical significance calculations, the transformed data were used. The original data were displayed in the graphs, while the significances following the ANOVA/Bonferroni approach were based on the transformed data. The comparison of active and inactive lever-pressing during the IVSA procedure was performed using a t-test on all analysed baseline data of the last three sessions before pretreatments.

In the main IVSA study, the statistical differences between the saline versus the JMV2959 or the ghrelin groups relative to the time/session and procedure related changes were calculated by two-way repeated measures analysis of variance (ANOVA RM), with the group (saline/JMV2959/ghrelin) and time/session (5.–7. baselines, 1.–3. pretreatments) as factors, followed by Bonferroni post-hoc tests. The lever-pressing difference between groups during the WIN55,212-2-seeking/relapse-like behavior testing session was analysed separately using the two-way ANOVA/Bonferroni with the group (saline/JMV2959/ghrelin) and lever-pressing type (active/inactive lever-pressing) as factors (again after logarithmic transformation of the data). When the drug-seeking active lever-pressing data were expressed in percentage of the baseline mean, the results failed the normality test. Thus, the Kruskal–Wallis one-way analysis followed by the Dunn´s test was used for analysing the differences among the groups. The percentage data were not transformed. In exception of the relapse-test session, all IVSA parameters were calculated as a total number of active and inactive lever-presses, infusions, and WIN55,212-2 consumption (mg/kg) during the 2-hour daily sessions. The last three WIN55,212-2 IVSA sessions/days prior to the pretreatments, the three pretreatment sessions (saline/JMV2959/ghrelin), and the relapse-test session were used in the statistical analyses.

In the additional IVSA study, a two-way repeated measures ANOVA/Bonferroni test was used for comparison of the WIN55,212-2 and vehicle IVSA (group factor) and the time/pretreatment effects (non-pretreated baselines, two JMV2959, one JMV2959 + ghrelin, two ghrelin pretreatment sessions); means of the last three baselines before the pretreatment were used in the statistical analyses (mean of 5.–7. baseline sessions). The WIN55,212-2 and the vehicle lever-pressing comparison was also conducted using a t-test within all baseline data of the last three sessions before pretreatments.

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). The mean of 3 baselines and the mean of 3 pretreatment sessions within the main IVSA study were illustrated as means ± SEMs (average results). The group means with 95% confidence intervals (95% CI) were used to present all subsequent results. Calculations for the 95% confidence interval (95% CI) adjustment were performed for small groups using the relevant t-values. As mentioned above, the data failed the

Lilliefors normality test in the IVSA studies, therefore, the data were subjected to a logarithmic transformation before the statistical analysis. Thus, the original results are depicted in the presented graphs, accompanied by the significance levels obtained from the ANOVA/Bonferroni tests conducted with transformed data.

The place preference scores were determined by calculating the percentage (%) difference in the total time spent in the THC-paired compartment (spontaneously least preferred) during the postconditioning session compared to the preconditioning session. The evaluated CPP data passed the normality test. The equal variance test passed in the CPP arrangement when the JMV2959 was administered repeatedly during conditioning. The equal variance test passed when the acute JMV2959 was administered at the lower 1 mg/kg dose and it failed with the higher 3 mg/kg acute dose in comparison with the saline group. The differences between the groups were assessed using a one-way ANOVA, followed by the Holm-Sidak post-hoc test for further analysis. The absolute values of time spent in the THC-paired compartment during the pre-conditioning and post-conditioning sessions were compared using a two-way repeated measures ANOVA followed by the Bonferroni's post hoc test for further analysis.

7. Results

7.1. Ghrelin and JMV2959 effects on the WIN55,212-2 Intravenous Self-Administration

In the main IVSA study, only rats with a minimum of 14 infusions per session were used during the baseline period for pretreatments and statistical evaluation. The rats were monitored and daily handled. Their weight was recorded during the whole IVSA experiment. All IVSA experiments were performed during the reverse/dark period of a 12 h light/dark cycle. The last three 120-min IVSA sessions from a total of about 14 sessions prior to the pretreatments were used as baseline data. The t-test revealed significant differences between baseline active and inactive lever-pressing $t\ (160) = 17.13$, $p < 0.001$. We observed distinct inter-individual differences within the basal WIN55,212-2 self-administration among all rats, however, the cannabinoid IVSA did not significantly differ among the groups that were pretreated with saline (n = 10), JMV2959 (n = 9), or ghrelin (n = 8) in all observed parameters: number of active and inactive lever-pressing, number of infusions, and the WIN55,212-2 intake consumption. The pretreatment with the GHS-R1A antagonist (before all three consequent 120-min sessions) significantly reduced the self-administration behavior below the achieved basal maintenance values of WIN55,212-2 self-administration, while ghrelin pretreatment significantly increased it (see Figure 6).

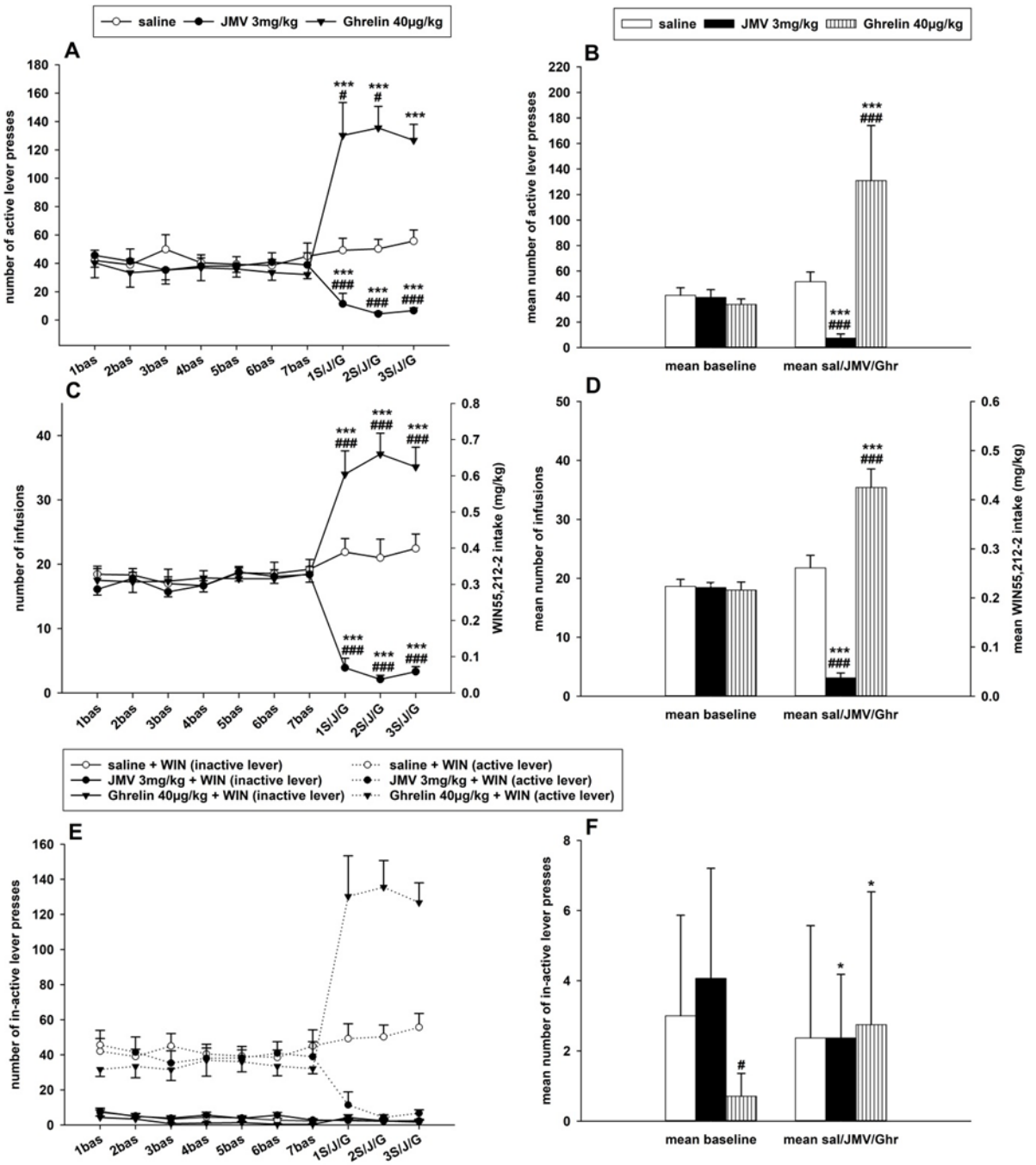

Figure 6. Effects of ghrelin and JMV2959 on WIN55,212-2 Intravenous Self-Administration. All administered intraperitoneally 20 min before the 120-min IVSA sessions: Saline (1 ml/kg) or JMV2959 (3 mg/kg) or ghrelin (40 µg/kg) (A) Active lever-pressings, (C) number of infusions, (E) numbers of inactive lever-pressings before pretreatments (1.–7. bas) and three days of pretreatments (1.–3. S/J/G). For statistical analysis, the last three baselines (5.–7. bas) were used using a two-way repeated measures ANOVA followed by the Bonferroni test. Before the statistical analysis, the data went through a logarithmic transformation. The original data together with the significances were calculated via the transformed ANOVA results (presented above). The means of saline/JMV2959/ghrelin (1.–3. S/J/G) active lever-pressing (B), infusions

*(D), and inactive lever-pressing (F) are illustrated together with the baseline means (5.–7. bas). The effects are presented as follows: Saline (open circle, open bar) (n = 9), JMV2959 (filled circle, filled bar) (n = 10), ghrelin (filled triangle, striped bar) (n = 8). Differences between the groups in comparison to saline group are expressed as # p < 0.05, ## p < 0.01, ### p < 0.001. Differences in the respective baseline mean within the group are expressed as *** p < 0.001. The results in graphs A, C, E are presented as group means with 95% confidence intervals. The results in graphs B, D, F are presented as means ± SEM.*

The active lever-pressing for WIN55,212-2 was significantly attenuated when the GHS-R1A antagonist (JMV2959 3 mg/kg) was administered 20 min before the three consequent 2-hour sessions in comparison to the saline group as well as to the baseline mean ($p < 0.001$) (see Figure 6A,6B). The two-way repeated measures ANOVA followed by the Bonferroni's post hoc test, used for analysing the JMV2959, ghrelin, and saline effects together, revealed an overall significant effect of time ($F1,150 = 15.55$; $p < 0.001$) and the group × time effect ($F2,150 = 5.03$; $p < 0.01$), and the effect of group was not significant ($F2,1 = 2.50$; n.s.). The basal active lever-pressing (mean of five to seven baselines) was 40.9 ± 6.1 in the saline and 39.3 ± 6.1 in the JMV2959 groups. JMV2959 pretreatment reduced the basal pressing to 7.4 ± 3.3 (mean of three pretreatment sessions), which represented 16.3% ± 4.4 of the baseline mean. Saline pretreatment resulted in 51.7 ± 7.6 (128.9% ± 7.8 of baseline mean) (the change was not significant in comparison to baseline mean). Ghrelin 40 µg/kg i.p. pretreatment (20 min before sessions) increased the active lever-pressing in all rats in comparison to the baseline mean 33.9 ± 4.3 up to an average of 130.8 ± 43.2 (343.2% ± 77.5 of the baseline mean). Extreme inter-individual differences were observed in response to ghrelin among rats from a minimum of 151% to a maximum of 716% of the baseline. The ghrelin-induced increase of active lever-pressing was significant in comparison to the baseline mean ($p < 0.001$) and to the saline group with $p < 0.001$ during the first and second pretreatment and $p < 0.01$ in the third pretreatment session. The apparent significant ghrelin-induced increase of active lever-pressing in comparison to the baseline mean ($p < 0.001$) and to the saline group ($p < 0.05$) is illustrated in Figure 6B, which illustrates comparisons of baseline and pretreatment means.

The number of infusions and the daily 2-hours WIN55,212-2 intake/doses in mg/kg are illustrated in Figure 6C and the comparison of the average basal (5–7. baseline) and mean pretreatment (1–3. pretreatment) results is illustrated in Figure 6D. A 12.5-µg/kg/infusion WIN55,212-2 dose was used in the FR1 self-administration schedule, and each infusion was followed by 15-s time-out period; the active lever-pressing during the time-out was not

rewarded. The average basal number of infusions and WIN55,212-2 intake (mean 5–7. baseline) was 18.6 ± 1.2 infusions and 0.235 ± 0.015 mg/kg within the saline group and 18.4 ± 0.9 infusions and 0.230 ± 0.012 mg/kg in the JMV2959 group. Pretreatment with JMV2959 significantly ($p < 0.001$) reduced the number of infusions/consumptions of WIN55,212-2 to 16.4% ± 4.2 of the baseline mean, while after the saline pretreatment, the number of infusions/WIN55,212-2 intake reached 115.7% ± 6.3 of the baseline mean (which was not significant in comparison to the baseline mean). The two-way repeated measures ANOVA followed by the Bonferroni test analysing the JMV2959, ghrelin, and saline effects together revealed overall significant differences among the groups ($F2,1 = 7.78$; $p < 0.001$), the group × time ($F2,150 = 5.04$; $p < 0.01$), and the effect of time ($F1,150 = 49.23$; $p < 0.001$). Pretreatment with JMV2959 always significantly ($p < 0.001$) reduced the number of infusions and the spontaneous WIN55,212-2 consumption also in comparison to the saline group. The ghrelin pretreatment almost doubled the number of infusions and the WIN55,212-2 intake from basal values 18.0 ± 1.4 infusions and 0.225 ± 0.017 mg/kg to 35.4 ± 3.1 infusions and 0.443 ± 0.039 mg/kg, respectively (199.8% ± 17.8 of baseline mean), which represented a significant increase in comparison to the baseline ($p < 0.001$) as well as to the saline group ($p < 0.01$ in the second pretreatment and $p < 0.05$ within the other pretreatments). Similarly, to the active lever-pressing, the ghrelin-induced increase of the number of infusions/WIN55,212-2 intake was significant relative to the baseline mean ($p < 0.001$) and to saline group ($p < 0.05$) when the baseline and pretreatment means were compared (see Figure 6D).

The inactive lever-pressing, illustrated in Figure 6E, F, showed low basal activity (mean 5–7. baseline): 4.07 ± 3.1 in the JMV2959 group, 3.00 ± 2.9 in the saline group, and 0.71 ± 0.65 in the ghrelin group, and pretreatments did not produce any significant changes in all the analyses using the two-way repeated measures ANOVA followed by the Bonferroni test.

The rats were single housed in their home cages during the forced abstinence and were monitored daily. On the twelfth day of the forced abstinence, the cannabinoid/WIN55,212-2-seeking/relapse-like behavior was tested back in the IVSA cages within a two-hour session under the standard IVSA conditions; however, the rats were not connected to the infusion pump. The active lever-pressing was not rewarded, but only recorded as well as the inactive lever-pressing, illustrated in Figure 7. Twenty minutes before the relapse-test session, JMV2959 or ghrelin or saline were administered to the appropriate animals. The two-way repeated measures ANOVA followed by the Bonferroni test (using the transformed data) was used for comparison of the active/inactive lever-pressing and the JMV2959/saline/ghrelin pretreatment effects (group), and it revealed significant differences among the groups ($F2,1 = 16.80$; $p < 0.001$), the

type of lever-pressing ($F_{1,48} = 45.21$; $p = 0.001$), and the group × lever-pressing type effect ($F_{2,48} = 5.01$; $p = 0.05$). The WIN55,212-2-seeking behavior was significantly decreased by the JMV2959 pretreatment ($p < 0.001$) in comparison to the saline-pretreated group. After the pretreatment with ghrelin, the relapse-like behavior was increased, however, the difference was not significant in comparison to the saline-pretreated group. When the WIN55,212-2-seeking active lever-pressing was expressed in a percentage to the baseline-pressing mean (see Figure 7), a decrease to 20.6% ± 4.5 within the JMV2959 group, an increase to 189.6% ± 52.6 within the saline group, and a distinct increase to 330.9% ± 88.2 in the ghrelin-pretreated group were observed. The Kruskal–Wallis one-way analysis followed by the Dunn's test comparison of active lever-pressing expressed in the percentage of baseline means during the relapse-test session (using the original data) revealed significant differences among the saline/JMV2959/ghrelin groups ($H = 19.30$ with 2 degrees of freedom; $p < 0.001$), specifically with the significant difference only between the saline and the JMV2959 groups ($p < 0.01$). The inactive lever-pressing was not expressed in a percentage because of zero occurring within the basal pressing.

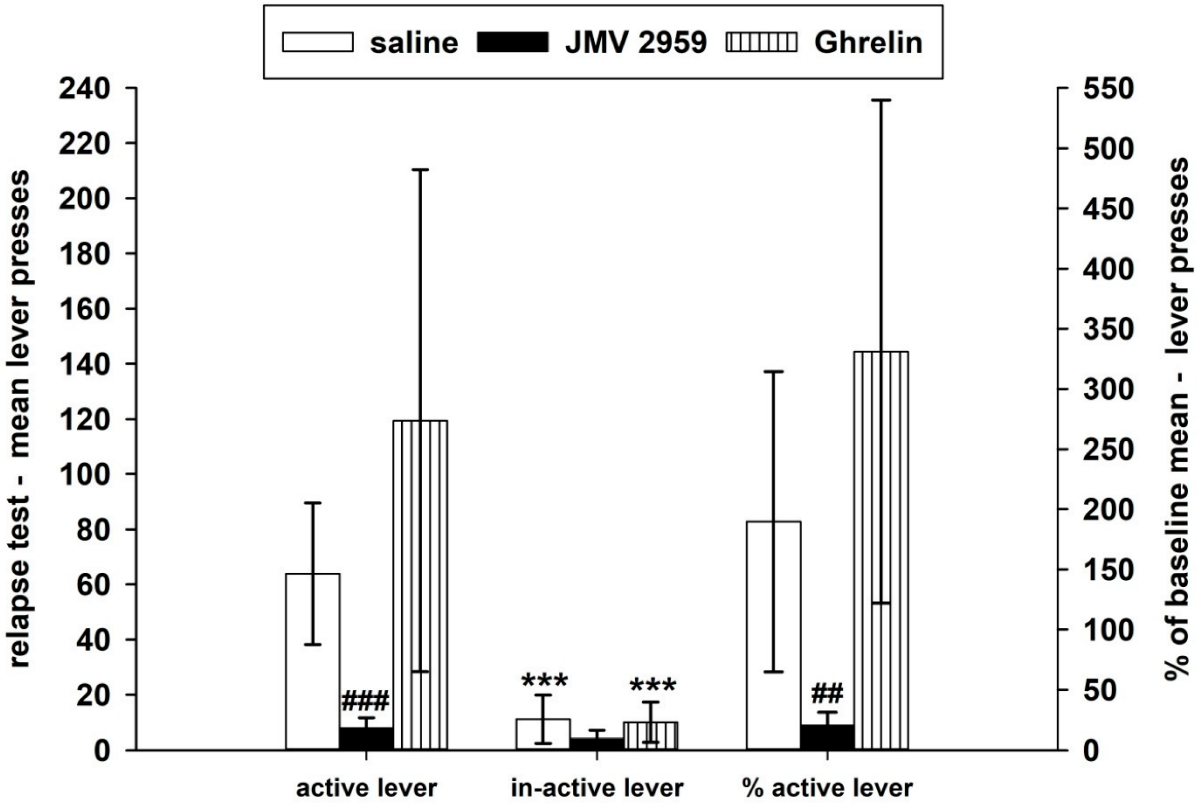

Figure 7 Effects of Ghrelin and JMV2959 on WIN55,212-2-seeking lever-pressing/relapse-like behavior.

*On the 12th day of the forced abstinence of the WIN55,212-2 IVSA, the active/inactive lever-pressing and percentage of the baseline mean (mean of the last three baselines before pretreatments, 5.-7. bas) effects were observed. 20 min before the 120-min session, saline (1 mL/kg) or JMV2959 (3 mg/kg) or ghrelin (40 µg/kg) were administered intraperitoneally while the rats were in the IVSA cages (cages not connected with the infusion pump). The IVSA relapse-test data went through logarithmic transformation before the statistical analysis. Figure 7 illustrates the original data together with significances calculated from the transformed ANOVA results. The percentage data were analysed directly and not transformed using the Kruskal–Wallis one-way analysis followed by a Dunn´s test. The means of active lever-pressing in the groups are presented as follows: Saline (open bar) (n = 9), JMV2959 (filled bar) (n = 10), ghrelin (striped bar) (n = 8). Differences between the groups in comparison to the saline group are expressed as ## $p < 0.001$, ### $p < 0.01$. Differences between active and inactive lever-pressing are expressed as *** $p < 0.001$. The results are presented as group means with 95% confidence intervals.*

During the IVSA experiments including the relapse-test session, the rat apparent individual differences in reactivity to the appropriate pretreatments are illustrated in Figure 8. These differences in WIN55,212-2 IVSA activity among the rats were observed during the whole study. For statistical evaluation, we have used the last three baselines before pretreatments as basal IVSA values. During the 3 days of saline pretreatment, the daily active lever-pressing ranged from 76 % to 194 % of baseline mean (see Figure 8A). During the 3 days of JMV2959 pretreatment, the active lever-pressing ranged from 0% to 91% of baseline mean. With two exceptions, once at the 91 % on the first pretreatment session and once at the 58 % on the third pretreatment sessions, the JMV2959 active lever pressing was below 37 %. Only in three sessions from all pretreatments the active lever-pressing was completely abolished by the JMV2959 pretreatment (0 %). During the 3 sessions of ghrelin pretreatment, the active lever-pressing ranged from 131 % to 767 % of baseline mean. This is mainly because two rats were extremely interested in the active lever after ghrelin pretreatment and pressed above 541% of baseline mean (541 % - 767 %). Another two rats in the ghrelin group pressed above 300 % with maximum 345 % of baseline mean in at least two sessions, the active lever-pressing of the remaining rats reached maximum 261 % of baseline mean. The two rats with the highest numbers of active lever-pressing during all three pretreatments showed no apparent signs of behavioral disturbances, such as frozen postures, sedation etc., no back leaning on the lever, they were fully attracted to the active lever. These two rats did not differ from the rest of the

rats considering the number of infusions (see Figure 8B). After ghrelin pretreatment, the number of infusions was ranging between 102% and 306% of baseline mean. Therefore, these rats achieved higher active lever-pressing during the time-out period. The JMV2959 pretreatment again reduced the number of infusions, thus increased the homogeneity of the values in the group.

Apparent differences in the individual reactivity of the rats to the pretreatments during the WIN55,212-2 seeking/relapse-like behavior (on the 12th day of forced abstinence period) are illustrated in Figure 8C. The JMV2959 pretreatment reduced the non-rewarded cannabinoid-seeking/relapse-like active lever-pressing to values within 3 – 51 % of the baseline mean; active lever-pressing was never completely abolished on the relapse-test session. Within the ghrelin pretreated group, the unreinforced active lever-pressing was within 110 – 642 % of the baseline mean. Within the saline-group, the mean active lever-pressing ranged during the relapse-test session from 77% to 564 % of baseline mean, with average 189.6 % ± 52.7 of baseline mean, which indicates craving incubation in accordance with the literature (Kirschmann, Pollock et al. 2017).

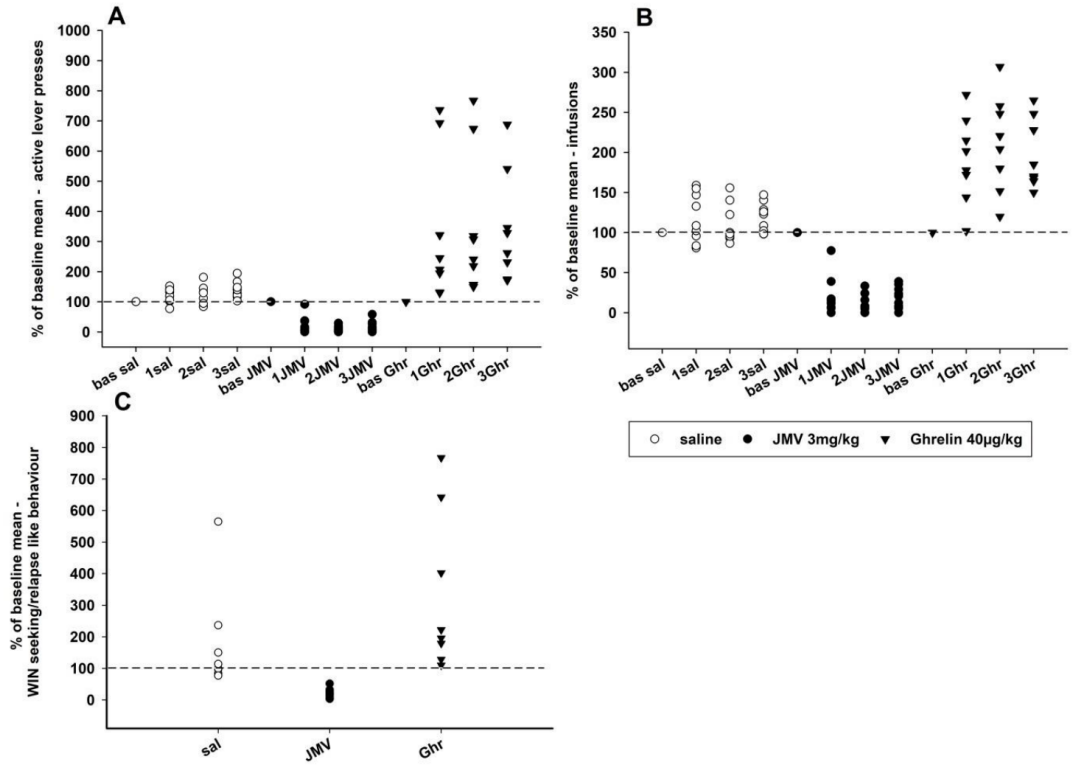

Figure 8 Effects of Ghrelin and JMV2959 on WIN55,212-2 Intravenous Self-Administration in single rats.

Percentage of baseline mean (mean of the last three baselines before pretreatments, 5.-7. bas). Saline (1 ml/kg) or JMV2959 (3 mg/kg) or ghrelin (40 µg/kg) were administered intraperitoneally 20 min before the 120-min IVSA sessions. The active lever-pressing is presented in the graph A, the number of infusions in the graph B and the WIN55,212-2 – seeking/relapse-like non-reinforced active lever-pressing on the 12th day of forced abstinence during the relapse-test session in the graph C. The results are illustrated as follows: saline (open circle), JMV2959 (filled circle), ghrelin (filled triangle). The dotted line shows the baseline mean level (bas, 100%).

7.2. Ghrelin and JMV2959 Effects on Vehicle and WIN55,212-2 Intravenous Self-Administration (additional IVSA study)

In the additional IVSA, a separate group of rats was used for comparison of the WIN55,212-2 IVSA, the vehicle IVSA and the appropriate pretreatments in changes of active lever-pressing illustrated in Figure 9. 4 rats self-administered the vehicle, another 4 the WIN55,212-2 again in a dose 12.5 µg/kg/infusion. The rats were randomly chosen without any minimum 14 daily infusions or other criterion demands; the IVSA arrangement was the same as in the main experiment (120-min sessions with schedule FR1, 15-s time-out, lights, etc.). The experimental schedule was as follows: the last 3 baseline 120-min sessions before pretreatments (from total 14 sessions) served as baseline values, then JMV2959 (3 mg/kg i.p.) was administered 20 min before two consequent sessions, before the third pretreatment session ghrelin (40 µg/kg i.p.) was applied together with JMV2959 in separate injections, and then, ghrelin (40 µg/kg i.p.) alone was injected 20 min before two consequent sessions. The t-test comparing all baseline data (3 baselines before pretreatments) revealed significant differences between the WIN55,212-2 (18.0 ± 1.9) and vehicle (6.5 ± 1.1) number of infusions $t(22) = 4.62$, $p < 0.001$, as well as the number of active lever presses (30.0 ± 4.6 versus 11.9 ± 2.4) ($t(22) = 4.62$, $p < 0.001$). Active versus inactive lever-pressing was significantly different within the WIN55,212-2 IVSA ($t(22) = 6.79$; $p < 0.001$) (basal inactive lever-pressing 4.1 ± 1.0) and also within the vehicle IVSA ($t(22) = 4.07$, $p < 0.01$) (basal inactive lever-pressing 4.2 ± 1.2), but there were no significant differences within inactive lever-pressing either after pretreatments, nor between the IVSA cannabinoid/vehicle groups. Using two-way repeated measures ANOVA followed by the Bonferroni test with factors IVSA type (WIN55.212-2/vehicle) (group) and pretreatments (baseline/JMV2959/JMV2959+ghrelin/ghrelin) (time), revealed significant differences among the groups ($F1,6 = 3,87$; $p < 0.05$), effect of time ($F3,18 = 18.49$; $p < 0.001$),

and the group × time effect (F3,18 = 9.03; p < 0.001) after the comparison of the number of active lever-pressing. The pretreatments had no significant influence on the vehicle IVSA. In the cannabinoid IVSA, a significant active lever-pressing reduction was observed after JMV2959 pretreatment to 17.4% ± 2.8 of baseline mean (p < 0.01 in comparison to baseline). The JMV2959 effect was attenuated by the ghrelin co-administration during the 3 pretreatment session to 49.2% ± 11.0 (n.s. to baseline) and ghrelin pretreatment increased the active lever-pressing to 182.4% ± 21.3 (p < 0.01 to baseline). When the changes were expressed in the percentage of the baseline mean (see Figure 9B), the two-way ANOVA RM/Bonferroni confirmed the significant pretreatment effects within the WIN55,212-2 IVSA groups and no significant effects within the vehicle IVSA groups. The JMV2959, co-administration JMV2959 + ghrelin, and ghrelin pretreatment percentage changes were significantly different between the WIN55,212-2 and vehicle IVSA (p < 0.05) (effect of time F2,12 = 20.62, p < 0.001; group × time effect F2,12 = 10.57, p = 0.002; the effect of group was not significant, F1,6 = 1.19).

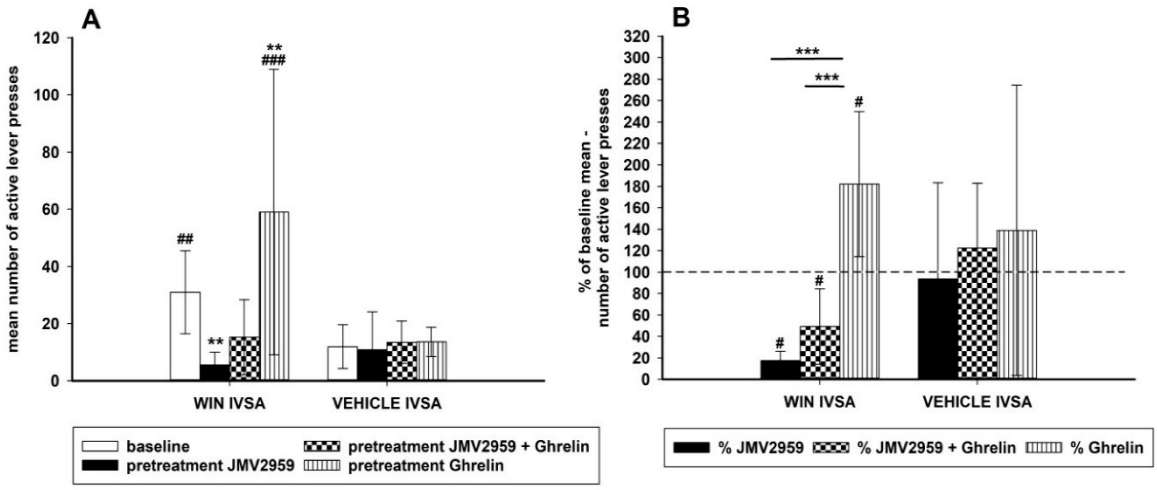

Figure 9. Effects of Ghrelin and JMV2959 Effects on Vehicle and WIN55,212-2 Intravenous Self-Administration – Active lever-pressing (additional IVSA experiment)

*The number of active lever-pressing for the vehicle and the WIN55,212-2 IVSA are illustrated in graph (A). Baseline pressing (mean of 3 sessions before pretreatment) was influenced by the administered intraperitoneally 20 min before the 120-min sessions pretreatment with JMV2959 (3 mg/kg) or JMV2959 + ghrelin or ghrelin (40 µg/kg). The means of the active lever-pressing are presented as follows: basal lever-pressing (open bar), JMV2959 (filled bar), JMV2959 + ghrelin (dotted bar), ghrelin (striped bar). Differences between WIN55,212-2 IVSA and vehicle IVSA are expressed as ## p < 0.01, ### p < 0.001. Differences of pretreatments to baseline lever-pressing are expressed as ** p < 0.01. The effects of pretreatments presented in the*

*percentage of the average baseline active lever-pressing (graph B) are presented as follows: percentage JMV2959 effect (filled bar), percentage JMV2959 + ghrelin effect (dotted bar), percentage ghrelin effect (striped bar). Differences between WIN55,212-2 IVSA and vehicle IVSA are expressed as # $p < 0.05$. Differences between pretreatments are expressed as *** $p < 0.001$. Dotted line shows the baseline active lever-pressing (100%). The additional IVSA data went through logarithmic transformation before the statistical analysis; thus, in the graphs are presented original data together with significances obtained from the transformed ANOVA results. The results are presented as group means with 95% confidence intervals (n = 4).*

Comparison of the number of infusions using the two-way repeated measures ANOVA followed by Bonferroni test with factors: groups/IVSA type (WIN55.212-2/vehicle) and time (non-pretreatment baseline/JMV2959/JMV2959+ghrelin/ghrelin) revealed significant differences among the groups ($F_{1,6} = 3.84$; $p < 0.05$), effect of the time ($F_{3,18} = 20.12$; $p < 0.001$) and the group x time effect ($F_{3,18} = 9.86$; $p < 0.001$). However, the pretreatments had no significant influence on the vehicle IVSA. Means of the three last baselines before pretreatments were used in the statistical analysis. Within the cannabinoid IVSA, we observed significant reduction of number of infusions after JMV2959 pretreatment to 23.9% ± 5.0 of baseline mean ($p < 0.001$ in comparison to baseline). This JMV2959 effect was attenuated by ghrelin co-administration during the third pretreatment session to 58.13% ± 12.1(n.s. to baseline) and ghrelin pretreatment increased the number of infusions to 141.5 % ± 22.1 ($p < 0.05$ to baseline). When the changes were expressed in percentage of baseline mean (see Figure 10B), two-way ANOVA RM/Bonferroni revealed again the significant pretreatment effects within the WIN55,212-2 IVSA group and no significant effects within the vehicle IVSA. Significant difference was found between WIN55,212-2 and vehicle IVSA in percentage of baseline means in number of infusions only in the co-administration (JMV2959 + ghrelin) session ($p < 0.05$) (effect of the time $F_{2,12} = 28.19$, $p = 0.001$; the group x time effect $F_{2,12} = 11.4$, $p = 0.03$; effect of the group was not significant, $F_{1,6} = 2.81$)

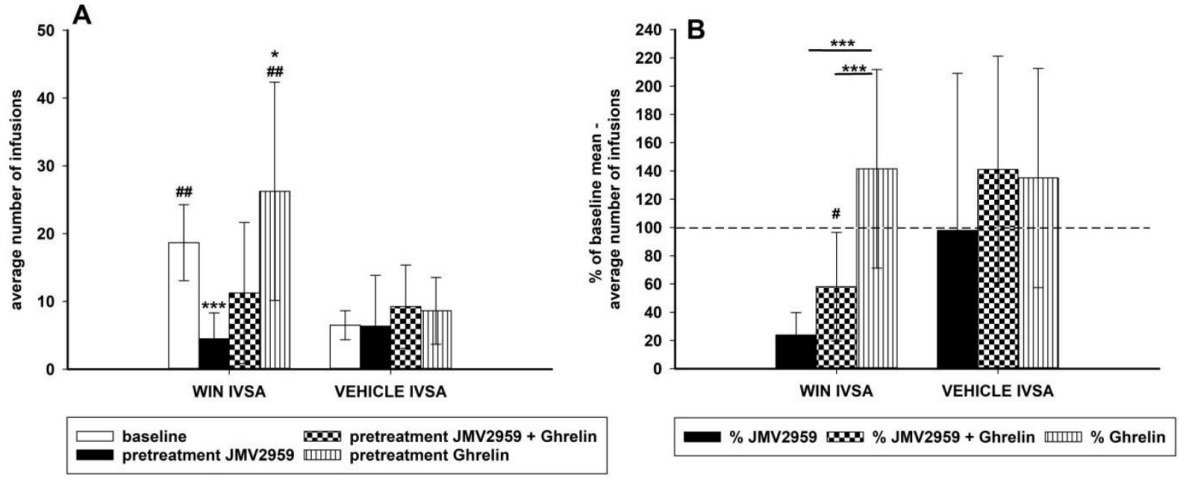

Figure 10. Effects of Ghrelin and JMV2959 Effects on Vehicle and WIN55,212-2 Intravenous Self-Administration – Number of Infusions (additional IVSA experiment)

*The number of infusions in the vehicle and WIN55,212-2 groups are illustrated in the graph A. The baseline number of infusions (mean of last 3 sessions before pretreatment) was influenced by pretreatment with JMV2959 (3 mg/kg) or JMV2959 + ghrelin or ghrelin (40 µg/kg) administered intraperitoneally 20 min before the 120-min sessions. The mean number of infusions are presented as follows: basal lever-pressing (open bar), JMV2959 (filled bar), JMV2959 + ghrelin (dotted bar), ghrelin (striped bar). Differences between WIN55,212-2 IVSA and vehicle IVSA are expressed as # $p < 0.05$, ## $p < 0.01$. Differences of pretreatments to baseline lever-pressing are expressed as * $p < 0.05$, *** $p < 0.001$. The effects of pretreatments illustrated in percentage of average baseline number of infusions (graph B) are presented as follows: percentage JMV2959 effect (filled bar), percentage JMV2959 + ghrelin effect (dotted bar), percentage ghrelin effect (striped bar). The statistical differences between the percentage of WIN55,212-2 IVSA and vehicle IVSA are expressed as # $p < 0.05$. Differences between pretreatments are expressed as *** $p < 0.001$. Dotted line shows the baseline active lever-pressing (100%). The additional IVSA data went through logarithmic transformation before the statistical analysis. The above figure presents the original data together with significances calculated from the transformed ANOVA results. The results are presented as group means with 95% confidence intervals (n = 4).*

7.3. Body mass within the Intravenous Self-Administration studies

A 20 g/d of a standard chow food and ad libitum water was given to the rats throughout the IVSA conditioning and experiments. The daily amount was always fully consumed by all

rats regardless of the treatments. In the IVSA study, the body mass of the rats was measured daily, and no significant changes were observed concerning JMV2959 or ghrelin administrations (see Table 1). The body mass changes were calculated within the main WIN55,212-2 IVSA experiment during the last week before pretreatments (bas 1 - 7), during the pretreatment days (JMV/sal/ghrelin 1 – 3), on the day of WIN55,212-2 -seeking/relapse-like behavior testing and during all evaluated periods together (7 baselines + 3 JMV/sal/ghrelin + WIN55,212-2-seeking/relapse-like behavioral day = total 11 days). According to the two-way ANOVA RM/Bonferroni test, there were no significant differences among the JMV2959, ghrelin and saline baseline groups as well as no influence of JMV2959/ghrelin/saline pretreatments. The only significant changes – weight gains were observed on the WIN55,212-2-seeking/relapse-like day in all three rat groups ($p < 0.001$), specifically 61 g within the JMV2959, 64 g within the saline and 59 g within the ghrelin group.

Changes of rat body mass within the main WIN55,212-2 IVSA study group means (g ± SEM)				
Group/interval	Baseline mean	Sal/JMV/ghrelin means	Relapse-test	Total mean
JMV2959 group	287.0 ± 3.0	292.2 ± 3.5	353.7 ± 2.8	294.5 ± 2.7
Saline group	288.1 ± 3.4	293.0 ± 6.5	357.0 ± 5.4	295.7 ± 3.7
Ghrelin group	287.4 ± 4.5	290.9 ± 6.0	349.7 ± 2.6	294.0 ± 4.3

Table 1. Changes of rat body mass within the IVSA study.

The body mass changes are shown as group means ± SEM (JMV2959 group n = 10, Saline group n = 9, Ghrelin group n = 8) during the chosen periods of the experiment as follows: mean of the last 7 days before pretreatment (Baseline mean), mean of the 3 pretreatment days (Sal/JMV/ghrelin means), the day of the WIN55,212-2 -seeking/relapse-like behavior test (Relapse-test), mean of 7 baseline days + 3 pretreatment days + day of WIN55,212-2 -seeking/relapse-behavior testing (Total mean). Two-way ANOVA RM difference among the groups: $F_{2,2} = 0.865$, $p = 0.42$ (n.s.), effect of the time: $F_{4,288} = 300.16$; $p < 0.001$, (difference of WIN55,212-2 seeking/relapse-like behavior in all groups - JMV2959, ghrelin and saline vs. other parameters), effect of the group x time: $F_{4,288} = 0.255$, $p = 0.91$ (n.s.).

7.4. JMV2959 Effects on Manifestation and Development of THC-Induced Conditioned Place Preference (CPP)

The CPP was calculated as the difference in the percentage of total (20 mins) time spent in the THC-paired/least preferred compartment during the post-conditioning session (day 10) and/minus the pre-conditioning session (day 1); eight days of THC-conditioning were used. The THC-induced CPP manifestation was significantly and dose-dependently attenuated by the 1 and 3 mg/kg JMV2959 when administered 20 min before testing on the post-conditioning day: $F_{2,24} = 8.65$, $p < 0.001$ (see Figure 11A). When the higher dose 3 mg/kg JMV2959 was repeatedly administered together with THC during conditioning, the development of THC-CPP was significantly reduced: $F_{2,25} = 9.52$, $p < 0.001$; the effect of the lower dose (1 mg/kg) was not significant (see Figure 11B). The JMV2959 doses (1 and 3 mg/kg i.p.) did not significantly influence the rat locomotor behavior within the tested period in our previous study (Jerabek, Havlickova et al. 2017). JMV2959 alone did not induce any CPP (Jerlhag, Egecioglu et al. 2009), therefore, it was not necessary to test it.

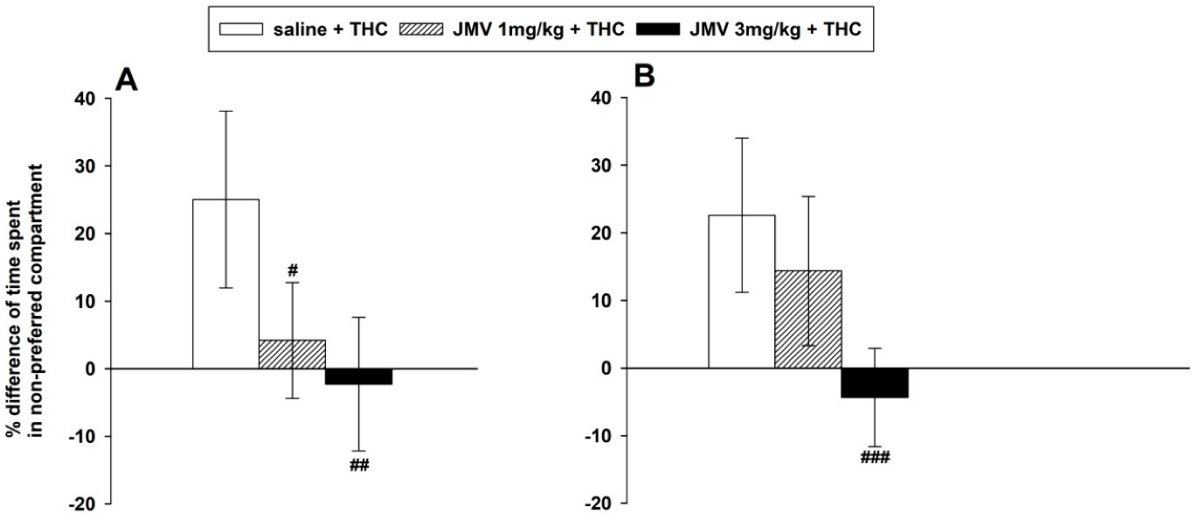

Figure 11. Effects of JMV2959 on THC-induced CPP in rats - percentage of total time spent in the THC-paired/least preferred compartment during the post-conditioning and/minus the pre-conditioning session.

JMV2959 (0, 1, 3 mg/kg i.p.) was administered in a single dose 20 min before the final testing after 8 days of conditioning with THC (0.3 mg/kg i.p.) (saline n = 11; JMV2959 groups n = 8; means ± SEM) in graph A. JMV2959 (0, 1, 3 mg/kg i.p.) was administered repeatedly during the 8 days conditioning together with THC (0.3 mg/kg i.p.) (saline n = 10; JMV2959 groups n = 9; means ± SEM)) in graph B. The results are presented as follows: Saline + THC (open bar), JMV2959 1 mg/kg + THC (striped bar), JMV2959 3 mg/kg + THC (filled bar). CPP was calculated as the difference in percentage of total (20 mins) time spent in the THC-paired (i.e., least preferred) compartment during the post-conditioning and/minus the pre-conditioning

session. The effects of JMV2959 pretreatments in comparison to the saline group are expressed as # p < 0.05, ## p < 0.01, ### p < 0.001. The results are presented with 95% confidence intervals as group means.

The effects of JMV2959 pretreatments on the THC- CPP were also calculated in a different way (for comparison), and similar results were obtained. The absolute values of time spent in the THC-paired/least preferred compartment before (pre-conditioned session, day 1) and after conditioning (post-conditioned session, day 10). These results were statistically evaluated using two-way repeated measures ANOVA/Bonferroni, with the group/treatment (0, 1, 3 mg/kg JMV2959) and the time/session as the factors. In both CPP arrangements the THC-CPP was established ($p < 0.001$). The acute JMV2959 administration after the THC conditioning significantly and dose dependently reduced the THC-CPP expression ($p < 0.05$ and $p < 0.001$) (see Figure 12A). The two-way ANOVA RM/Bonferroni revealed an overall effect of the time ($F_{1,24} = 9.32$, $p = 0.005$) and the group x time interaction ($F_{2,24} = 8.65$, $p = 0.001$), the effect of group was not significant ($F_{2,24} = 1.70$, n.s.). The repeated JMV2959 administration with the THC during conditioning together significantly reduced the THC-CPP development only when the higher 3 mg/kg JMV2959 dose was used; the lower 1 mg/kg JMV2959 dose was not significant (see Figure 12B). The two-way ANOVA RM/Bonferroni revealed overall effect of the group ($F_{2,25} = 4.51$, $p = 0.02$), the time ($F_{1,25} = 17.60$, $P < 0.001$) and the group x time interaction ($F_{2,25} = 9.52$, $p < 0.001$).

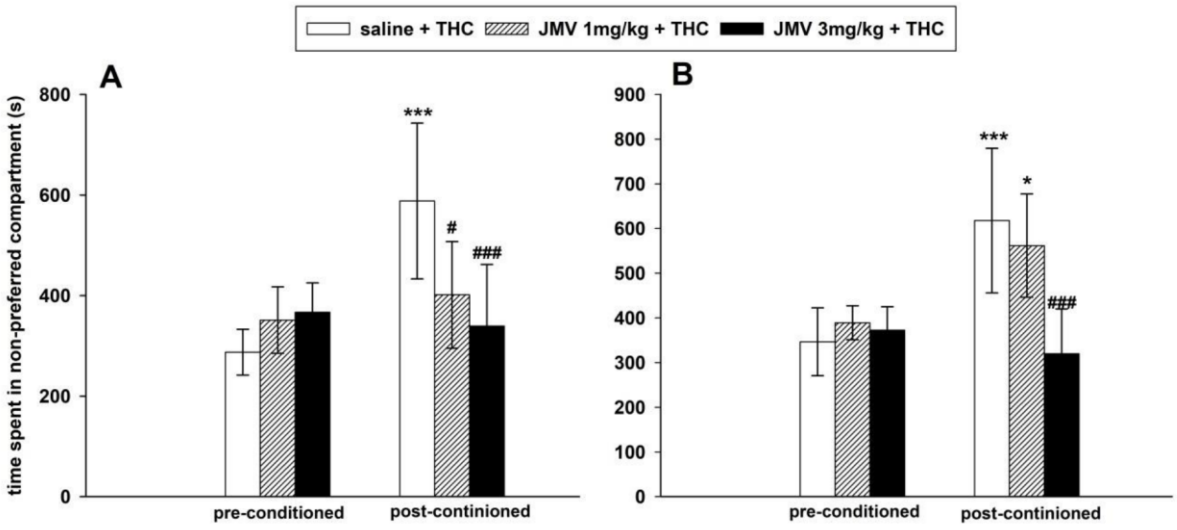

Figure 12. Effects of JMV2959 on THC-induced CPP in rats – absolute values.

*The graphs show mean time spent by the rats in the THC-paired (thus spontaneously non-preferred) compartment before (pre-conditioned/day 1) and after 8 days of conditioning with THC 0.3 mg/kg (post-conditioned/ day 10). In the graph A, JMV2959 (0, 1, 3 mg/kg) was administrated in a single dose 20 min before final testing after conditioning with THC (n = 8 – 11). In the graph B, JMV2959 was administered repeatedly together with THC during conditioning (n = 9 – 10). The results are presented as follows: saline + THC (open bar), JMV2959 1 mg/kg + THC (striped bar), JMV2959 3 mg/kg + THC (filled bar). The effect of conditioning with THC, thus the difference between pre- and post-conditioned measurements are expressed as * $p < 0.05$, *** $p < 0.001$. The effects of JMV2959 pretreatments in comparison to the saline group are expressed as # $p < 0.05$, ### $p < 0.001$. The results are presented with 95% confidence intervals as group means.*

8. DISCUSSION

The outcome from our previous research indicated a significant involvement of the central ghrelin signalling in the cannabinoid-induced dopamine as well as the endocannabinoid and GABA changes in the NACSh in rats. The currently presented results demonstrated that GHS-R1A antagonist JMV2959 significantly reduced the cannabinoid/WIN55,212-2 intravenous self-administration (IVSA) and reduced the cannabinoid/WIN55,212-2-seeking/relapse-like behavior. Furthermore, JMV2959 significantly reduced development, as well as expression of the cannabinoid/THC-induced conditioned place preference (CPP). Thus, our research confirmed, that the GHS-R1A antagonism can reduce the cannabinoid reinforcement effects in several addiction models.

The CPP model is focused on the association and conditioning of environmental cues and in this research, on THC effect. This plays an important role in the acquisition and maintenance of addiction (Bardo and Bevins 2000). It is already identified that cannabinoids are known for their general biphasic/dual effects (Lupica, Riegel et al. 2004). THC doses as low as 0.1 to 0.3 mg/kg are linked with rewarding and stimulatory effects, while doses higher than 1 mg/kg show hypoactivity and aversion (Le Foll, Wiggins et al. 2006).

Single administration of 1 and 3 mg/kg JMV2959 dose-dependently and significantly reduced the THC-CPP expression. Though, the higher dose/3 mg/kg induced a highly significant effect ($p < 0.001$). Evidently, JMV2959 significantly reduced the manifestation of the developed place conditioning with THC interactions which suggests that GHS-R1A antagonism attenuated the anticipation of the previously retained reward which is an attribute of craving. The rewarding/reinforcing effects of cannabinoids are probably mediated through mesolimbic CB1 receptors via dopamine release trigger within the nucleus accumbens, similarly to other drugs of abuse (Tanda, Pontieri et al. 1997, Volkow, Hampson et al. 2017, Manzanares, Cabanero et al. 2018, Zehra, Burns et al. 2018, Charalambous, Lapka et al. 2020). This is supported on our previous research where JMV2959 reduced the WIN55,212-2-induced accumbens dopamine release (Charalambous, Havlickova et al. 2021).

Studies in rodents reported that ghrelin antagonism reduced expression of CPP induced by alcohol (Jerlhag, Egecioglu et al. 2009), stimulants (Jerlhag, Egecioglu et al. 2010, Jerlhag and Engel 2011, Havlickova, Charalambous et al. 2018), and opioids (Engel, Nylander et al. 2015, Jerabek, Havlickova et al. 2017, Sustkova-Fiserova, Puskina et al. 2019). Other studies reported that JMV2959 did not alone induce the CPP (Jerlhag, Egecioglu et al. 2009) or conditioned taste aversion (Rodriguez, Fehrentz et al. 2018). Furthermore, in our previous study, JMV2959 alone (1, 3, and 6 mg/kg) did not significantly influence the rat locomotion in

the activity cage, when monitored within 25 to 45 min after JMV2959 administration (Jerabek, Havlickova et al. 2017). Importantly, our further recent study documented, that administration of JMV2959 alone did not significantly affect the memory functions in the Morris water maze as well as did not influence the biological molecular markers of memory and addiction processes in selected brain structures, although we used JMV2959 doses which effectively reduced the reinforcement effects of cannabinoids, stimulants and opioids in our previous experiments (Lapka, Charalambous et al. 2023).

The IVSA model is focused on the drug rewarding/reinforcing abilities and evaluate the principal treatment goal of reducing or even eliminating the drug-taking behavior. Even though there is clear evidence of the addictive potential of cannabis use in humans (Volkow, Hampson et al. 2017, Zehra, Burns et al. 2019), the utilization of the IVSA model in cannabinoids is still unclear. IVSA of the aminoalkylindole cannabinoid WIN55,212-2 was performed in rats and mice (Fattore, Cossu et al. 2001, Amchova, Kucerova et al. 2014, Lefever, Marusich et al. 2014). The substantial role of the CB1R in the cannabinoid reinforcing effects was supported in a self-administration using the CB1 receptor antagonist rimonabant (Fattore, Cossu et al. 2001, Lefever, Marusich et al. 2014). THC is considered as a partial CB1R and CB2R agonist (Spiller, Bi et al. 2019, Zehra, Burns et al. 2019), while WIN55.212-2 acts as a full CB1R/CB2R agonist (D'Ambra, Estep et al. 1992). In extent, a WIN55,212-2 IVSA rat model is fully suitable for testing the general cannabinoid/CB1R agonist reinforcing effects and the involved mechanisms and, in this research, the possible GHS-R1A involvement. The potentially suggested mechanisms might help reducing the CB1R-agonist drug-taking behavior.

Taking into consideration the knowledge, literature, and the biphasic characteristic/effects of cannabinoids, a WIN55,212-2 dose of 12.5 µg/kg/infusion was chosen, which according to the literature had the most reinforcing effects. During the maintenance period, both WIN55,212-2 IVSA studies were in accordance with the literature (Fattore, Cossu et al. 2001, Amchova, Kucerova et al. 2014, Lefever, Marusich et al. 2014). The inactive lever-pressing was significantly lower than the active lever-pressing. Moreover, the vehicle IVSA was significantly lower than the WIN55,212-2 IVSA in both studies, which visibly confirmed the reinforcing effects of the cannabinoid (Lefever, Marusich et al. 2014, Volkow, Hampson et al. 2017, Zehra, Burns et al. 2019). The pretreatment with the GHS-R1A antagonist significantly reduced the basal WIN55,212-2 IVSA maintenance in both studies and in all monitored parameters (number of active lever-pressing, number of infusions, daily consumptions in mg/kg; the inactive lever-pressing was mainly not significantly influenced. The pretreatment with the 3 mg/kg i.p. JMV2959 reduced the basal WIN55,212-2 IVSA in the

main study to average 16.4% ± 4.2 (infusions) and 16.3% ± 4.4 (active lever-pressing), and in the additional study to average 23.9% ± 5.0 (infusions) and 17.4% ± 2.8 (active lever-pressing). The cannabinoid self-administration was eliminated in three sessions (in two different rats), and in nine sessions the rats seek only one infusion. This suggests that the GHS-R1A antagonist significantly reduced the WIN55,212-2/cannabinoid-induced reinforcing/rewarding effects. Furthermore, JMV2959 pretreatment also significantly reduced the WIN55,212-2-seeking/relapse-like behavior tested in the IVSA cage on the twelfth day of forced abstinence, when the non-reinforced active lever-pressing decreased to 20.6% ± 4.5 of the baseline mean. Within the saline group, the non-reinforced active lever-pressing during the relapse-test session achieved a 189.6% ± 52.6 of the baseline, which indicates the incubation of the cannabinoid craving, similarly to other another study (Kirschmann, Pollock et al. 2017). In the IVSA experimental schedule, the same animals were pretreated with JMV2959/ghrelin during the maintenance IVSA period and during the relapse-test session. Thus, it should be noted that the previous pretreatment history might have influenced the rat behavior during the drug-seeking session. These WIN55,212-2 IVSA results are in accordance with other self-administration studies dealing with GHS-R1A-antagonism in the alcohol, sucrose (Landgren, Simms et al. 2011, Landgren, Simms et al. 2012, Suchankova, Steensland et al. 2013), fentanyl (Sustkova-Fiserova, Puskina et al. 2020), and methamphetamine (Havlickova, Charalambous et al. 2018) rodent models.

It is important to mention that JMV2959 did not influence the vehicle IVSA. According to literature, these results are consistent with other studies when the JMV2959/GHS-R1A antagonism significantly reduced reinforcing effects, such as ghrelin/hexarelin-provoked food intake, increased weight gain and fat mass, the sucrose self-administration, and consumption of rewarding food (Landgren, Simms et al. 2011, Moulin, Brunel et al. 2013). However, when JMV2959 was administered alone, it did not significantly influence the standard food consumption and body mass in rodents (Landgren, Simms et al. 2011, Moulin, Brunel et al. 2013), the locomotor activity, memory functions or the accumbens dopamine in rats/mice (Jerlhag, Egecioglu et al. 2009, Sustkova-Fiserova, Jerabek et al. 2014, Engel, Nylander et al. 2015, Jerabek, Havlickova et al. 2017, Lapka, Charalambous et al. 2023). In this IVSA study, the JMV2959 treatments did not affect the rat body mass.

Evidently, the ghrelin (40 µg/kg i.p.) administration significantly increased the number of infusions and active lever-pressing to 199.8% ± 17.8 and 343.2% ± 77.5 of the baseline mean, respectively. The observed noticeable inter-individual differences in the rats' active lever-pressing after the ghrelin pretreatment (from minimum 151% to maximum 716% of

baseline mean) indicate the heterogenous sensitivity of the rats to the ghrelin-increasing effect on motivation to the cannabinoid self-administration. Furthermore, pretreatment with ghrelin during the relapse-test session augmented the non-reinforced cannabinoid-seeking active lever-pressing to 330.9% ± 88.2 of the baseline mean and the active lever-pressing tend to be higher in comparison to the saline group. The craving incubation during the period of abstinence increased the active lever-pressing within the saline group. The values within the ghrelin group were rather spread (110–642% of the baseline mean) similarly to the saline group (77–565% of the baseline mean) and the comparison between the saline and ghrelin groups did not reach statistical significance in the relapse-test session. All the results propose that ghrelin supported/enhanced the cannabinoid attraction and motivation of rats to seek the active lever-pressing. This is in accordance with the literature, when intracerebral administration of ghrelin increased alcohol intake (Jerlhag, Egecioglu et al. 2009) and heroin IVSA (Maric, Sedki et al. 2012) and peripheral administration of ghrelin increased cocaine-induced potentiation of alcohol consumption (Cepko, Selva et al. 2014) in rats. In the additional IVSA study, ghrelin co-administration together with JMV2959 eliminated the significant JMV2959-induced attenuation of WIN55,212-2 IVSA in the active lever-pressing parameter (from $p < 0.01$ to n.s. in comparison to baseline) and the number of infusions (from $p < 0.001$ to n.s. relatively to baseline) suggesting the involvement of the GHS-R1A mechanisms.

For completeness of information on cannabinoid research it may also be mentioned, that in our previously published experiments the JMV2959 pretreatment also reduced the cannabinoids (THC, WIN55,212-2) induced behavioral stimulation observed in the Laboras cage. Cannabinoids in low doses, as most drugs of abuse tend to induce behavioral stimulation, which is considered to be a sign of the nigrostriatal dopaminergic pathway activation, which may become sensitized and contribute to drug addiction (Wise 1988, Katsidoni, Kastellakis et al. 2013).

Overall, the above discussed IVSA results demonstrated the important involvement of ghrelin/GHS-R1A in the rewarding/reinforcing effects of WIN55,212-2, which complements the behavioral studies with THC-CPP; thus, there is strong indication that the central ghrelin system crucially participates in the rewarding/reinforcing pro-addictive effects of cannabinoids similarly to alcohol, stimulants, and opioids (Engel and Jerlhag 2014, Panagopoulos and Ralevski 2014, Zallar, Farokhnia et al. 2017, Sustkova-Fiserova, Charalambous et al. 2022). For a more specific investigation of the GHS-R1A antagonist/acyl-ghrelin pretreatment effects on the WIN55,212-2 IVSA, the employment of a randomized schedule or prolonged free/non-pretreated session intervals between pretreatments might be more appropriate. Certainly,

further research of potential employment of the GHS-R1A antagonism to reduce signs of cannabinoid addiction behavior should carefully consider the usual mode of cannabinoid administration (inhalation), the distinct differences among the cannabinoid types, the particularities of cannabis, and other factors. Our presented results document that GHS-R1A plays a significant role in the THC/WIN55,212-2/cannabinoid rewarding/reinforcing effects, which encourages further research of the GHS-R1A antagonism as a potential novel approach to cannabinoid addiction treatment.

IV. CONCLUSION

Our presented experimental research on natural (THC) and synthetic (WIN55,212-2) cannabinoids with the GHS-R1A antagonist JMV2959 in rats documented the important role of GHS-R1A in several mechanisms of cannabinoid dependence and significantly contributed to understanding the role of ghrelin / GHS-R1A in the mechanisms of this dependence. We further corroborated previously observed significant interaction between ghrelin / GHS-R1A and (endo)cannabinoid systems using (i) the intravenous self-administration (IVSA) paradigm to provide valuable information about the addictive potential of cannabinoids and the GHS-R1A involvement in neural mechanisms of cannabinoid reward and motivation/ seeking and (ii) the conditioned place preference (CPP) paradigm to study the GHS-R1A involvement in the rewarding and conditioning effects of cannabinoids Our proposed hypotheses were confirmed: (ad 1) the systemic pretreatment with the JMV2959 reduced the WIN55,212-2 intravenous self-administration and the tendency to relapse/ drug-seeking behavior, while (ad 2) systemic pretreatment with acyl-ghrelin enhanced the WIN55,212-2 induced IVSA and seeking behaviors, (ad 3) co-administration of JMV2959 together with acyl-ghrelin reduces the ghrelin antagonism effects on the WIN55,212-2 induced IVSA, which confirmed involvement of the GHS-R1A in the observed effects. Also, (ad 4) our cannabinoid WIN55,212-2 intravenous self-administration model confirmed the cannabinoid reinforcement effects in comparison to the saline self-administering group of rats. Further, (ad 5) the JMV2959 pretreatments during the IVSA experiment did not significantly influence the body weight and (ad 6) the GHS-R1A antagonist JMV2959 reduced the tetrahydrocannabinol (THC)-induced conditioned place preference expression as well as development.

These 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.

LIST OF FIGURES

LIST OF TABLES

LIST OF PUBLISHED ARTICLES

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

1. Charalambous, Ch.; Havlíčková, T.; Lapka, M.; Puskina, N.; Slamberova R.; Kuchar M.; Sustkova-Fiserova, M.: Cannabinoid-Induced Conditioned Place Preference, Intravenous Self-Administration, and Behavioral Stimulation Influenced by Ghrelin Receptor Antagonism in Rats. International Journal of Molecular Sciences, 2021, 22 (5): Article 2397. IF: 5,923/2021

IF publications of the Department of Pharmacoloft/3.LF, which support the Ghrelin antagonism hypothesis.

1. Lapka, M., Charalambous, C., Khryakova, A., Certilina, A., Novotny, J., Hejnova, L., Sustkova-Fiserova, M. Ghrelin/GHS-R1A antagonism in memory test and its effects on central molecular signaling involved in addiction in rats. Pharmacology Biochemistry and Behavior. 2023, 224. 173528. IF: 3.697/2023; Q2/2021
2. Sustkova-Fiserova, M., Charalambous, C., Khryakova, A., Certilina, A., Lapka, M., Šlamberová, R. The Role of Ghrelin/GHS-R1A Signaling in Nonalcohol Drug Addictions. Int J Mol Sci. 2022, 23(2), 761. IF: 5,924/2022
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LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
CB1R	cannabinoid receptor type 1
CBD	cannabidiol
CPP	Conditioned place preference
CUD	cannabis use disorder
GABA	γ-aminobutyric acid
GHS-R1A	growth hormone secretagogue receptor type A1
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
i.p.	intraperitoneal
ICD	International Classification of Diseases
IVSA	intravenous self-administration
LABORAS	automated animal behavior recognition system
mPFC	medial prefrontal cortex
NACSh	nucleus accumbens shell
NAc	nucleus accumbens
THC	tetrahydrocannabinol
VTA	ventral tegmental area
WHO	World Health Organization

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