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THE EFFECTS OF NICOTINE, NICOTINE WITHDRAWAL, GHRELIN AND GHRP-6 ON LOCOMOTOR ACTIVITY AND DOPAMINE RELEASE IN RATS

PhD Thesis Summary

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ABBREVIATIONS

[^{3}H]DA = tritium-labelled dopamine

BBB = blood-brain barrier

BNST = bed nucleus of the stria terminalis

CNS = central nervous system

CPM = counts per minute

CRF = corticotropin-releasing factor

CRFR1 = corticotropin-releasing factor receptor type 1

CRFR2 = corticotropin-releasing factor receptor type 2

DA = dopamine

DSM-5 = Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition

GH = growth hormone

GHRP-6 = growth hormone releasing peptide-6

GHSR = growth hormone secretagogue receptor

GHSR1a = growth hormone secretagogue receptor type 1a

GHSR1b = growth hormone secretagogue receptor type 1b

GPCR = G-protein coupled receptor

icv = intracerebroventricular

ip = intraperitoneal

LDT = laterodorsal tegmental nucleus

NAcc = nucleus accumbens

nAChR = nicotinic acetylcholine receptor

sc = subcutaneous

VTA = ventral tegmental area

WHO = World Health Organization

1. INTRODUCTION

1.1. The effects of nicotine and nicotine withdrawal

Nicotine is the primary psychoactive component of tobacco and is responsible for its addictive properties. Tobacco addiction leads to the harmful habit of smoking, which is associated with high morbidity and mortality worldwide. According to the World Health Organization (WHO), in 2018 more than 6 million people died due to active smoking, while 1 million were non-smokers who had been exposed to second-hand smoke. Nicotine is a naturally occurring alkaloid found in tobacco leaves. A single cigarette contains about 10–12 mg of nicotine, but the average smoker absorbs roughly 1 mg from it. During smoking, nicotine is rapidly absorbed through the alveoli into arterial blood and reaches the brain within seconds. It easily crosses the blood-brain barrier (BBB), binds nicotinic acetylcholine receptors (nAChRs) - particularly in the ventral tegmental area (VTA) - and increases dopamine (DA) release in the mesolimbic pathway, producing reward and fostering addiction. Besides its psychostimulant effect, nicotine induces tachycardia, vasoconstriction, bronchospasm and contraction of both skeletal and smooth muscles by stimulating the release of acetylcholine. The actions of nicotine are mediated by nAChRs which are ligand-gated ion channels composed of pentameric combinations of α and β subunits. These receptors bind nicotine, but normally respond to acetylcholine. After binding to these pentameric ligand-gated ion channels, nicotine causes a conformational change in the receptor that results in the opening of the integral cation channel. Activation of nAChRs leads to increased permeability to both Na^+ and Ca^{2+}, which results in local depolarization and induces the release of various neurotransmitters.

Tobacco addiction, also known as nicotine dependence, is the most common type of substance dependence globally, causing more than seven million deaths each year. Nicotine dependence is characterized by a compulsion to seek out and consume nicotine, a loss of control over intake, and an emergence of a negative emotional state when access to the drug is prevented. According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), tobacco addiction or nicotine dependence is classified as ”tobacco-use disorder”. Clinically, a diagnosis requires any two of eleven criteria to be met within a 12-month period and is graded as mild (2–3 criteria), moderate (4–5 criteria), or severe (six or more criteria). From a neuroendocrinological perspective, nicotine dependence has been conceptualized as a dynamic, evolving disorder that consists of three stages: binge/intoxication, withdrawal/negative affect, and preoccupation/anticipation (craving). The basis of nicotine dependence is a combination of positive reinforcement, provided by the rewarding and euphoric effects of nicotine, and negative reinforcement, maintained by the avoidance of the aversive,

dysphoric effects of nicotine withdrawal. Under experimental conditions, the nicotine withdrawal syndrome can be induced by mecamylamine, a non-selective and non-competitive nAChR antagonist that is usually administered orally in humans and subcutaneously (sc) or intracerebroventricularly (icv) in rodents, precipitating the symptoms of nicotine withdrawal.

1.2. The effects of ghrelin and GHRP-6

Ghrelin is a 28-amino-acid peptide that stimulates growth hormone (GH) release via activation of the ghrelin receptor. Originally purified from the rat stomach, ghrelin was identified as the first peripheral hormone with potent orexigenic activity to regulate appetite and food intake in mammals, including humans, and was therefore named the "hunger hormone". Moreover, over the past few decades, studies have demonstrated that ghrelin regulates many other physiological processes, and dysfunction of ghrelin can lead to multiple pathologies in the endocrine, metabolic, cardiovascular, and central nervous systems (CNS). In contrast, growth hormone-releasing peptide 6 (GHRP-6), also known as growth hormone-releasing hexapeptide, is a synthetic Met-enkephalin derivative that lacks opioid activity but can stimulate GH release via the ghrelin receptor. GHRP-6 is usually considered an antagonist of the ghrelin receptor, but it can also act as an agonist, stimulating GH release and even food intake. The ghrelin receptor is a seven-transmembrane G-protein-coupled receptor (GPCR) that was previously named the growth hormone secretagogue receptor (GHSR) and classified into two subtypes (GHSR1a and GHSR1b), with GHSR1a regulating food intake and energy balance, and GHSR1b inhibiting the activity of GHSR1a However, due to subsequent discoveries, the hormone ghrelin is now considered the receptor's natural endogenous ligand, and GHSR1a is now simply referred to as the ghrelin receptor. It can be found both in the CNS and the periphery. In the CNS, the ghrelin receptor is expressed in regions where fenestrated capillaries are present, such as the arcuate nucleus of the hypothalamus (which can be reached by peripherally circulating ghrelin). It is also expressed in brain regions that are activated chiefly by centrally administered ghrelin, such as the laterodorsal tegmental nucleus (LDT) of the brainstem, as the passage of peptides is limited by the BBB. In the periphery, ghrelin receptors have been detected in many organs, including the gastrointestinal tract, pancreas, heart, lungs, vasculature, kidneys, and gonads, indicating that peripheral ghrelin may play a role in regulating the digestive, reproductive, and cardiovascular systems.

In addition to its physiological role in the rewarding effect of food, ghrelin and its receptor have been implicated in reward sensation induced by natural and synthetic drugs, including nicotine, alcohol, amphetamine, and cocaine. Indeed, several lines of evidence

demonstrate an interaction between ghrelin and nicotine. In animals, the evidence is limited, but most studies demonstrate that administration of nicotine increases acyl ghrelin levels, and that administration of a GHSR1a antagonist attenuates some of the behavioural and biochemical effects of nicotine. In humans, findings are mixed, but most studies indicate that smokers have higher ghrelin levels than non-smokers, and that smoking cessation leads to a decrease in ghrelin levels. Moreover, studies in habitual smokers suggest that endogenous ghrelin levels correlate positively with years of smoking and may predict the risk of smoking relapse. However, no clear association has been found between ghrelin levels and the severity of nicotine dependence, withdrawal, or craving.

2. AIM OF STUDY

To address the aims, we conducted two sets of experiments: (1) *in vivo* behavioural testing of nicotine-treated rats receiving icv injections, and (2) *in vitro* DA-release measurements using brain-slice superfusion.

The first aim of this work was to investigate the effects of ghrelin and GHRP-6 on the horizontal and vertical locomotor activity in rats exposed to chronic nicotine treatment followed by acute nicotine withdrawal. In one of our previous studies, we investigated the role of corticotropin-releasing factor (CRF) receptors - type 1 (CRFR1) and type 2 (CRFR2) - in the changes in locomotor activity and striatal DA release observed in rats under similar *in vivo* conditions.

The second aim of this work was to investigate the effects of ghrelin and GHRP-6 on DA release in the bed nucleus of stria terminalis (BNST), which could mediate the changes in locomotor activity observed during chronic nicotine treatment and acute nicotine withdrawal. In our previous *in vitro* superfusion studies, we demonstrated that ghrelin and nicotine equally stimulate DA release in the rat amygdala, and that ghrelin amplifies nicotine-induced DA release in the rat striatum. However, less attention has been paid to the actions of ghrelin and nicotine in the BNST. Therefore, in the most recent study nicotine and ghrelin were superfused into the BNST of male Wistar rats, and DA release from the BNST was measured *in vitro*. To determine which receptors mediated these effects, mecamylamine, a non-selective nAChR antagonist, and GHRP-6, a selective GHSR1a antagonist, were also superfused into the rat BNST.

3. MATERIALS AND METHODS

3.1. *In vivo* Conducta system experiments

The male and female Wistar rats used were provided by Toxi-Coop, Toxicological Research Center Zrt., Budapest, Hungary. The rats were adolescent (approximately 6-7 weeks old) but already sexually mature, weighing 150-250 g upon arrival. Before the experiments, the rats were housed together and kept at a constant temperature on a standard illumination schedule with 12-h light and 12-h dark periods (lights on from 06:00). Commercial food and tap water were available ad libitum. To minimise the effects of non-specific stress, the rats were handled daily. During the experiments, the rats were treated in accordance with the instructions of the Ethical Committee for the Protection of Animals in Research, University of Szeged, Hungary.

The rats were implanted with a stainless steel Luer cannula (10 mm long) aimed at the right lateral cerebral ventricle (LCV) under anaesthesia with 60 mg/kg pentobarbital sodium (Euthanasol, CEVA-Phylaxia, Budapest, Hungary). The stereotaxic coordinates were 0.2 mm posterior and 1.7 mm lateral to the bregma, and 3.7 mm deep from the dural surface, according to the stereotaxic atlas of the rat brain. Cannulas were secured to the skull with acrylate and dental cement (Spofa Dental Adhesor, Prague, Czech Republic). The rats were allowed to recover for 7 days before the experiments started. After the experiments, the rats were decapitated. A volume of 10 μl of methylene blue (Reanal Ltd., Budapest, Hungary; 1 g/100 ml) was injected icv to verify the cannula position by visual inspection. Animals without the dye (3 out of 100) in the LCV were excluded from the final statistical analysis. No animals were lost following anaesthesia or surgery.

The rats were administered daily by intraperitoneal (ip) injection of 2 mg/kg nicotine (Sigma-Aldrich Inc., St. Louis, USA) or 0.9% saline solution (B. Braun Inc., Melsungen, Germany) twice daily for 7 days (at 08:00 and 20:00). This dose and schedule of nicotine administration produces a plasma nicotine level in rats similar to that found in individuals who smoke 1-2 packs of cigarettes a day. In parallel, the rats the rats received a daily icv injection of either 1 μg/2 μl ghrelin, 1 μg/2 μl GHRP-6, or 2 μl of 0.9% saline, for 7 days (once daily at 8:00). On the morning of the 8th day (12 hours after the last ip injection) and the 9th day (24 hours after the last ip injection), horizontal and vertical activities were monitored using the Conducta system. The Conducta system (Experimetria Ltd., Budapest, Hungary) is based on the principle of the open-field test as described in our previous studies. The horizontal and vertical activity of the rats was monitored by a 5×5 grid of photocell beams and recorded by a computer for 10 min each. The horizontal activity reflects overall activity and arousal, while vertical activity indicates exploratory and stereotypic behaviour.

The statistical analysis of the results was performed using GraphPad Prism 7 (GraphPad Inc., USA). Differences between groups were determined by one-way ANOVA followed by Tukey's post-hoc test, or by the Kruskal-Wallis test followed by Dunn's post-hoc test, which were preceded by a Shapiro-Wilk normality test. A probability level of 0.05 or less ($p \leq 0.05$) was considered statistically significant.

3.2. *In vitro* superfusion experiments

Male Wistar rats weighing 150–250 g (N = 6) were used for each *in vitro* experiment. The rats were treated in accordance with the ARRIVE guidelines, and the experiments were carried out in compliance with the EU Directive 2010/63/EU for animal experiments. The agonists nicotine and ghrelin were provided by B. Braun Inc. (Melsungen, Germany) and Bachem Inc. (Bubendorf, Switzerland), respectively. The antagonists mecamylamine and GHRP-6 were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The Krebs solution contained 113 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.5 mM glucose, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, all obtained from Reanal Ltd. (Budapest, Hungary). The tritium-labelled DA ([^{3}H]DA) and the Ultima Gold scintillation fluid were ordered from Perkin-Elmer Inc. (Waltham, MA, USA). The superfusion system was purchased from MDE Ltd. (Heidelberg, Germany), and the method was originally described by Gaddum.

The BNST slices obtained were incubated in 8 ml of Krebs solution, submerged in a water bath at 37 ˚C and gassed through a single-use needle with a mixture of 5% CO2 and 95% O2 for 30 min. During the incubation, 5 µl of [^{3}H]DA (1 mCi/ml; 60 Ci/mmol) was added to the incubation medium with a Hamilton Microliter syringe. Six BNST slices were transferred to each of the cylindrical chambers of the superfusion system and superfused with Krebs solution for 30 min. A multichannel peristaltic pump (Gilson Minipuls 2) was used to maintain a constant superfusion rate of 300 µl/min. After 30 min of superfusion, the slices were superfused for an additional 32 min, during which fractions were collected into Eppendorf tubes by a multichannel fraction collector (Gilson FC 203B). The BNST slices were treated with 100 µM nicotine and/or 1 µM ghrelin 20 min after superfusion started; where indicated, slices were pre-treated with 100 µM mecamylamine and/or 1 µM GHRP-6 (a selective GHSR1a antagonist) 10 min after superfusion started. Electrical stimulation was applied 32 min after the start of superfusion (i.e., 2 min after fraction collection began). The stimulation consisted of square-wave impulses with a voltage of 100 V, a pulse length of 5 ms, and a frequency of 10 Hz. The electrical impulses were delivered to each of the four chambers for 2 min via gold electrodes attached to the chamber halves and connected to an ST-02 electrical stimulator. After the next

30 min of superfusion, the remaining BNST slices were removed and solubilised in 200 µl of Krebs solution using an ultrasonic homogeniser (Branson Sonifier 250). The homogenised slices and the collected samples were each mixed with 3 ml of Ultima Gold scintillation fluid in glass vials. Radioactivity was measured with a Tri-Carb 2100TR liquid scintillation spectrometer (Packard, USA). Fractional [^{3}H]DA release was calculated as the percentage of radioactivity in the collected samples relative to that remaining in the tissue, with radioactivity expressed in counts per minute (CPM).

Statistical analysis was performed using Stata13 (StataCorp, College Station, TX, USA). A repeated-measures ANOVA followed by Tukey's post-hoc test was used for pairwise comparisons. A probability level of 0.05 or less ($p \leq 0.05$) was considered statistically significant.

4. RESULTS

4.1. Locomotor activity

On the 8th day (12 hours after the last ip administration), nicotine-treated rats exhibited significant hyperactivity ($F(5,45) = 17.28$; $p < 0.001$ for horizontal activity and ($F(5,45) = 1.97$; $p = 0.024$ for vertical activity). This hyperactivity was significantly reduced by ghrelin and GHRP-6. When the rats were separated in male and female groups, the horizontal activity increased more significantly in males ($F(5,25) = 9.04$; $p = 0.001$) than in females ($F(5,20) = 7.86$; $p = 0.008$), and accordingly, ghrelin and GHRP-6 significantly reduced horizontal activity only in males but not in females. In comparison, vertical activity increased significantly in both males $F(5,25) = 11.67$; $p = 0.004$) and females $F(5,20) = 10.872$; $p = 0.050$), but ghrelin and GHRP-6 had no significant effect on vertical activity in either sex.

On the 9th day (24 hours after the last ip administration), nicotine-treated rats exhibited significant hypoactivity ($F(5,45) = 19.11$; $p < 0.001$ for horizontal activity and ($F(5,45) = 4.94$; $p = 0.013$ for vertical activity). This hypoactivity was significantly reversed by ghrelin and GHRP-6. When the rats were separated by sex, horizontal activity decreased significantly in both male ($F(5,25) = 7.94$; $p < 0.001$) and female ($F(5,20) = 12.05$; $p < 0.001$) nicotine-treated rats. Ghrelin and GHRP-6 significantly reversed this hypoactivity in both sexes. In contrast, although vertical activity decreased more in males ($F(5,25) = 2.81$; $p = 0.045$) than in females ($F(5,20) = 2.48$; $p = 0.079$) on the 9th day, ghrelin and GHRP-6 had no significant effect on vertical activity in either sex.

4.2. Dopamine release

Nicotine significantly increased the fractional [^{3}H]DA release from rat BNST after electrical stimulation ($F(3,31) = 19.78$; $p < 0.001$), an effect that was significantly inhibited by mecamylamine ($F(3,31) = 19.78$; $p < 0.001$). Ghrelin increased the fractional [^{3}H]DA release even more than nicotine did ($F(3,31) = 16.58$; $p < 0.001$), an effect that was significantly inhibited by GHRP-6 ($F(3,31) = 16.58$; $p < 0.001$). Moreover, when administered together, ghrelin significantly amplified the nicotine-induced fractional [^{3}H]DA release from rat BNST after electrical stimulation ($F(3,31) = 13.19$; $p < 0.001$). The additive effect of nicotine and ghrelin was partially reversed by mecamylamine ($F(3,31) = 16.58$; $p < 0.001$) and by GHRP-6 ($F(3,31) = 13.5$; $p < 0.05$). Our previous studies have demonstrated that ghrelin and nicotine stimulate DA release in the rat amygdala to a similar extent, and that ghrelin amplifies nicotine-induced DA release in the rat striatum.

5. DISCUSSION

5.1. The effects of nicotine, nicotine withdrawal, ghrelin and GHRP-6 *in vivo*

On the 8th day, horizontal and vertical activities increased in rats exposed to nicotine for 7 days. This finding is concordant with previous studies in which locomotor hyperactivity was reported on the 4th, the 8th and the 10th day of chronic nicotine exposure. The locomotor hyperactivity observed can be explained by the increased concentration of DA and the density of DA receptors (D1-type and D2-type) in the striatum, as well as the supersensitivity of midbrain DA receptors that usually develops after a few days in response to nicotine. On the 9th day, horizontal and vertical activities declined in rats exposed to one day of acute nicotine withdrawal. The nicotine withdrawal syndrome comprises a somatic component - characterised by locomotor hypoactivity, increased appetite, and weight gain - and an affective component, manifested as dysphoria, anxiety, and depression. The physical signs start promptly within a few hours, and peak around 24 hours following nicotine cessation. The affective symptoms may start early, but can persist from days to months, resulting in chronic nicotine withdrawal characterized by craving and increased risk of relapse. The locomotor hyperactivity observed in nicotine-treated rats on the 8th day was significantly reduced by ghrelin and GHRP-6. Additionally, the locomotor hypoactivity observed in nicotine-treated rats on the 9th day was significantly reversed by both the natural and synthetic peptides acting through the ghrelin receptor. GHRP-6 is usually considered an antagonist of the ghrelin receptor, but it can also act as an agonist in many respects, including stimulation of GH release and food intake; thus, the similar effects of ghrelin and GHRP-6 on the locomotor responses to nicotine are not surprising,

and could be related to the neuroprotective effects of GH secretagogues demonstrated in the hypothalamus and cerebellum. Therefore, the interaction among ghrelin, nicotine, and GHRP-6 may occur in various brain regions, including the hypothalamus and cerebellum, but it is most likely mediated by the cholinergic-dopaminergic reward link. This link encompasses the afferent cholinergic projection from the LDT to the VTA and the mesolimbic dopaminergic pathway.

5.2. The effects of nicotine, mecamylamine, ghrelin and GHRP-6 *in vitro*

Nicotine significantly amplified the release of DA from the rat BNST after electrical stimulation, and this effect was inhibited significantly by mecamylamine, a non-selective nAChR antagonist. This finding is concordant with previous *in vivo* and *in vitro* studies, which indicated that nicotine stimulates DA release in several subcortical and cortical brain regions. This stimulatory effect of nicotine on DA is mediated by different nAChR subtypes expressed on dopaminergic terminals in the BNST, and may contribute to the reward sensation produced by nicotine. Multiple studies have demonstrated that DA signalling in the BNST is implicated in the sensation of reward produced by addictive drugs, such as nicotine. Dose-dependent increases in extracellular DA in the BNST were observed after administration of synthetic drugs, such as nicotine. Likewise, increased release of DA in the BNST was described following exposure to natural rewarding substances, such as sucrose. Furthermore, blocking DA D_1 receptors in the BNST reduced ethanol and sucrose self-administration. Nevertheless, several studies suggest that DA signalling in the BNST may also play a role in the negative affect induced by nicotine withdrawal. The stimulatory effect of ghrelin on DA and locomotion is mediated by GHSR1a receptors scattered along the cholinergic-dopaminergic reward link, which consists of the afferent cholinergic projection that starts in the LDT and projects to the VTA, and the mesocorticolimbic dopaminergic pathway that emerges from the VTA and, among others, sends projections to the ventral striatum represented by the nucleus accumbens (NAcc). Activation of the cholinergic-dopaminergic reward link by ghrelin may lead to stimulation of DA release from dopaminergic terminals in the BNST, and may induce a similar reward sensation to that produced by nicotine. When administered together, ghrelin significantly amplified the nicotine-induced release of DA from the rat BNST after electrical stimulation, and this effect was partially reversed by mecamylamine and partially by GHRP-6.

6. CONCLUSIONS

In conclusion, the present findings, together with previous studies, demonstrate that changes in horizontal and vertical activity observed after 12 and 24 hours of nicotine withdrawal are mediated by midbrain DA and can be attenuated by ghrelin and GHRP-6. This study provides the first *in vivo* evidence that ghrelin and GHRP-6 attenuate both the locomotor hyperactivity produced by chronic nicotine treatment and the locomotor hypoactivity induced by acute nicotine withdrawal, with possible sex differences. *In vitro,* this study provides the first evidence that nicotine significantly increases DA release in the BNST, an effect that is significantly inhibited by mecamylamine. Ghrelin alone enhances DA release in the BNST even more than nicotine does, and this effect is significantly inhibited by GHRP-6. When ghrelin and nicotine are administered together, ghrelin amplifies the nicotine-induced DA release in the BNST; this additive effect is partly reversed by mecamylamine and partly by GHRP-6. However, the precise mechanism by which ghrelin interacts with nicotine remains to be elucidated. Our previous studies have demonstrated that ghrelin and nicotine stimulate DA release in the rat amygdala to a similar extent, and that ghrelin amplifies nicotine-induced DA release in the rat striatum. These preclinical findings may have clinical implications: they suggest that ghrelin could be used as a prognostic tool (for instance, by measuring ghrelin levels in smokers to predict relapse risk) and that the ghrelin receptor could serve as a pharmacological target for the treatment of nicotine dependence.

PUBLICATIONS

Original articles on which the present work is based:

Ayman J, Palotai M, Dochnal R, Bagosi Z. Ghrelin Amplifies the Nicotine-Induced Release of Dopamine in the Bed Nucleus of Stria Terminalis (BNST). Biomedicines 2023 Sep, 11(9), 2456. doi: 10.3390/biomedicines11092456

Ayman J, Buzás A, Dochnal R, Palotai M, Jászberényi M, Bagosi Z. Changes in Locomotor Activity Observed During Acute Nicotine Withdrawal Can Be Attenuated by Ghrelin and GHRP-6 in Rats. Biomedicines 2025 Jan, 13(1), 143. doi.org: 10.3390/biomedicines13010143

Oral presentations related to the present work:

Ayman J, Bagosi Z. A ghrelin és a GHRP-6 hatásai a nikotin és a nikotinmegvonás okozta lokomóció és dopamin változásokra. MÉT, 29-31 May 2024, Debrecen, Hungary

Poster presentations related to the present work:

Ayman J, Megyesi K, Dochnal R, Csabafi K, Bagosi Z. A ghrelin és a [D-Lys3]-GHRP-6 hatásai a krónikus nikotin adást követő akut nikotin megvonásra. FAMÉ, 7-9 June 2023, Mátraháza, Hungary

Ayman J, Dochnal R, Bagosi Z. Ghrelin amplifies the nicotine-induced dopamine release in the rat amygdala, bed nucleus of stria terminalis (BNST) and striatum. INC, 25-26 January 2024, Pécs, Hungary

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