Charles University

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Dissertation Thesis Summary

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

Úloha ghrelinové signalizace v neurobiologických mechanismech odměňovacích účinků kanabinoidů a opioidů

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ABSTRACT

Background: Ghrelin, an orexigenic appetite stimulating peptide, in addition to promoting energy balance, contributes to the rewarding effects associated with overeating. It also seems to play an important role in the rewarding/reinforcing effects of alcohol and addictive stimulants. The involvement of the ghrelin mechanisms in cannabinoid and opioid misuse and addiction have been under-researched.
Aims: The principal aim of this research thesis was to investigate whether the pretreatment with the growth hormone secretagogue receptor 1A (GHS-R1A) antagonist (JMV2959) could reduce the cannabinoid receptor type 1 (CB1R) agonist WIN55,212-2–induced dopamine efflux in the nucleus accumbens shell (NACSh), which is considered a crucial trigger impulse of the addiction process. Also, test whether JMV2959 can influence the WIN55,212-2 and fentanyl-induced effects on the endocannabinoids N-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) and the gama-aminobutyric acid (GABA) content in the NACSh, and in extend, to specify the involvement of GHS-R1A located in the ventral tegmental area (VTA) and the NACSh in the observed accumbens changes. Furthermore, to test whether the JMV2959 pretreatment could reduce the cannabinoid [tetrahydrocannabinol (THC) and WIN55,212-2] induced behavioural stimulation.
Methods: In vivo microdialysis was used to determine the changes of dopamine and its metabolites in the NACSh in rats following the synthetic aminoalklylindol cannabinoid WIN55,212-2 administration into the posterior VTA with and without the ghrelin antagonist pretreatment (JMV2959, 3 mg/kg i.p. 20 min before WIN55,212-2 administration) and also to determine the WIN55,212-2 and fentanyl effects on anandamide, 2-AG and GABA accumbens content. The behavioural changes in rats were observed on the fully automated behaviour recognition system (LABORAS) apparatus which monitored the effects of JMV2959 on the THC and WIN55,212-2.
Findings: The WIN55,212-2 administration induced significant accumbens dopamine release, which was significantly reduced by the 3 mg/kg i.p. JMV2959 pretreatment. Simultaneously, the cannabinoid-increased accumbens dopamine metabolic turnover was significantly augmented by the JMV2959 pretreatment. The intracerebral WIN55,212-2 administration also increased the endocannabinoid anandamide and the 2-AG extracellular levels in the NACSh, which was moderately but significantly attenuated by the JMV2959 pretreatment. Moreover, the cannabinoid-induced decrease in accumbens GABA levels was reversed by the JMV2959 pretreatment. The pretreatment with JMV2959 (administered systemically, into the NACSh or VTA) reversed the dose dependent fentanyl-induced anandamide increase in the NACSh, resulting in a significant anandamide decrease and intensified the fentanyl-induced decrease in accumbens 2-AG levels. The behavioural study in the LABORAS apparatus showed that JMV2959 pretreatment significantly and dose-dependently reduced the systemic THC/WIN55,212-2-induced behavioural stimulation in rats.
Conclusions: The overall findings on this research documented the significant contribution of ghrelin / GHS-R1A in the cannabinoid's and opioid's pro-addictive effects and supported further research into ghrelin antagonism as a potential new therapeutic direction in these addictions.

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

ABSTRAKT

Úvod: Orexigenní peptid stimulujícího chuť k jídlu, vedle podpory energetické rovnováhy také navozuje odměňující účinky spojené s přejídáním. Mimo jiné se ukazuje, že ghrelin hraje důležitou roli v odměňujících/posilujících účincích alkoholu a návykových stimulantů. Nicméně zapojení ghrelinového mechanismu ve zneužívání a závislosti na opioidech a kanabinoidech není dosud dostatečně prozkoumáno.
Cíle: Hlavním cílem této výzkumné práce bylo zjistit, zda premedikace antagonistou GHS-R1A (JMV2959) může snížit vyplavení dopaminu z nucleus accumbens shell (NACSh), indukovaný agonistou CB1R (kanabinoidní receptor typu 1) WIN55,212-2, což je považováno za klíčový impuls procesu závislosti. Bylo také otestováno, zda JMV2959 může ovlivnit účinky WIN55,212-2 a fentanylu na endokanabinoidy N-arachidonoylethanolamin (anandamid) a 2-arachidonoylglycerol (2-AG) a obsah kyseliny gama-aminomáselné (GABA) v NACSh a dále specifikovat zapojení GHS-R1A umístěného ve ventrální tegmentální oblasti (VTA) a NACSh do pozorovaných změn v NAC. Dále bylo testováno, zda premedikace JMV2959 může snížit kanabinoidy (tetrahydrokanabinol/THC a WIN55,212-2) indukovanou behaviorální stimulaci.
Metody: In vivo mikrodialýza byla použita ke stanovení změn dopaminu a jeho metabolitů v NACSh u potkanů po podání syntetického aminoalklylindolového kanabinoidu WIN55,212-2 do zadní VTA s premedikací antagonisty ghrelinu (JMV2959, 3 mg/kg i.p., 20 min před WIN55,212-2) a bez ní. Stejná metodika byla použita za účelem stanovení účinků WIN55,212-2 a fentanylu na obsah anandamid, 2-AG a GABA v NAC. K pozorování účinků JMV2959 na změnu chování potkanů způsobené THC a WIN55,212-2 bylo použito automatizované zařízení pro rozpoznávání chování (LABORAS).
Zjištění: Podání WIN55,212-2 vyvolalo významné uvolnění dopaminu v NAC, které bylo významně sníženo premedikací JMV2959 v dávce 3 mg/kg i.p. Současně byl metabolický zvrat dopaminu v NAC, který byl zvýšen kanabinoidy, významně zvýšen premedikací JMV2959. Intracerebrální podání WIN55,212-2 zvýšilo anandamid a extracelulární hladiny 2-AG v NACSh, což bylo mírně, ale významně sníženo premedikací JMV2959. Mimo to byl kanabinoidy indukovaný pokles hladin GABA v NAC zvrácen premedikací JMV2959. Premedikace JMV2959 (podávaný systémově i do NACSh nebo VTA) zvrátila na dávce závislé zvýšení anandamid vyvolané fentanylem v NACSh, což vedlo k významnému poklesu anandamid a zesílilo fentanylem vyvolaný pokles hladin 2-AG v NAC. Behaviorální studie pomocí LABORAS ukázala, že premedikace JMV2959 významně a v závislosti na dávce snížila systémovou stimulaci chování vyvolanou THC/WIN55,212-2.
Závěry: Výsledky ukázaly významný vliv ghrelinu/GHS-R1A na závislostních účincích opioidů a kanabinoidů a podporují další výzkum ghrelinového antagonismu jako potenciálního nového terapeutického směru u těchto závislostí.

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

I. INTRODUCTION

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

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

This dissertation research thesis summarises the important findings of a neurobehavioral research of the mesolimbic ghrelin signalling involvement in the cannabinoids [tetrahydrocannabinol (THC) and WIN55,212-2] pro-addictive effects. Furthermore, the endocannabinoid changes induced by opioids (fentanyl) in the rat NAc, with or without the GHS-R1A antagonist pretreatment, were rigorously described as well. Besides other findings, these achieved innovative results, which were also published in international journals with high IF (average IF=5.924), demonstrated that the ghrelin/GHS-R1A system importantly participates in the accumbens-neurotransmitter and exhibit behavioural changes that are associated with the rewarding/reinforcing effects of cannabinoids as well as opioids. This encourages further research of the GHS-R1A antagonism as a potential novel approach to cannabinoid and opioid addiction treatment and the possibility to decrease the craving and in extent, relapse.

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

II. HYPOTHESIS AND AIM

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

A study in mice described that the CB1 antagonist reduced the ghrelin-induced locomotor stimulation and dopamine overflow in the NAc (Kalafateli, Vallof et al. 2018). Collectively with our above-mentioned studies with GHS-R1A antagonist and morphine in rats, these results indicated, that the ghrelin signalling and the CB1 receptors in the NAc might be significantly involved not only in the in the opioid, but also in the cannabinoid reinforcing effects possibly through a dopamine-independent mechanism (Caille and Parsons 2003, Vigano, Valenti et al. 2004, Caille, Alvarez-Jaimes et al. 2007). The involvement of ghrelin signalling in the accumbens changes induced by cannabinoids/cannabis have not been previously tested.

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

1. The systemic pretreatment with the JMV2959 could reduce the intrategmentally (into the VTA) administered synthetic CB1R agonist WIN55,212-2–induced dopamine efflux in the NACSh.
2. The systemic pretreatment with the JMV2959 could reduce the intrategmentally (into the VTA) administered synthetic CB1R agonist WIN55,212-2–induced endocannabinoid and GABA changes in the NACSh.
3. The systemic pretreatment with the GHS-R1A antagonist (JMV2959) could reverse/reduce the anandamide increase and influence the 2-AG decrease in the

rat NACSh induced by the selective μ-receptor agonist (fentanyl) systemic administration, similarly to the previously described situation with non-selective opioid (morphine).

4. The central pretreatment with the JMV2959 administered into the VTA and/or the NACSh could influence the fentanyl-induced endocannabinoid (anandamide and 2-AG) changes in the NACSh.
5. The systemic pretreatment with the JMV2959 could also reduce the tetrahydrocannabinol (THC) and/or WIN55,212-2–induced locomotor stimulation in the LABORAS system.

III. MATERIALS AND METHODS

Animals used in the experimental research

Male adult Wistar rats provided by Velaz, Praha-Lysolaje, Czech Republic, initially aged 8 weeks were used in all the experiments. During the habituation period for at least seven days before the initiation of all experiments and during the experimental days the rats were given free access to food and water and were housed in polycarbonate cages in singles (microdialysis) or threes (LABORAS) per cage with constant humidity (50–60%), room temperature (22–24 C) and reversed 12 h light/dark cycle (6 a.m.–6 p.m.). After the drug administration during the experiments, the food was removed. (Charalambous, Lapka et al. 2021) (Sustkova-Fiserova, Charalambous et al. 2017).

Drugs and Chemicals used in the experiments research

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

Fentanyl was always dissolved in saline and administered subcutaneously (s.c.) 0.1 mL/100 g of body weight; 30 μg/kg was selected following the literature as a reliable analgesic and discriminative dose increasing accumbens dopamine (Di Chiara and Imperato 1988, Megens, Artois et al. 1998, Zhang, Walker et al. 2000). Saline was used as a placebo. The JMV2959 was dissolved in saline, when administered intraperitoneally (i.p.) 20 min before fentanyl; the selected dose 3 mg/kg JMV2959 s.c. was determined based on previous studies in Wistar rats (Clifford, Rodriguez et al. 2012, Sustkova-Fiserova, Jerabek et al. 2014, Sustkova-Fiserova, Jerabek et al. 2016). The intracerebral JMV2959 doses were in accordance with the literature (Hansson, Shirazi et al. 2012, Skibicka, Hansson et al. 2012). When JMV2959 was administered intra-cerebrally, JMV2959 was dissolved in the Ringer's solution (adjusted to pH = 7.0) and Ringer's solution was used as a placebo. Doses 2 or 10 μg of JMV2959 were administered into the rat VTA at a volume of 0.5 μL for 1 min; the cannula stayed in place for another minute and after was retracted (5 μL microsyringe; Innovative Labor System, Stutzerbach, Germany). The administration sites were verified following the end of the experiment. The dialysis probe was used for the administration of JMV2959 into the NAc. During perfusion with Ringer's solution (always 2 μL/min) the inlet tube was switched to tube filled with 8 mM or 40 mM solution of JMV2959 in the Ringer's solution for 15 min, starting 5 min before fentanyl administration; thereafter, the inlet tube was switched back to Ringer's solution. The position of each dialysis probe was histologically verified after the completion of each microdialysis experiment and only animals with correct probe positions were included into the statistical evaluations.

Both THC and WIN55,212-2 were firstly dissolved in one drop of Polysorbate 80 (Tween 80) and then diluted in saline. Instead of THC/WIN55,212-2 as the vehicle (saline with one drop of Tween 80) and instead of JMV2959/ghrelin pretreatments, saline served as the placebo/control. THC was used in a stimulatory/rewarding 0.1 mg/kg dose in LABORAS and administered intraperitoneally (i.p.) in volumes of 0.1 mL/100 g of body weight. WIN55,212-2 was diluted in saline or Ringer's solution in microdialysis experiments. Saline/Ringers solution with the drop of Tween 8 (vehicle)

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

In-vivo microdialysis and chemical analysis assay

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

Post-surgery, the rats were kept in individual cages. In accordance with Sustkova-Fiserova, 48 h after implantation, the probe (MAB4, 2 mm active cuprophane membrane, Agnthos, Sweden) was inserted into the guide cannula and artificial cerebrospinal fluid (Ringer´s solution; 147 mM NaCl, 2. 2 mM CaCl2 and 4.0 mM KCl; adjusted to pH 7.0) was flushed through the probe at a constant rate of 2.0 µl/min (Univentor 864 Syringe Pump, Agnthos, Sweden) (Sustkova-Fiserova, Jerabek et al. 2014, Sustkova-Fiserova, Jerabek et al. 2016). After minimum 60 min of habituation, 20 µL samples were collected at 20 min intervals in small polyethylene tubes containing 7 µL HCl 0.1 mM to prevent monoamine degradation. The further 20 µL dialysate samples of each 20 min interval were collected in empty small polyethylene tubes for detection of the other neurotransmitters (endocannabinoids and GABA). After three consecutive baseline samples, rats were injected with saline or JMV2959 (i.p.), which was followed (20 min later) by the administration with fentanyl (s.c)/ WIN55,212-2 (VTA) or saline (s.c.). In the fentanyl experiments with JMV2959 administration into the VTA/NAc four baseline samples and 5 min before fentanyl were collected, JMV2959 was administered into the VTA or NAc perfusion started and samples were further collected starting with fentanyl administration. Samples were collected for 3 h following injection of fentanyl/ WIN55,212-2 or saline. Immediately following collection, the samples were frozen at −70 °C. The amount of anandamide, 2-AG and

GABA in the dialysate were quantified using HPLC-MS. The appropriate HPLC-MS determination methods were described in detail earlier (Syslova, Rambousek et al. 2011, Sustkova-Fiserova, Jerabek et al. 2016). The endocannabinoids in the dialysate went under lyophilization in freeze dryer (Labconco Free Zone, USA) to concentrate the substances from the dialysates, and detection using liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) which consisted of a chromatograph Accela 1250 (Thermo Scientific, USA), autosampler Accela (Thermo Scientific, USA) and a TSQ Vantage mass spectrometer (Thermo Scientific, USA). The data were acquired and processed using Xcalibur 2.1.0 software (Thermo Scientific, USA).

Behavioural Testing in LABORAS

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

Statistical Analysis

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

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

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

IV. RESULTS

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

Pretreatment with the GHS-R1A antagonist/JMV2959 3 mg/kg i.p. 20 min before WIN55,212-2 significantly reduced the cannabinoid-induced dopamine efflux in the NACSh. The same 3 mg/kg i.p. JMV2959 dose "per se" did not influence accumbens dopamine, as it was already described (Jerlhag, Egecioglu et al. 2009, Engel, Nylander et al. 2015, Sustkova-Fiserova, Puskina et al. 2019). Also, the lower JMV2959 dose 1 mg/kg alone did not induce significant changes in accumbens dopamine/metabolites.

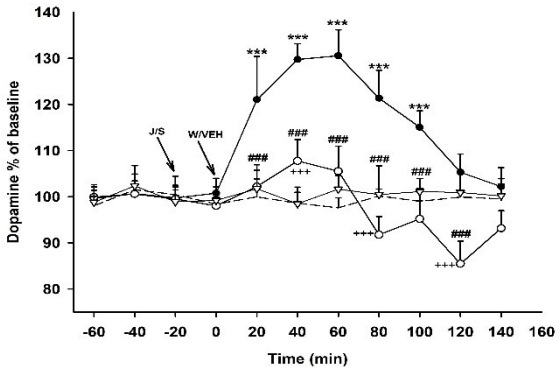

Figure 1. Effects of growth hormone secretagogoue receptors (GHS-R1A) antagonist (JMV2959) on WIN55,212-2-induced accumbens dopamine increase.

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

The accumbens extracellular dopamine metabolic turnover increase, induced by intracerebral WIN55,212-2 during its maximal effect (40 and 60 min intervals) in the present study, was significantly augmented after JMV2959 pretreatment in comparison to the WIN55,212-2 + saline group, measured with metabolic ratios HVA/DA, DOPAC/DA and 3-MT/DA ($p < 0.001$).

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

Pretreatment with JMV2959 transiently significantly reduced the WIN55,212-2-induced anandamide increase within a 20 min interval ($p < 0.05$) and 40–100 min intervals ($p < 0.001$) to a maximum of 151.4% of the baseline mean; thus, no more than by 12.7%. Despite the JMV2959 pretreatment, the cannabinoid-induced anandamide increase remained significantly relative to the vehicle + saline group from the 40 min interval until the end of the experiment (140 min interval). The JMV2959 pretreatment transiently significantly reduced the cannabinoid-induced 2-AG elevation within 20 min and 60–80 min intervals ($p < 0.001$) and 40 min and 120 min intervals ($p < 0.05$) by 5.5% to a maximum of 114.5% of the baseline. Despite JMV2959 pretreatment, the WIN55,212-2-induced accumbens 2-AG elevation remained significant relative to the vehicle + saline group within 40–140 min intervals. When JMV2959 was injected i.p. 20 min before the cannabinoid, we observed an initial moderate diminution of GABA in the NACSh significant relatively to vehicle + saline group within 20 min and 40 min intervals ($p < 0.01$ and $p < 0.001$, respectively) with a maximum of 92.6% of the baseline. However, the drop in accumbens GABA was transformed into a mild but significant increase at the 60 min interval ($p < 0.05$) to a maximum of 106.6% of the

baseline at the 80 min interval, and then the GABA levels decreased again back to baseline mean levels (non-significant relative to the vehicle + saline group).

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

Pre-treatment with the GHS-R1A antagonist JMV2959 administered intraperitoneally (i.p.) turned the fentanyl-induced accumbens anandamide increase and induced a significant decrease with the maximum drop 50% of baseline mean level. Pre-treatment with JMV2959 intensified the fentanyl-induced accumbens 2-AG decrease. The GHS-R1A antagonist, JMV2959 i.p. administration 20 min before fentanyl prevented the fentanyl-induced accumbens GABA increase maintaining its concentration almost on the baseline level

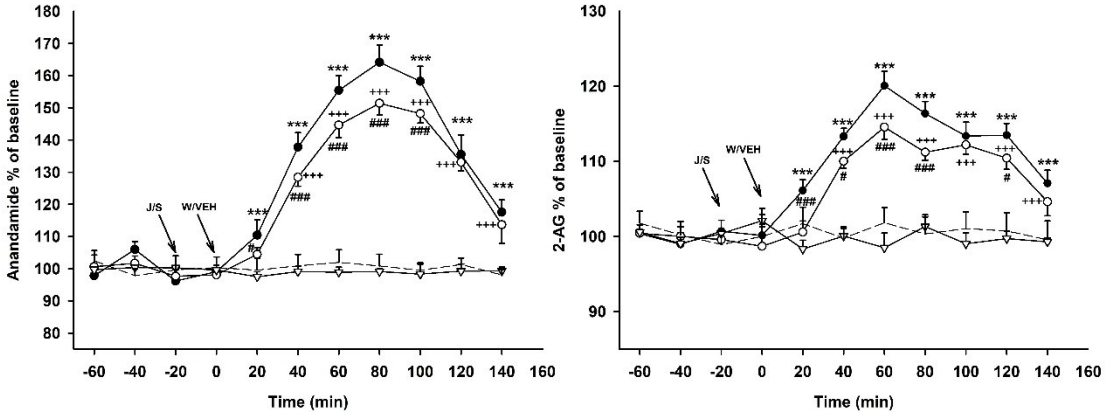

Figure 2. Effects of growth hormone secretagogoue receptors (GHS-R1A) antagonist (JMV2959) on WIN55,212-2-induced accumbens endocannabinoids changes.

The JMV2959 effects on the μ-selective opioid fentanyl-induced changes of anandamide and 2-AG when administered in the VTA and/or NACSh

Pre-Treatment with JMV2959 Administered into the VTA

The lower dose (2 μg) pre-treatment caused a drop of anandamide accumbens levels to the baseline concentration and the higher dose (10 μg) induced significant anandamide decrease. Pre-treatment with JMV2959 influenced the fentanyl-induced accumbens 2-AG decrease differently depending on the given dose. The 2 μg JMV2959/VTA dose slightly but significantly attenuated and simultaneously also prolonged the fentanyl-induced 2-AG decrease. The 10 μg JMV2959/VTA dose significantly deepened the accumbens fentanyl-induced 2-AG decrease.

Pre-Treatment with JMV2959 Administered into the NAc

Pre-treatment with both JMV2959 doses into the NACSh dose dependently reversed the fentanyl-induced accumbens anandamide increase. The JMV2959/NAc pre-treatment induced decrease/reversal of accumbens anandamide after fentanyl administration were observed only during first intervals, the anandamide levels returned to baseline levels at about 90 min (lower JMV2959 dose) and 120 min (higher JMV2959 dose) after fentanyl administration. Pre-treatment with both JMV2959 doses into the NACSh dose dependently reversed the fentanyl-induced accumbens anandamide increase. The JMV2959/NAc pre-treatment induced decrease/reversal of accumbens anandamide after fentanyl administration were observed only during first

intervals, the anandamide levels returned to baseline levels at about 90 min (lower JMV2959 dose) and 120 min (higher JMV2959 dose) after fentanyl administration.

Pre-treatment with both JMV2959 doses into the NAc significantly and dose dependently deepened the fentanyl-induced accumbens 2-AG extracellular concentrations. Both JMV2959 pre-treatments with fentanyl induced significant 2-AG decrease in comparison to saline + saline. Single lower JMV2959 dose administered into the NAc and saline i.p. did not significantly influence the accumbens 2-AG. Administration of single higher JMV2959 dose 40 mM/15 min/NAc induced slight but significant 2-AG decrease.

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

The 1 or 3 mg/kg JMV2959 administered 20 min before THC, significantly and dose-dependently reduced the THC-induced changes in all monitored parameters (locomotion, rear, distance travelled, and average speed). Also, the 1 or 3 mg/kg JMV2959 administered 20 min before WIN55,212-2, dose-dependently reduced the WIN55,212-2-induced changes in all monitored parameters.

V. DISCUSSION

The present thesis demonstrates that the central ghrelin signalling system, involving GHS-R1A, is required for indirect measures of the rewarding/reinforcing effects of the cannabinoid/CB1 agonist/WIN55,212-2 and μ-opioid agonist/fentanyl that participate in addiction processing. The GHS-R1A, CB1 and μ-opioid receptors are expressed within the NACSh as well as the VTA, thus, interaction in these signalling systems within these brain structures were considered. (Pickel, Chan et al. 2004, Fattore, Deiana et al. 2005, Maldonado, Valverde et al. 2006, Ferrini, Salio et al. 2009, Gomes, Fujita et al. 2013, Befort 2015). Beside others, the suggested hypotheses and the presented results imply interactions among the GHS-R1A, the CB1R, and the μ-opioid receptors within the brain mesolimbic system. Midbrain GHS-R1As are co-localized with dopaminergic and cholinergic receptors (Guan, Yu et al. 1997, Ferrini, Salio et al. 2009) and presumably interact in amplification of the dopaminergic signalling in the VTA neurons, and stimulate accumbens dopamine efflux (Jerlhag, Egecioglu et al. 2006, Jerlhag, Egecioglu et al. 2011). The brain endocannabinoid system is important for the regulation of the dopamine signalling during reinforcement processes (Lupica and Riegel 2005, Solinas, Goldberg et al. 2008). Also, possible interactions between endocannabinoids and central ghrelin signalling and the functional cooperation of CB1/endocannabinoids and GHS-R1A/ghrelin within hypothalamus, may contribute to ghrelin's orexigenic effects (Cani, Montoya et al. 2004)Kola (Tucci, Rogers et al. 2004, Kola, Farkas et al. 2008, Folgueira, Seoane et al. 2014, Al Massadi, Lopez et al. 2017). Cannabinoids including the main cannabis constituent tetrahydrocannabinol and synthetic cannabinoids (e.g., WIN55,212-2) are likely to mediate their pleasurable, anxiolytic, and rewarding/reinforcing effects through the CB1Rs located within the central brain reward circuits, particularly the VTA and the NAc (Matsuda, Lolait et al. 1990, Herkenham 1991, Parsons and Hurd 2015).

In the cannabinoids experiments, the low dose of WIN55,212-2 (2.4 mM/0.5 μL) administered into the posterior ventral tegmental area triggered significant accumbens dopamine release together with extracellular endocannabinoids, anandamide, and 2-AG, increase and transient GABA decrease. The low 2.4 mM/ 0.5 μL dose of WIN55,212-2 effect is in accordance with the literature (Zangen, Solinas et al. 2006, Polissidis, Chouliara et al. 2009, Parsons and Hurd 2015, Bloomfield, Ashok et al. 2016, Volkow, Hampson et al. 2017, Zehra, Burns et al. 2019). All the observed cannabinoid-induced accumbens changes were significantly reduced by the GHS-R1A antagonist/JMV2959 3 mg/kg i.p. pretreatment 20 min before the cannabinoid. The maximal microdialysate concentration values were used for calculating the extracellular dopamine metabolic turnover ratios. The accumbens extracellular dopamine metabolic turnover increase, induced by the intracerebral WIN55,212-2, was significantly increased after the JMV2959 pretreatment in comparison to the WIN55,212-2/saline group, measured with metabolic ratios HVA/DA, DOPAC/DA and 3-MT/DA. The significant increase formation of HVA (HVA/DA ratio) was produced by an enhanced DOPAC production (DOPAC/DA ratio). This was observed in our previous opioid microdialysis studies when a significant increased formation of HVA and DOPAC in the NACSh after the JMV2959 pretreatment before the morphine and fentanyl administration (Sustkova-Fiserova, Jerabek et al. 2014, Sustkova-Fiserova, Puskina et al. 2019). Thus, a significant impact of JMV2959 pretreatment was observed on cannabinoid- and opioid-provoked accumbens dopamine metabolism due to the monoamine oxidase (MAO) (the significant HVA and DOPAC/DA increase). Further, the behavioural stimulation observed within 20–40 min after the 0.1 mg/kg

THC/WIN55,212-2 intraperitoneal administration was significantly reduced by the JMV2959 3 mg/kg pretreatment. These results indicate substantial participation of ghrelin/GHS-R1A mechanisms in the rewarding/reinforcement effects of the CB1R agonists- THC/synthetic cannabinoid WIN55,212-2, which are involved in cannabinoid addiction.

In the fentanyl experiments, pretreatment with JMV2959 in all doses and types of administration (i.p., into the VTA or NAc) significantly affected the fentanyl-induced accumbens anandamide increase and the 2-AG decrease. Pre-treatment with the GHS-R1A antagonist JMV2959 administered intraperitoneally (3 mg/kg) reversed the fentanyl-induced anandamide increase. Pre-Treatment with JMV2959 administered into the NAc (perfusion with 8 or 40 mM for 15 min) reversed the fentanyl-induced anandamide increase, however, the observed significant dose-dependent anandamide decrease had a sharper drop and shorter duration in comparison with the intraperitoneal JMV2959 effect, which was possibly due to the local (NAc) type of JMV2959 administration. Pre-Treatment with JMV2959 administered into the VTA (2 or 10 μg) prevented the fentanyl-induced accumbens anandamide increase, but only the higher dose reversed the anadamide levels to partly significant decrease. It is difficult to compare effects of differently administered various doses, but considering the effects of the pretreatment JMV2959 doses, which alone did not significantly influence the accumbens anandamide, it can be suggested that GHS-R1A receptors of both VTA and NAc brain structures participate in the significant JMV2959 reversal effects on the fentanyl/opioid-induced anandamide increase in the NASh, with major involvement of the NAc structure.

The fentanyl-induced 2-AG decrease was significantly intensified by the 3 mg/kg i.p. JMV2959. This is fully in accordance with the Sustkova-Fiserova et al. 2016 study with morphine and the intraperitoneal JMV2959 pretreatment (Sustkova-Fiserova, Jerabek et al. 2016). Similarly to the 3 mg/kg JMV2959 i.p. administered effects, intra-accumbens administration of JMV2959 (perfusion with 8 or 40 mM for 15 min) also deepened significantly and dose-dependently the fentanyl-induced 2-AG accumbens decrease. When JMV2959 was administered into the VTA (2 or 10 μg), only the higher JMV2959 dose significantly deepened the fentanyl-induced 2-AG decrease. The lower JMV2959/VTA dose attenuated but prolonged the fentanyl-induced accumbens 2-AG decrease. Thus, both types of JMV2959 administration significantly changed the fentanyl-induced accumbens 2-AG decrease, but the pre-treatment effects seemed more expressed with the administration into the NACSh.

The pre-treatment with ghrelin antagonist in all given doses and types of administration prevented the opioid/fentanyl-induced accumbens GABA efflux in a very similar way. These findings suggest that ghrelin antagonism may inhibit opioid-induced dopamine release, which was also described previously in other studies (Engel, Nylander et al. 2015, Sustkova-Fiserova, Jerabek et al. 2016), by attenuating opioid-evoked GABA release in the NACSh. VTA GABA-A/GABAergic system importantly contributes to the dopamine-independent opioid reinforcement (Laviolette and van der Kooy 2001, Ting and van der Kooy 2012). The opioid-induced GABA efflux in the NACSh contributes to the opioid reinforcing properties (Aono, Saigusa et al. 2008). GABA elevated concentrations potentiate opioid-induced accumbens dopamine release. Interactions between central ghrelin and GABAergic systems have been implicated within the central nucleus of amygdala (CeA) (Cruz, Herman et al. 2013) and hypothalamus (Lopez Soto, Agosti et al. 2015) hereby the observed significant ghrelin and accumbens GABA interaction.

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

In the LABORAS experiment, a significant increase in locomotion, rearing, distance travelled, and the overall average speed of behaviour was observed in comparison to the vehicle treated group, within 20–40 min after the WIN55,212-2 as well as the THC administration. The JMV2959 (1 and 3 mg/kg) administration 20 min before the cannabinoid significantly and dose-dependently reduced the monitored cannabinoid-stimulated behavioural parameters; however, in case of WIN55,212-2 the effect was significant only when the higher dose 3 mg/kg JMV2959 was used ($p < 0.001$). These results are in accordance with previous studies, when JMV2959 in mice or rats reduced locomotor hyperactivity induced by fentanyl (Sustkova-Fiserova, Charalambous et al. 2017). When JMV2959 was administered alone, no significant influence on rat behaviour was observed within the relevant period in LABORAS, which corresponds well with the Jerabek et al. 2017 study in activity cage/open field (Jerabek, Havlickova et al. 2017). To summarize, the behavioural stimulation observed within 20–40 min after the 0.1 mg/kg THC as well as WIN55,212-2 intraperitoneal administration was significantly reduced by the GHS-R1A antagonist (JMV2959) pretreatment.

VI. CONCLUSION

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

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

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

These results signify a strong participation of accumbens endocannabinoids, particularly anandamide, but also GABA in the neural opioid/fentanyl reinforcing processes and suggest that ghrelin antagonism may play an important role in the NACSh endocannabinoid/anandamide and GABA changes possibly related to opioid/μ-receptor agonist reinforcement. Although GHS-R1A receptors within both NACSh as well as VTA have been found to contribute significantly to these effects, administration of ghrelin antagonist into the NACSh seemed to have stronger impact on the accumbens endocannabinoid opioid-induced changes. Our findings further suggest substantial involvement of ghrelin/GHS-R1A central signalling in the cannabinoid rewarding/ reinforcement pro-addictive effects, which encourages further investigation of the GHS-R1A antagonism as a potential approach to cannabinoid addiction treatment. Further investigation to assess if substances affecting GABA or endocannabinoid concentrations and action, such as GHS-R1A antagonists, can be used to prevent cannabis/opioid -seeking behaviour and as a potential new therapeutic direction in these addictions.

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LIST OF ABBREVIATIONS

(2-AG) 2-arachidonoylglycerol
(3-MT) 3-methoxytyramine
(anandamide) N-arachidonoylethanolamine
(CB1R) Cannabinoid Receptor Type 1
(CeA) Central nucleus of Amygdala
(DOPAC) 3,4-dihydroxyphenylacetic acid
(GABA) Gama-AminoButyric Acid
(GHS-R1A) Growth Hormone Secretagogue Receptor 1A
(HVA) Homovanillic acid
(ICD/ICD-10) International Classification of Diseases
(NAc) Nucleus Accumbens
(NACSh) Nucleus Accumbens shell
(THC) Tetrahydrocannabinol
(VTA) Ventral Tegmental Area
(WHO) World Health Organization

LIST OF PUBLICATIONS

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.
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7. 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