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Dissertation research summary

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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

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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 behavioural 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 behaviour 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 behaviour. 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 is a chronic mental and physical condition that is characterized by the loss of control of the individual over a certain type of behaviour. It is a relapsing disease/disorder with complex negative effects on the individual and in extend, on society. Substance dependence involves the urge to use the substance/drug repeatedly (constantly or intermittently) in order to achieve the expected psychological effect(s) (excessive satisfaction/well-being/reward) or to prevent the occurrence of unpleasant conditions that arise in the absence of the substance/drug in the body (withdrawal symptoms); the substance/drug use occur despite clear evidence of harmful consequences (NIDA 2018).

Dopamine is a key component in drug reward (Di Chiara and Imperato 1988, Koob and Bloom 1988). The acute intake of all substances that are known to cause addiction increases the extracellular dopamine concentration in the nucleus accumbens (NAc) (Weiss, Paulus et al. 1992). All addictive drugs significantly activate dopaminergic transmission in the nucleus accumbens shell (NACSh), which is considered an important initial impulse of the addiction processes, linked with reward, reinforcement, and disruption of salience attribution (Nestler 2005, Hyman, Malenka et al. 2006, Koob and Volkow 2010). The addictive drug-induced dopamine efflux in the NAc triggers consequent conditioning processes in the brain which form associations of drug reward with particular conditions/cues and reinforce the drug-seeking behaviour (Adinoff 2004).

Cannabinoids are the most widely used illegal drugs in Europe. Abused cannabinoids beside the natural constituents of Cannabis sativa/cannabis also include several synthetic cannabinoids used in several ways, such as "spice" in herbal mixtures, infused papers, or even as adulterating cannabis with synthetic cannabinoids. From 2002 to 2019, more than 180 synthetic cannabinoids of various chemical structures, including aminoalkylindoles, were detected on the drug market by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (EMCDDA 2020). Social, medical, and legal acceptance of cannabis has grown dramatically during the past 15 years in Europe and North America. The medical and recreational use of cannabis is also increased, but the public proportion that perceives important harms from cannabis/cannabinoids use is decreased (Hasin 2018, EMCDDA 2020). In Europe, including the Czech Republic, a prevailing supply of high-potent tetrahydrocannabinol (THC) strains of cannabis has existed over the last few years, linked with increased risks of cannabis use disorder, which includes uncontrolled drug-seeking and withdrawal symptoms, psychotic disorders, dysphoria, sleep and eating disorders, etc. (Zehra, Burns et al. 2018, EMCDDA 2020). It was estimated that about 9% of chronic cannabis users display characteristic signs and symptoms 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 have also been associated with many new synthetic cannabinoids, including aminoalkylindole derivatives, which have been during last 15 – 20 years broadly abused in Europe and elsewhere (EMCDDA 2020).

This dissertation research thesis summarises the important findings of our rigorous research of the ghrelin involvement in the cannabinoids (THC and WIN55,212-2) pro-addictive effects. 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 (average IF=4,759/2021). Nevertheless, this dissertation is focused only on the cannabinoid experimental results. The theoretical part of the thesis documents a reflection of the problematic around the use of addictive substances with focus on the cannabinoids and suggests how it affects the human brain in a physiological and molecular point of view. The experimental part of the thesis summarizes the most important experimental findings of the ghrelin/GHS-R1A and cannabinoid addiction relationships, which were obtained during a rigorous investigation. The obtained and published results encourage further research of the GHS-R1A antagonism as a potential novel approach to cannabinoid addiction treatment with the promise of the ability to decrease the cannabinoid craving and in extent, relapse. Currently, no specific pharmacotherapies have been approved for cannabinoid/cannabis use disorder and dependence; thus, cannabinoid addiction treatment remains exclusively symptomatic, unsatisfactory, and with a low relapse prevention with a great need to find new effective therapeutical approaches (Kondo, Morasco et al. 2020).

II. 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 ventral tegmental area (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 overall findings of 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 cannabinoid addiction. (Charalambous et al 2021)

For this recent research thesis, the intravenous self-administration (IVSA) 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 (CPP) 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 behavioural 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.

III. MATERIALS AND METHODS

Animals used in the experimental research

Male adult Wistar rats initially aged 8 weeks were used in all the experiments. At least seven days before the beginning of the experiments, the rats were given free access to food and water, and they were housed in polycarbonate cages with a constant humidity (50–60%) and room temperature (22–24 °C). Throughout the IVSA conditioning and tests, the rats 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.). The rats were accommodated individually (IVSA), or 3 per cage (CPP). (Charalambous, Lapka et al. 2021) (Sustkova-Fiserova, Charalambous et al. 2017).

Drugs and Chemicals used in the experiments research

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). 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.

WIN55,212-2 Intravenous Self-Administration

Forty-four naive male rats were used in this study; groups of 10 (JMV2959), 9 (saline group), and 8 (ghrelin group) were used in the statistical analyses in the main WIN55,212-2 IVSA study; four rats self-administered vehicle and a further four rats WIN55,212-2 in the additional IVSA experiment. The self-administration sessions started on the sixth day after the catheter implantation surgery. Changes in general behaviour, catheter patency, the body weight, and food intake of each animal were recorded daily. 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. After a stabile drug consumption for at least seven sessions (above 70% preference of the active lever, minimum 14 infusions during a session), 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, an 11 day abstinence period started. On the 12 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 behaviour. 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 1A.

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, 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.

During 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 1B.

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

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 behaviour day, and during all evaluated periods (7 baselines + 3 pretreatment days + relapse-like behaviour day = 11 days) (Charalambous, Havlickova et al. 2021).

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 experiment consisted of pre-conditioning (day 1), conditioning (days 2–9), and post-conditioning (day 10). On day 1 (pre-conditioning), each rat was injected i.p. with saline 20 min prior testing, then placed in the central compartment with both gates open, and initial/spontaneous place preference was determined during the 20 min. Conditioning was performed using a repetitive procedure in which THC (0.3 mg/kg i.p.) was paired to the spontaneously least preferred compartment. 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). CPP was calculated as the difference in the percentage of the total time spent in the THC-paired (i.e., least spontaneously preferred) compartment during the post-conditioning and pre-conditioning sessions (see Figure 2). It was previously described that the application 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 2. Timeline schedules of the CPP experiments.

IV. RESULTS

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

The active lever-pressing for WIN55,212-2 (demonstrated in Figure 3A,3B) was significantly attenuated by the GHS-R1A antagonist (JMV2959 3 mg/kg) when was administered 20 min before the three consequent 120-min sessions in comparison to the saline group as well as to the baseline mean. Pretreatment with JMV2959 significantly reduced the

basal pressing and the saline pretreatment was not significantly changed in comparison to the baseline mean. Pretreatment with ghrelin 40 μg/kg i.p. (20 min before) increased the active lever-pressing in all rats in comparison to the baseline mean; however, extreme inter-individual differences were observed in response to ghrelin among the rats. The ghrelin-induced increase of active lever-pressing was significant in comparison to the baseline mean and to the saline group during the first and second pretreatment session, and less during the third pretreatment session. The significant ghrelin-induced increase of active lever-pressing in comparison to the baseline mean and to the saline group is apparent also in Figure 3B, which illustrates comparisons of baseline and pretreatment means. The number of infusions and the daily 120-min WIN55,212-2 intake/doses in mg/kg are illustrated in Figure 3C and the comparison of the average basal (5–7. baseline) and mean pretreatment (1–3. pretreatment) results is presented in Figure 3D.

The average basal number of infusions and WIN55,212-2 intake (mean of five to seven baselines) was increased within the saline group and JMV2959 group. Pretreatment with JMV2959 significantly reduced the number of infusions/consumptions of WIN55,212-2, 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). Pretreatment with JMV2959 always significantly reduced the number of infusions and the spontaneous WIN55,212-2 consumption also in comparison to the saline group. Pretreatment with ghrelin almost doubled the number of infusions and relevant WIN55,212-2 intake from basal values, which represented a significant increase in comparison to the baseline as well as to the saline group. 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 and to saline group when the baseline and pretreatment means were compared (see Figure 3D).

The inactive lever-pressing, illustrated in Figure 3E,3F, showed low basal activity (mean of five to seven baselines) in the JMV2959, saline, and ghrelin group, and pretreatments did not produce any significant changes in all the analyses.

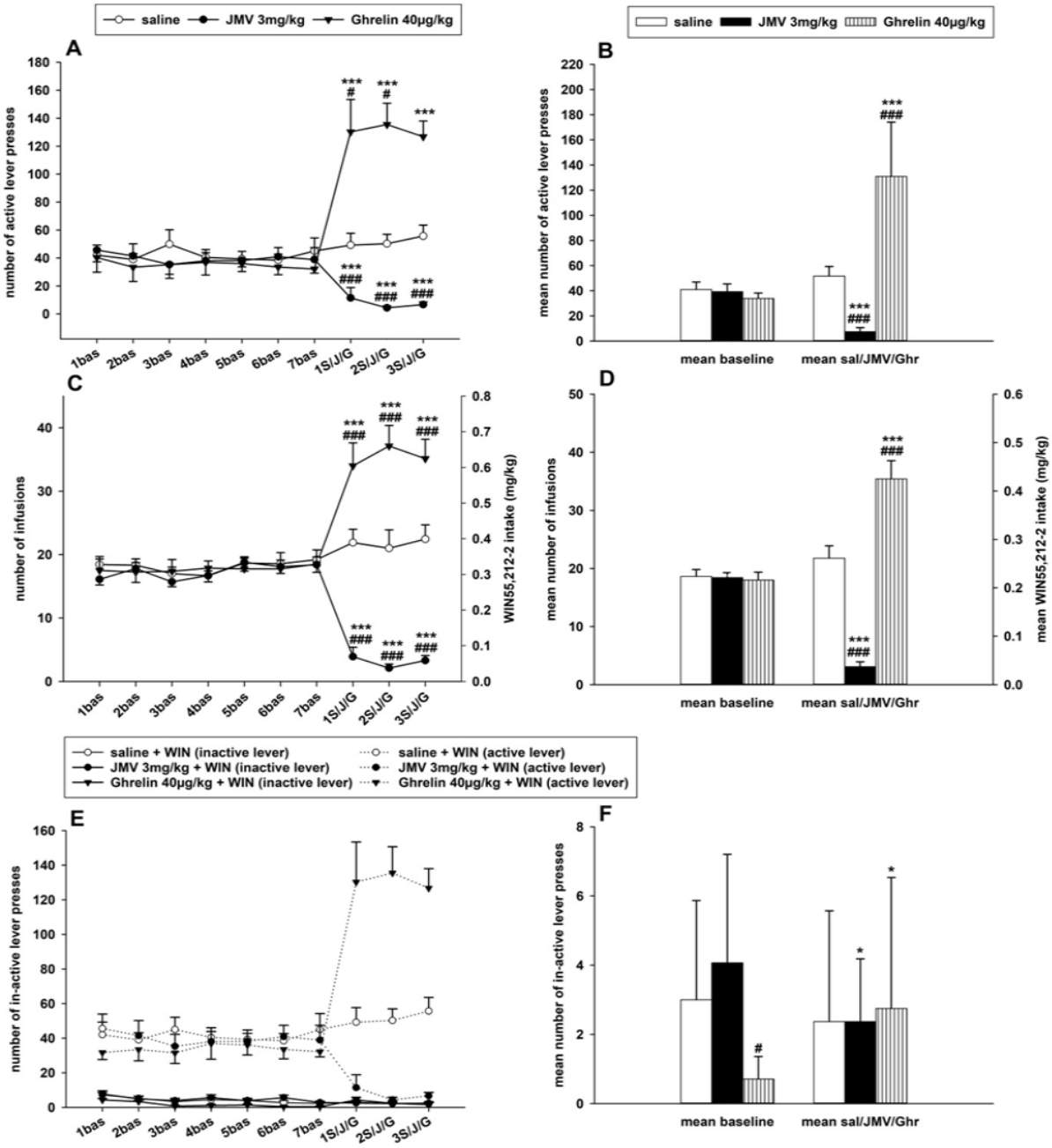

Figure 3. Effects of JMV2959 and ghrelin on WIN55,212-2 Intravenous Self-Administration.
*Saline (1 ml/kg) or JMV2959 (3 mg/kg) or ghrelin (40 µg/kg) were administered intraperitoneally 20 min before the 120-min IVSA sessions. Illustrated in graphs A,C,E are the daily active lever-pressings (A), number of infusions (C), and numbers of inactive lever-pressings (E) during the last week before pretreatments (1.–7. bas) and during three days of pretreatments (1.–3. S/J/G). Only the last three baselines (5.–7. bas) were used for statistical analysis by two-way repeated measures ANOVA followed by the Bonferroni test. The 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. In graphs B, D, F, 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 WIN55,212-2-seeking behaviour was significantly decreased by the JMV2959 pretreatment in comparison to the saline-pretreated group. After the ghrelin pretreatment, the relapse-like behaviour 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 4), a decrease within the JMV2959 group, an increase within the saline group, and a distinct increase in the ghrelin-pretreated group were observed. The inactive lever-pressing was not expressed in a percentage because of zero occurring within the basal pressing.

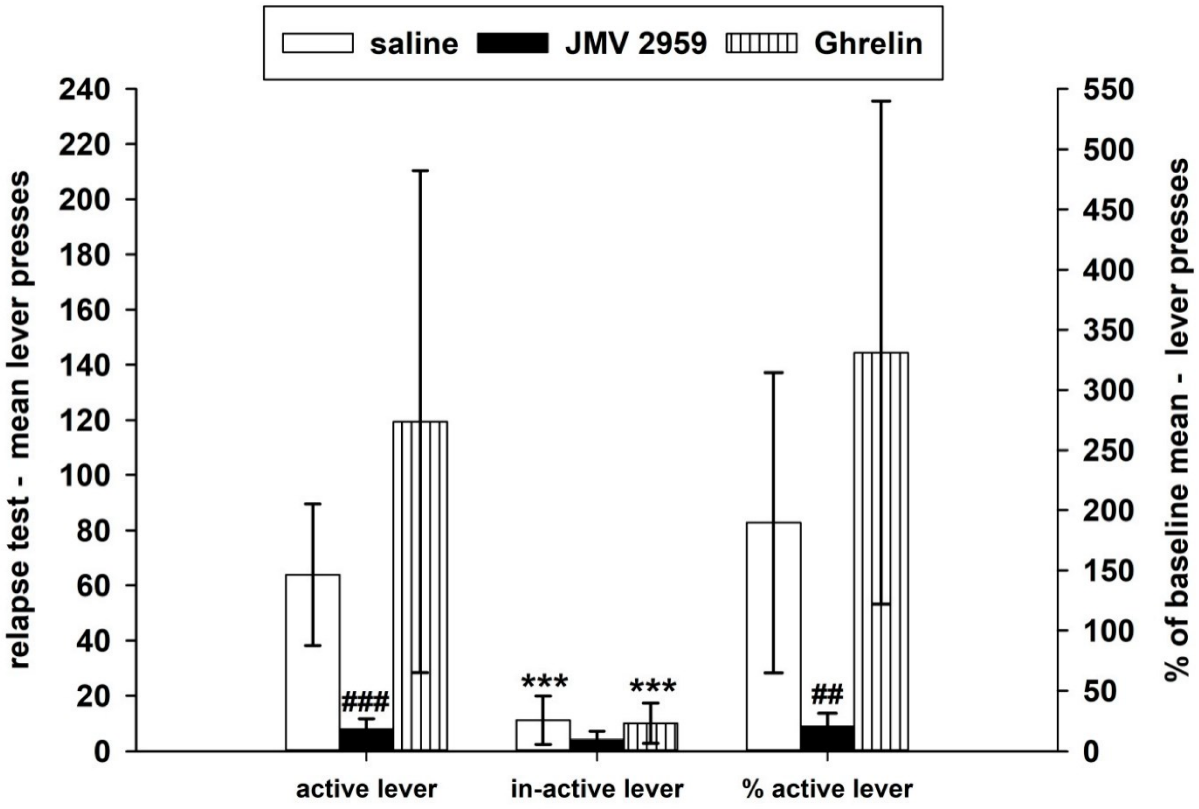

Figure 4 Effects of JMV2959 and Ghrelin on WIN55,212-2-seeking lever-pressing/relapse-like behaviour.

*Observed on the 12 day of forced abstinence of the WIN55,212-2 intravenous self-administration (IVSA) in active/inactive lever-pressing and percentage of the 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 session, when the rats were in the IVSA cages not connected with the infusion pump. The IVSA relapse-test 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. However, the percentage data were analysed directly/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.*

The apparent individual differences in reactivity of the rats to the appropriate pretreatments during the IVSA experiments including the relapse-test session are illustrated in Figure 5. During the 3 days of saline pretreatment, the daily active lever-pressing ranged from 76 % to 194 % of baseline mean (the Figure 5A). 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 behavioural 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 5B). 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 behaviour (on the 12th day of forced abstinence period) are illustrated in Figure 5C. The JMV2959 pretreatment reduced the non-rewarded cannabinoid-seeking/relapse-like active lever-pressing 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).

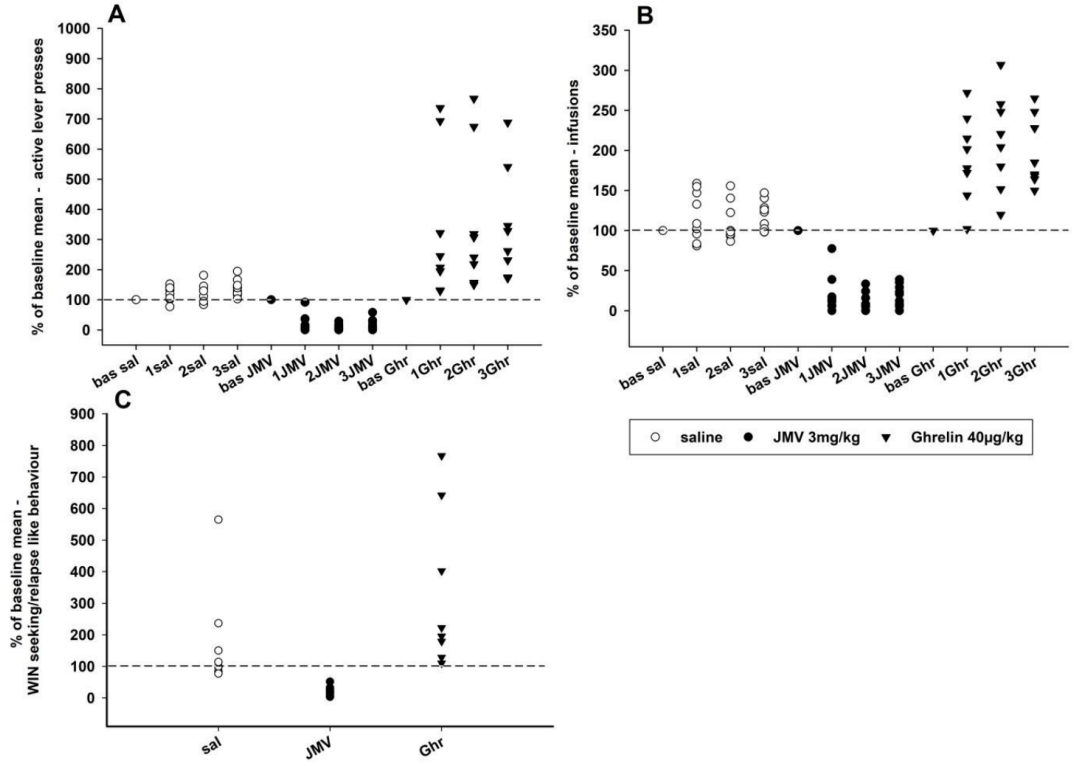

Figure 5 Effects of JMV2959 and Ghrelin on WIN55,212-2 Intravenous Self-Administration in single rats.

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%).

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

A separate group of rats was used in the additional IVSA for comparison of WIN55,212-2 IVSA with intravenous self-administration of the vehicle and the appropriate pretreatments, which is illustrated in Figure 6 in changes of active lever-pressing. Four rats self-administered the vehicle, another four the WIN55,212-2 again in a dose 12.5 µg/kg/infusion. Here the rats were chosen randomly with no demand for the minimum 14 daily infusions and other criterions; 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 three 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 (three baselines before pretreatments) revealed

significant differences between the WIN55,212-2 and vehicle number of infusions, as well as the number of active lever presses. Active versus inactive lever-pressing was significantly different within the WIN55,212-2 IVSA and also within the vehicle IVSA, but there were no significant differences within inactive lever-pressing either after pretreatments, or between the IVSA cannabinoid/vehicle groups. However, the pretreatments had no significant influence on the vehicle IVSA. Within the cannabinoid IVSA, a significant reduction of active lever-pressing was observed after JMV2959 pretreatment of baseline mean. This JMV2959 effect was attenuated by ghrelin co-administration during the third pretreatment session and ghrelin pretreatment increased the active lever-pressing. When the changes were expressed in the percentage of the baseline mean (see Figure 6B), a significant pretreatment effects within the WIN55,212-2 IVSA groups and no significant effects within the vehicle IVSA groups was observed. The JMV2959, co-administration JMV2959 + ghrelin, and ghrelin pretreatment percentage changes were significantly different between the WIN55,212-2 and vehicle IVSA.

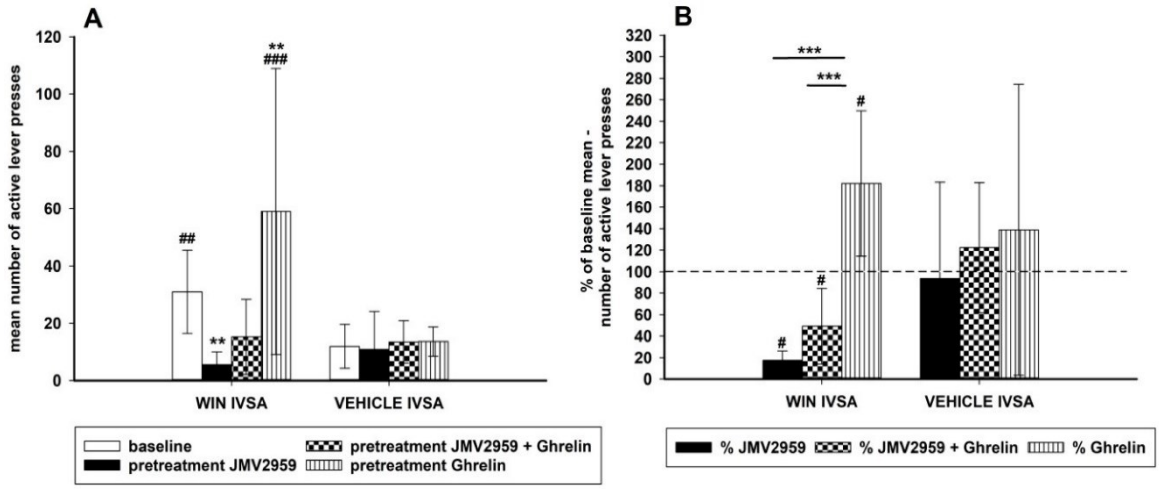

Figure 6. Effects of JMV2959 and Ghrelin Effects on Vehicle and WIN55,212-2 Intravenous Self-Administration – Active lever-pressing (additional IVSA experiment)

*The number of active lever-pressing for vehicle and for WIN55,212-2 are illustrated in graph (A). Baseline pressing (mean of three 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 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 illustrated 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).*

The pretreatments had no significant influence on the vehicle IVSA. Within the cannabinoid IVSA, we observed significant reduction of number of infusions after JMV2959 pretreatment of baseline mean. This JMV2959 effect was attenuated by ghrelin co-

administration during the third pretreatment session and ghrelin pretreatment increased the number of infusions. When the changes were expressed in percentage of baseline mean (see Figure 7B), significant pretreatment effects within the WIN55,212-2 IVSA group were observed and no significant effects within the vehicle IVSA were observed. 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.

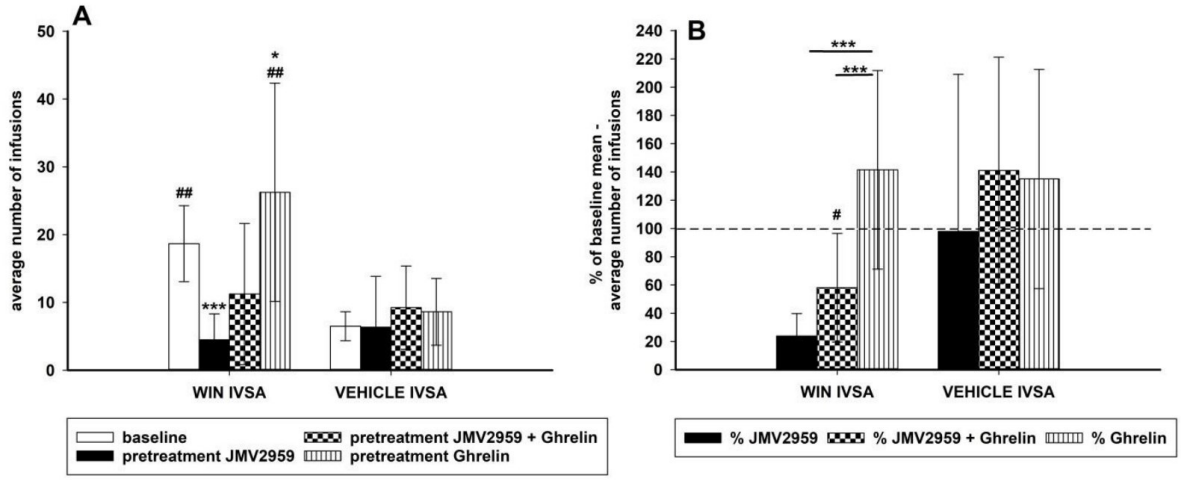

Figure 7. Effects of JMV2959 and Ghrelin Effects on Vehicle and WIN55,212-2 Intravenous Self-Administration – Number of Infusions (additional IVSA experiment)

*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 three 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, 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).*

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 min) 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 established THC-induced CPP manifestation was significantly and dose-dependently attenuated by 1 and 3 mg/kg JMV2959 when administered 20 min before testing on the post-conditioning day (see Figure 8A). When the higher dose 3 mg/kg JMV2959 was repeatedly administered together with THC during conditioning, the development of THC-CPP was significantly reduced, the effect of the lower dose (1 mg/kg) was not significant (see

Figure 8B). 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.

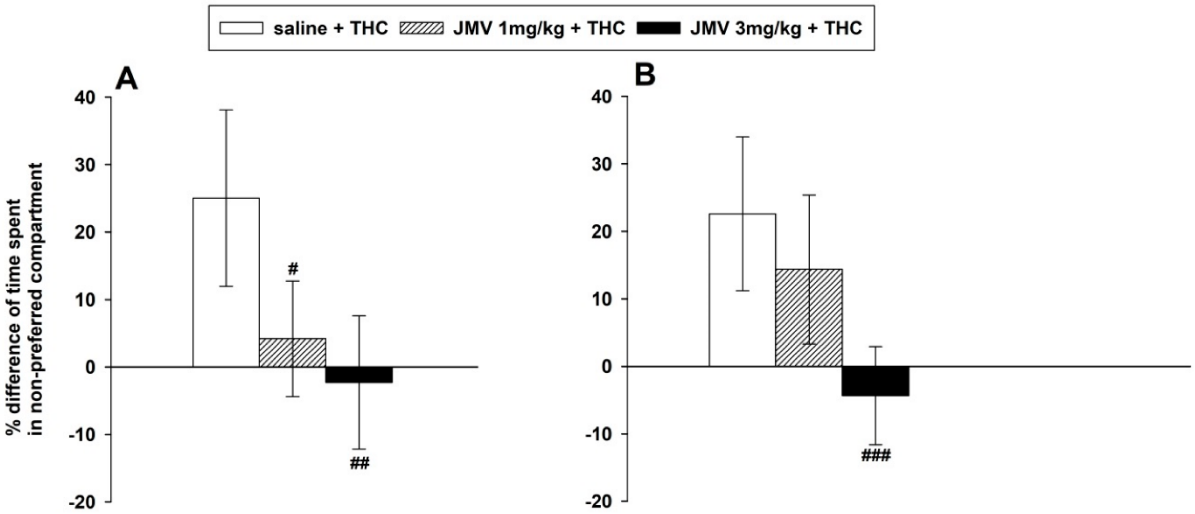

Figure 8. Effects of JMV2959 on tetrahydrocannabinol (THC)-induced conditioned place preference (CPP) in rats - percentage of total time spent in the THC-paired/least preferred compartment during the post-conditioning and/minus the pre-conditioning session.

In graph (A), 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 (B), 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). 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 min) 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 as group means with 95% confidence intervals.

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). In both CPP arrangements the THC-CPP was established. The acute JMV2959 administration after the THC conditioning significantly and dose dependently reduced the THC-CPP expression (see Figure 9A). 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 9B).

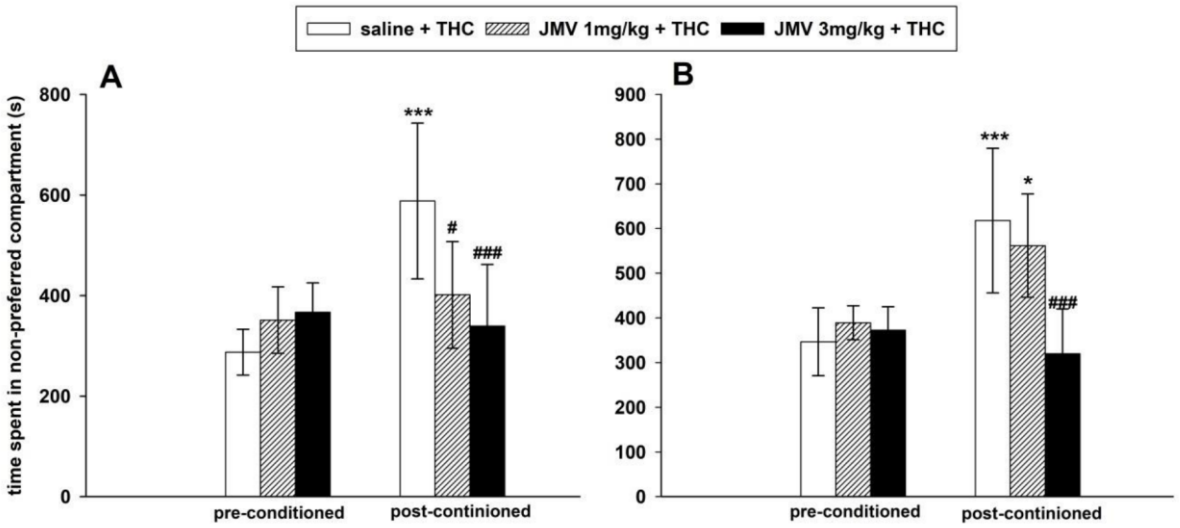

Figure 9. Effects of JMV2959 on tetrahydrocannabinol (THC)-induced conditioned place preference (CPP) in rats – absolute values.

*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 as group means with 95% confidence intervals.*

V. DISCUSSION

Within the IVSA experiment, 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 study arrangements 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 in both studies. Moreover, the vehicle IVSA was significantly lower than the WIN55,212-2 IVSA, which visibly confirmed the reinforcing effects of the cannabinoid WIN55,212-2 (Lefever, Marusich et al. 2014, Volkow, Hampson et al. 2017, Zehra, Burns et al. 2019). The pretreatment with the GHS-R1A antagonist JMV2959 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 both IVSA studies. In the main IVSA study (N=8 - 10) the cannabinoid self-administration was eliminated in three sessions (in two different rats), and in nine sessions the rats pressed 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 behaviour 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 mean of the baseline active lever-pressing. Within the saline group, the non-reinforced active lever-pressing during the relapse-test session indicated the incubation of the cannabinoid craving, in accordance with other studies (Kirschmann, Pollock et al. 2017). In the IVSA experimental schedule, the same animals were pretreated with JMV2959 or ghrelin or saline 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 behaviour 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 IVSA 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 reinforced 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 also did not affect the rat body mass.

Evidently, the administration of ghrelin (40 µg/kg i.p.) significantly increased the number of infusions and active lever-pressing of the baseline mean. The observed noticeable inter-individual differences in the rats' active lever-pressing after the ghrelin pretreatment indicate the heterogenous sensitivity of the rats to the ghrelin-increasing effect on motivation to the cannabinoid self-administration. In addition, ghrelin pretreatment during the relapse-test session augmented the non-reinforced cannabinoid-seeking active lever-pressing in comparison to the baseline mean and the active lever-pressing tend to be higher in comparison to the saline group. The craving incubation during the abstinence period increased the active lever-pressing within the saline group. The values within the ghrelin group were rather spread to the saline group and the comparison between the saline and ghrelin groups did not reach statistical significance in the relapse-test session. These results suggest that ghrelin supported/enhanced the cannabinoid attraction and motivation of rats to seek the cannabinoid hence increase 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 and the number of infusions suggesting/confirming the involvement of the GHS-R1A mechanisms.

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 behavioural 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 behaviour should carefully consider the usual mode of cannabinoid administration (inhalation), the distinct differences among the cannabinoid types, the particularities of cannabis, and other factors.

The 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. 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).

Altogether 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.

VI. 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 behaviour, while (ad 2) systemic pretreatment with acyl-ghrelin enhanced the WIN55,212-2 induced IVSA and seeking behaviours, (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 ABBREVIATIONS

CPP - conditioned place preference
EMCDDA - European Monitoring Centre for Drugs and Drug Addiction
GHSR1A - growth hormone secretagogue receptor
IVSA - intravenous self-administration
NAc - nucleus accumbens
NACSh - nucleus accumbens shell
THC - tetrahydrocannabinol
WHO/DSM-IV - Diagnostic and Statistical Manual of Mental Health of the World Health Organization

LIST OF PUBLICATIONS

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 Pharmacology/3.LF, which support the Ghrelin antagonism hypothesis.

1. Šustková-Fišerová, M.; Charalambous, C.; Havlíčková, T.; Lapka, M.; Jeřábek, P.; Puškina, N.; Syslová, K.: Alterations in Rat Accumbens Endocannabinoid and GABA Content during Fentanyl Treatment: The Role of Ghrelin. International Journal of Molecular

Sciences, 2017, 18(11): Article 2486. IF: 3,687/2017; Q2/2017; Aktuální IF časopisu: 4,183/2018; Q2/2018

2. Jeřábek, P.; Havlíčková, T.; Pushkina, N.; Charalambous, Ch.; Lapka, M.; Kačer, P.; Šustková-Fišerová, M. (K): Ghrelin receptor antagonism of morphine-induced conditioned place preference and behavioral and accumbens dopaminergic sensitization in rats. Neurochemistry International, 2017, 110(November): 101-113. IF: 3,603/2017; Q2/2017; Aktuální IF časopisu: 3,994/2018; Q2/2018

3. Sustkova-Fiserova, M.; Puskina, N.; Havlíčková, T.; Lapka, M.; Syslova, K.; Pohorala, V.; Charalambous, Ch.: Ghrelin receptor antagonism of fentanyl-induced conditioned place preference, intravenous self-administration and dopamine release in the nucleus accumbens in rats. Addiction Biology, 2019; IF: 4,223/2018; Q1/2018

4. Havlickova, T.; Charalambous, Ch.; Lapka, M.; Puskina, N.; Jerabek, P.; Sustkova-Fiserova, M.: Ghrelin Receptor Antagonism of methamphetamine-induced conditioned place preference and intravenous self-administration in rats. International Journal of Molecular Sciences, 2018, 19: Article 2925. IF: 4,183/2018; Q2/2018

5. Charalambous, Ch.; Lapka, M.; Havlíčková, T.; Syslova K.; Sustkova-Fiserova, M.: Alterations in Rat Accumbens Dopamine, Endocannabinoids and GABA Content During WIN55,212-2 Treatment: The Role of Ghrelin. International Journal of Molecular Sciences, 2021, 22 (1): Article 210. IF: 5,923/2021

6. 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

7. 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

VII. REFERENCES

1. Adinoff, B. (2004). "Neurobiologic processes in drug reward and addiction." Harv Rev Psychiatry **12**(6): 305-320.
2. Amchova, P., J. Kucerova, V. Giugliano, Z. Babinska, M. T. Zanda, M. Scherma, L. Dusek, P. Fadda, V. Micale, A. Sulcova, W. Fratta and L. Fattore (2014). "Enhanced self-administration of the CB1 receptor agonist WIN55,212-2 in olfactory bulbectomized rats: evaluation of possible serotonergic and dopaminergic underlying mechanisms." Front Pharmacol **5**: 44.
3. Cepko, L. C., J. A. Selva, E. B. Merfeld, A. I. Fimmel, S. A. Goldberg and P. J. Currie (2014). "Ghrelin alters the stimulatory effect of cocaine on ethanol intake following mesolimbic or systemic administration." Neuropharmacology **85**: 224-231.
4. Di Chiara, G. and A. Imperato (1988). "Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats." Proc Natl Acad Sci U S A **85**(14): 5274-5278.
5. EMCDDA (2020). "European Drug Report 2020: Trends and Developments." Publications Office of the European Union: 88.

6. Engel, J. A. and E. Jerlhag (2014). "Role of appetite-regulating peptides in the pathophysiology of addiction: implications for pharmacotherapy." CNS Drugs **28**(10): 875-886.
7. Engel, J. A., I. Nylander and E. Jerlhag (2015). "A ghrelin receptor (GHS-R1A) antagonist attenuates the rewarding properties of morphine and increases opioid peptide levels in reward areas in mice." Eur Neuropsychopharmacol **25**(12): 2364-2371.
8. Fattore, L., G. Cossu, C. M. Martellotta and W. Fratta (2001). "Intravenous self-administration of the cannabinoid CB1 receptor agonist WIN 55,212-2 in rats." Psychopharmacology (Berl) **156**(4): 410-416.
9. Hasin, D. S. (2018). "US Epidemiology of Cannabis Use and Associated Problems." Neuropsychopharmacology **43**(1): 195-212.
10. Havlickova, T., C. Charalambous, M. Lapka, N. Puskina, P. Jerabek and M. Sustkova-Fiserova (2018). "Ghrelin Receptor Antagonism of Methamphetamine-Induced Conditioned Place Preference and Intravenous Self-Administration in Rats." Int J Mol Sci **19**(10).
11. Hyman, S. E., R. C. Malenka and E. J. Nestler (2006). "Neural mechanisms of addiction: the role of reward-related learning and memory." Annu Rev Neurosci **29**: 565-598.
12. Charalambous, C., T. Havlickova, M. Lapka, N. Puskina, R. Slamberova, M. Kuchar and M. Sustkova-Fiserova (2021). "Cannabinoid-Induced Conditioned Place Preference, Intravenous Self-Administration, and Behavioral Stimulation Influenced by Ghrelin Receptor Antagonism in Rats." Int J Mol Sci **22**(5).
13. Charalambous, C., M. Lapka, T. Havlickova, K. Syslova and M. Sustkova-Fiserova (2020). "Alterations in Rat Accumbens Dopamine, Endocannabinoids and GABA Content During WIN55,212-2 Treatment: The Role of Ghrelin." Int J Mol Sci **22**(1).
14. Charalambous, C., M. Lapka, T. Havlickova, K. Syslova and M. Sustkova-Fiserova (2021). "Alterations in rat accumbens dopamine, endocannabinoids and GABA content during WIN55,212-2 treatment: the role of ghrelin." International Journal of molecular Sciences **22**: 210.
15. Jerabek, P., T. Havlickova, N. Puskina, C. Charalambous, M. Lapka, P. Kacer and M. Sustkova-Fiserova (2017). "Ghrelin receptor antagonism of morphine-induced conditioned place preference and behavioral and accumbens dopaminergic sensitization in rats." Neurochem Int **110**: 101-113.
16. Jerlhag, E., E. Egecioglu, S. L. Dickson and J. A. Engel (2010). "Ghrelin receptor antagonism attenuates cocaine- and amphetamine-induced locomotor stimulation, accumbal dopamine release, and conditioned place preference." Psychopharmacology (Berl) **211**(4): 415-422.
17. Jerlhag, E., E. Egecioglu, S. Landgren, N. Salome, M. Heilig, D. Moechars, R. Datta, D. Perrissoud, S. L. Dickson and J. A. Engel (2009). "Requirement of central ghrelin signaling for alcohol reward." Proc Natl Acad Sci U S A **106**(27): 11318-11323.
18. Katsidoni, V., A. Kastellakis and G. Panagis (2013). "Biphasic effects of Delta9-tetrahydrocannabinol on brain stimulation reward and motor activity." Int J Neuropsychopharmacol **16**(10): 2273-2284.

19. Kirschmann, E. K., M. W. Pollock, V. Nagarajan and M. M. Torregrossa (2017). "Effects of Adolescent Cannabinoid Self-Administration in Rats on Addiction-Related Behaviors and Working Memory." Neuropsychopharmacology **42**(5): 989-1000.
20. Kondo, K. K., B. J. Morasco, S. M. Nugent, C. K. Ayers, M. E. O'Neil, M. Freeman and D. Kansagara (2020). "Pharmacotherapy for the Treatment of Cannabis Use Disorder: A Systematic Review." Ann Intern Med **172**(6): 398-412.
21. Koob, G. F. and F. E. Bloom (1988). "Cellular and molecular mechanisms of drug dependence." Science **242**(4879): 715-723.
22. Koob, G. F. and N. D. Volkow (2010). "Neurocircuitry of addiction." Neuropsychopharmacology **35**(1): 217-238.
23. Landgren, S., J. A. Simms, P. Hyytia, J. A. Engel, S. E. Bartlett and E. Jerlhag (2012). "Ghrelin receptor (GHS-R1A) antagonism suppresses both operant alcohol self-administration and high alcohol consumption in rats." Addict Biol **17**(1): 86-94.
24. Landgren, S., J. A. Simms, D. S. Thelle, E. Strandhagen, S. E. Bartlett, J. A. Engel and E. Jerlhag (2011). "The ghrelin signalling system is involved in the consumption of sweets." PLoS One **6**(3): e18170.
25. Lapka, M., C. Charalambous, A. Khryakova, A. Certilina, J. Novotny, L. Hejnova and M. Sustkova-Fiserova (2023). "Ghrelin/GHS-R1A antagonism in memory test and its effects on central molecular signaling involved in addiction in rats." Pharmacol Biochem Behav **224**: 173528.
26. Lefever, T. W., J. A. Marusich, K. R. Antonazzo and J. L. Wiley (2014). "Evaluation of WIN 55,212-2 self-administration in rats as a potential cannabinoid abuse liability model." Pharmacol Biochem Behav **118**: 30-35.
27. Manzanares, J., D. Cabanero, N. Puente, M. S. Garcia-Gutierrez, P. Grandes and R. Maldonado (2018). "Role of the endocannabinoid system in drug addiction." Biochem Pharmacol **157**: 108-121.
28. Maric, T., F. Sedki, B. Ronfard, D. Chafetz and U. Shalev (2012). "A limited role for ghrelin in heroin self-administration and food deprivation-induced reinstatement of heroin seeking in rats." Addict Biol **17**(3): 613-622.
29. Moulin, A., L. Brunel, D. Boeglin, L. Demange, J. Ryan, C. M'Kadmi, S. Denoyelle, J. Martinez and J. A. Fehrentz (2013). "The 1,2,4-triazole as a scaffold for the design of ghrelin receptor ligands: development of JMV 2959, a potent antagonist." Amino Acids **44**(2): 301-314.
30. Nestler, E. J. (2005). "Is there a common molecular pathway for addiction?" Nature Neuroscience **8**(11): 1445-1449.
31. NIDA. (2018). "The Science of Drug Use and Addiction: The Basics." 2022, from https://archives.drugabuse.gov/publications/media-guide.
32. Panagopoulos, V. N. and E. Ralevski (2014). "The role of ghrelin in addiction: a review." Psychopharmacology (Berl) **231**(14): 2725-2740.
33. Sanchis-Segura, C. and R. Spanagel (2006). "Behavioural assessment of drug reinforcement and addictive features in rodents: an overview." Addict Biol **11**(1): 2-38.
34. Sanudo-Pena, M. C., J. Romero, G. E. Seale, J. J. Fernandez-Ruiz and J. M. Walker (2000). "Activational role of cannabinoids on movement." Eur J Pharmacol **391**(3): 269-274.

35. Suchankova, P., P. Steensland, I. Fredriksson, J. A. Engel and E. Jerlhag (2013). "Ghrelin receptor (GHS-R1A) antagonism suppresses both alcohol consumption and the alcohol deprivation effect in rats following long-term voluntary alcohol consumption." PLoS One **8**(8): e71284.
36. Sustkova-Fiserova, M., C. Charalambous, T. Havlickova, M. Lapka, P. Jerabek, N. Puskina and K. Syslova (2017). "Alterations in Rat Accumbens Endocannabinoid and GABA Content during Fentanyl Treatment: The Role of Ghrelin." Int J Mol Sci **18**(11).
37. Sustkova-Fiserova, M., C. Charalambous, A. Khryakova, A. Certilina, M. Lapka and R. Slamberova (2022). "The Role of Ghrelin/GHS-R1A Signaling in Nonalcohol Drug Addictions." Int J Mol Sci **23**(2).
38. Sustkova-Fiserova, M., P. Jerabek, T. Havlickova, P. Kacer and M. Krsiak (2014). "Ghrelin receptor antagonism of morphine-induced accumbens dopamine release and behavioral stimulation in rats." Psychopharmacology (Berl) **231**(14): 2899-2908.
39. Sustkova-Fiserova, M., N. Puskina, T. Havlickova, M. Lapka, K. Syslova, V. Pohorala and C. Charalambous (2020). "Ghrelin receptor antagonism of fentanyl-induced conditioned place preference, intravenous self-administration, and dopamine release in the nucleus accumbens in rats." Addict Biol **25**(6): e12845.
40. Tanda, G., F. E. Pontieri and G. Di Chiara (1997). "Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common mu1 opioid receptor mechanism." Science **276**(5321): 2048-2050.
41. Volkow, N. D., A. J. Hampson and R. D. Baler (2017). "Don't Worry, Be Happy: Endocannabinoids and Cannabis at the Intersection of Stress and Reward." Annu Rev Pharmacol Toxicol **57**: 285-308.
42. Weiss, F., M. P. Paulus, M. T. Lorang and G. F. Koob (1992). "Increases in extracellular dopamine in the nucleus accumbens by cocaine are inversely related to basal levels: effects of acute and repeated administration." J Neurosci **12**(11): 4372-4380.
43. Zallar, L. J., M. Farokhnia, B. J. Tunstall, L. F. Vendruscolo and L. Leggio (2017). "The Role of the Ghrelin System in Drug Addiction." Int Rev Neurobiol **136**: 89-119.
44. Zehra, A., J. Burns, C. K. Liu, P. Manza, C. E. Wiers, N. D. Volkow and G. J. Wang (2018). "Cannabis Addiction and the Brain: a Review." J Neuroimmune Pharmacol **13**(4): 438-452.
45. Zehra, A., J. Burns, C. K. Liu, P. Manza, C. E. Wiers, N. D. Volkow and G. J. Wang (2019). "Cannabis Addiction and the Brain: a Review." Focus (Am Psychiatr Publ) **17**(2): 169-182.