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

PhD Thesis

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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

Other original articles on which the present work is based:

Jászberényi M, Bujdosó E, Bagosi Z, Telegdy G. Mediation of the behavioral, endocrine and thermoregulatory actions of ghrelin. Horm Behav. 2006 Aug; 50(2):266-73. doi: 10.1016/j.yhbeh.2006.03.010.

Palotai M, Bagosi Z, Jászberényi M, Csabafi K, Dochnal R, Manczinger M, Telegdy G, Szabó G. Ghrelin and nicotine stimulate equally the dopamine release in the rat amygdala. Neurochem Res. 2013 Oct; 38(10):1989-95. doi: 10.1007/s11064-013-1105-1.

Palotai M, Bagosi Z, Jászberényi M, Csabafi K, Dochnal R, Manczinger M, Telegdy G, Szabó G. Ghrelin amplifies the nicotine-induced dopamine release in the rat striatum. Neurochem Int. 2013 Oct; 63(4):239-43. doi: 10.1016/j.neuint.2013.06.014.

Buzás A, Bokor P, Balangó B, Pintér D, Palotai M, Simon B, Csabafi K, Telegdy G, Szabó G, Bagosi Z. Changes in striatal dopamine release and locomotor activity following acute withdrawal from chronic nicotine are mediated by CRF1, but not CRF2, receptors. Brain Res. 2019 Mar 1; 1706:41-47. doi: 10.1016/j.brainres.2018.10.028.

Oral presentations related to the present work:

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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. Magyar Kísérletes és Klinikai Farmakológiai Társaság, Magyar Anatómus Társaság, Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság és Magyar Élettani Társaság Vándorgyűlés (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. International Neuroscience Conference (INC), 25-26 January 2024, Pécs, Hungary

TOTAL IMPACT FACTOR: 7.8
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ABBREVIATIONS

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

ACTH = adrenocorticotropic hormone

AgRP = agouti-related peptide

Arc = arcuate nucleus

AVP = arginine vasopressin

BBB = blood-brain barrier

BNST = bed nucleus of the stria terminalis

CeA = central nucleus of the amygdala

CNS = central nervous system

CP = caudate-putamen

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

GABA = gamma-aminobutyric acid

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

HPA = hypothalamic-pituitary-adrenal

icv = intracerebroventricular

ip = intraperitoneal

LC-NE = locus coeruleus-norepinephrine

LDT = laterodorsal tegmental nucleus

MCH = melanin-concentrating hormone

NAcc = nucleus accumbens

nAChR = nicotinic acetylcholine receptor

NE = norepinephrine

NPY = neuropeptide Y

PAG = periaqueductal gray

PVN = paraventricular nucleus

sc = subcutaneous

SN = substantia nigra

vs. = versus

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 [1]. Tobacco addiction leads to the harmful habit of smoking, which is associated with high morbidity and mortality worldwide [1]. 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 [1]. 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 [1]. 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 [2, 3]. Besides its psychostimulant effect, nicotine induces tachycardia, vasoconstriction, bronchospasm, and contraction of both skeletal and smooth muscles by stimulating the release of acetylcholine [1] (**Figure 1.**).

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 [4]. 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 [4]. 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 [1, 5]. Based on their primary sites of expression, nAChRs are classified into two subtypes: muscle-type nicotinic receptors found in neuromuscular junctions and neuronal-type nicotinic receptors found on neuronal cell bodies and nerve terminals [4]. In the brain, there are nine isoforms of the neuronal α-subunit (α2–α10) and three isoforms of the β-subunit (β2–β4). These receptors are assembled as either heteropentamers comprising two α and three β subunits, or as homopentamers composed of five α7 subunits. Each configuration exhibits distinct pharmacological properties, particularly with respect to nicotine sensitivity and desensitisation rate [1, 5]. The most abundant neuronal nAChRs are α4β2, α3β4 and α7 located both pre- and postsynaptically, whereby they can influence the release of other neurotransmitters, such as DA, glutamate, and gamma-aminobutyric acid (GABA) [1, 5] (**Figure 2.**).

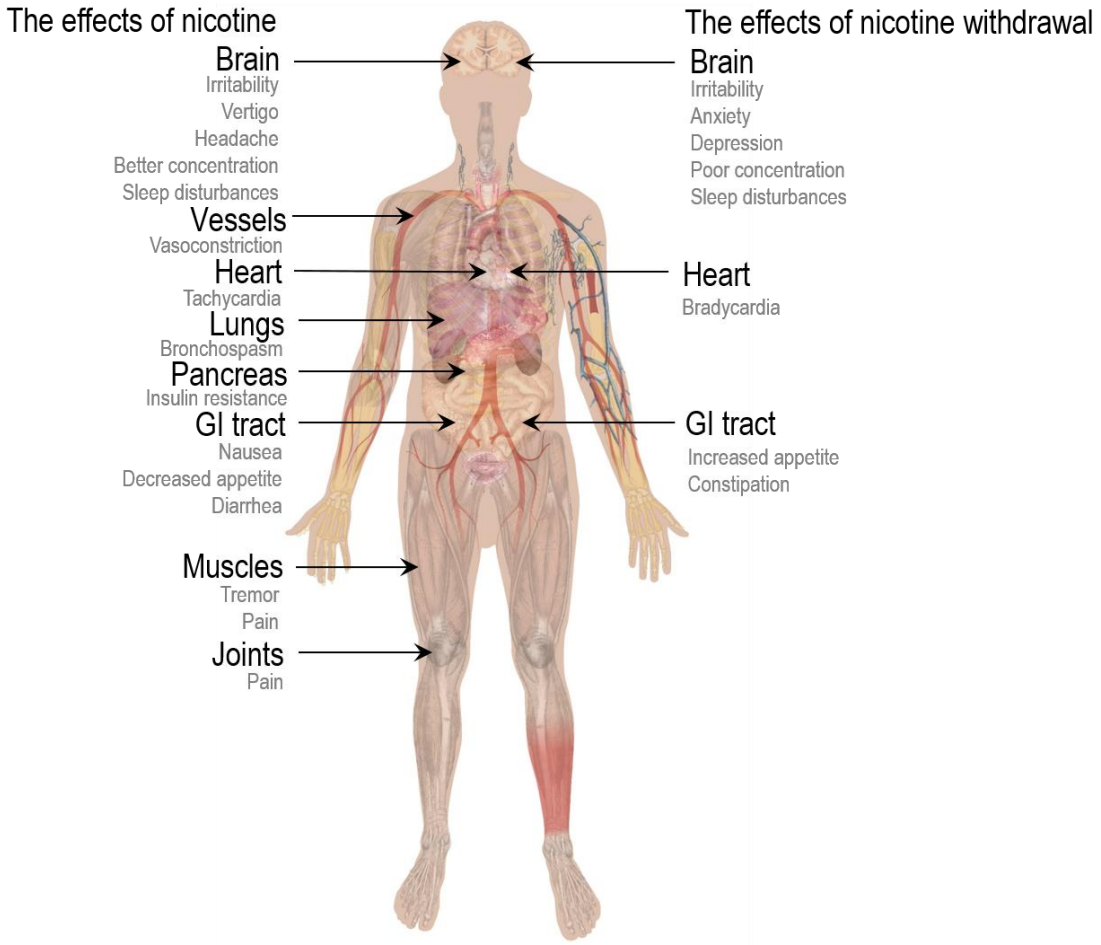


Figure 1. The effects of nicotine and nicotine withdrawal on the human body

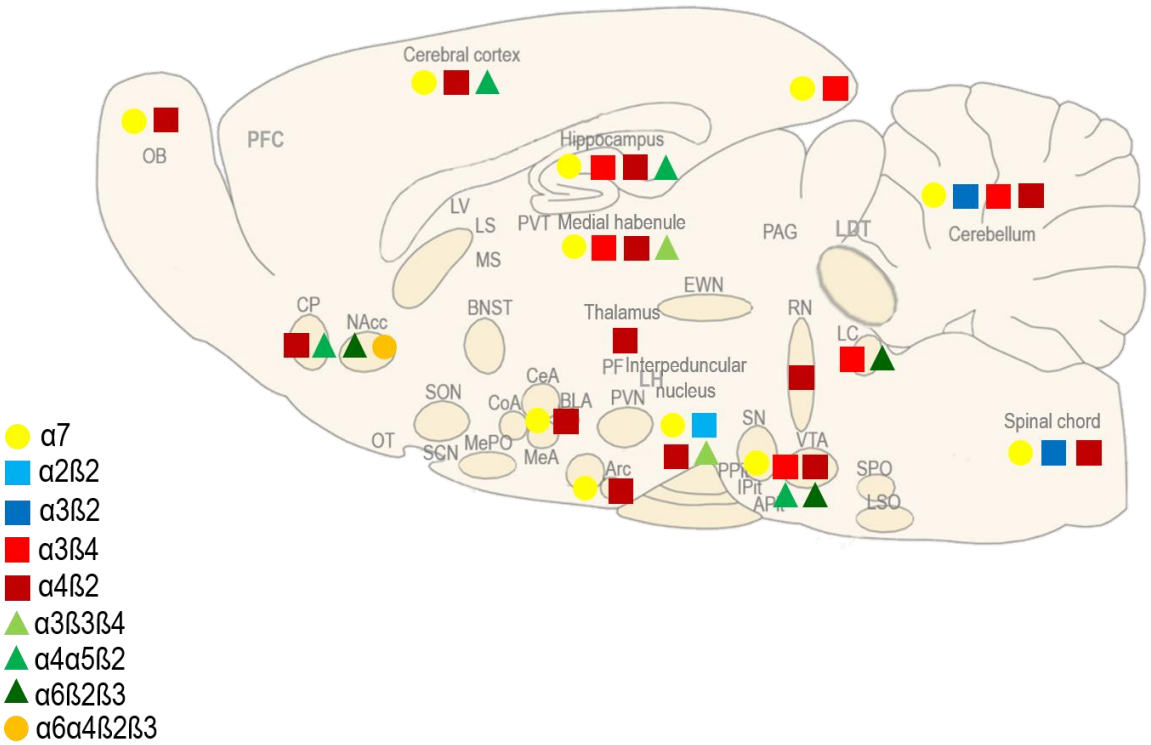


Figure 2. The sites of nAChR expression in the rat brain

Tobacco addiction, also known as nicotine dependence, is the most common type of substance dependence globally, causing more than seven million deaths each year [1]. Nicotine dependence is characterized by a compulsion to seek out and consume nicotine, a loss of control over intake, and the emergence of a negative emotional state when access to the drug is prevented [6]. 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” [7]. 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 (6 or more criteria) [8]. 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) [6].

Acute nicotine administration increases the release of DA in the midbrain, which is associated with the sensation of reward, euphoria, and locomotor hyperactivity in rats [9, 10]. The pursuit of reward and euphoria represents a positive reinforcement that leads to nicotine addiction [11]. Chronic nicotine administration further elevates midbrain DA release, although nicotine’s impact depends upon the dose and the schedule of administration [12, 13]. Usually, continuous infusion of nicotine several times a day may induce tolerance, while repetitive injection once a day may produce behavioural sensitisation to the effects of nicotine [14, 15]. Nicotine also enhances glutamate release, which stimulates further DA liberation, while simultaneously increasing GABA release, which inhibits DA secretion. With long-term exposure to nicotine, some nAChRs become desensitised, but others do not [4, 16]. As a result, GABAergic inhibitory tone diminishes, while glutamatergic excitation persists, leading to increased excitation of dopaminergic neurons and enhanced responsiveness to nicotine [4, 16]. This binge/intoxication stage is mediated by the nigrostriatal and mesolimbic pathways, [14, 15]. The nigrostriatal pathway arises from dopaminergic neurons in the substantia nigra (SN) and projects to the caudate-putamen (CP), together forming the dorsal striatum. This pathway modulates motor behaviour and posture, contributing to the learning of motor programmes and habits [14, 15]. The mesolimbic pathway arises from dopaminergic neurons in the VTA and terminates in the nucleus accumbens (NAcc), a major component of the ventral striatum. These VTA neurons also project to the amygdala and prefrontal cortex, mediating reward sensation, emotion, and motivation [14, 15].

Classically, the nigrostriatal pathway controls motor behaviour, posture, and learning of motor programmes and habits, whereas the mesolimbic pathway contributes to motor behaviour by mediating reward, emotion, and motivation [17]. Nevertheless, manipulations of DA release

in the dorsal and ventral striatum affect motor behaviour in distinct but parallel ways, which depend on the nature of the cortical and limbic input to these brain structures [17]. Acute withdrawal following chronic nicotine administration causes a nicotine withdrawal syndrome that starts within a few hours, peaks at around 24 hours, and lasts a few days after the cessation of chronic nicotine intake [18]. The nicotine withdrawal syndrome in rats consists of a somatic component, characterized by locomotor hypoactivity, increased appetite, and weight gain, and an affective component, characterized by anxiety, depression, and reward deficit [19]. The somatic symptoms in humans include bradycardia, gastrointestinal discomfort, and increased appetite; in rodents they correspond to locomotor hypoactivity, increased appetite, weight gain, and stereotypic behaviour [1, 5, 19]. The affective symptoms in humans include dysphoria, anxiety, depression, irritability, and difficulty concentrating; in rodents they correlate with anhedonia, anxiety- and depression-like behaviour [1, 5, 19]**.** This stage of withdrawal/negative affect is mediated by the extended amygdala, a functional unit consisting of three anatomically distinct structures: the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST) and the shell of the NAcc [11]. The extended amygdala can be regarded as an interface between stress systems and reward systems, the activation of which produces a negative emotional state mediated by corticotropin-releasing factor (CRF), norepinephrine (NE), and dynorphin [11]. The avoidance of the dysphoria, anxiety, and depression represents a negative reinforcement that maintains nicotine addiction [11]. Some of the affective symptoms, such as anxiety and depression, may persist during chronic nicotine withdrawal, leading to the third stage of nicotine addiction, characterized by craving [11].

The craving for drug use is hypothesized to play a key role in relapse in humans. It contributes to the characterization of addiction as a "chronic relapsing disorder" [11]. Exposure to stressors is one of the primary triggers of nicotine relapse, involving the activation of anti-reward and stress systems for months or even years after cessation [20, 21]. Nevertheless, the sensation of craving does not always align with relapse and is challenging to measure clinically [11]. The key brain regions implicated in this final stage include the basolateral amygdala, which mediates conditioned reinforcement, and the hippocampus, which processes contextual information [11]. Executive control during this phase relies on the prefrontal cortex, integrating signals from the orbital, medial, and cingulate cortices that collectively mediate the subjective experience of craving [11]. The primary neurotransmitter involved in the preoccupation/anticipation phase is glutamate [11].

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 [4]. As mentioned before, nAChRs can be classified into two major subtypes: muscle-type nAChRs, found at neuromuscular junctions, and neuronal-type nAChRs [4]. The psychoactive effects of nicotine are mediated by neuronal-type nAChRs – pentameric ligand-gated ion channels assembled from specific combinations of α (α2–α10) and β (β2–β4) subunits [4]. Rewarding and euphoric effects are mediated chiefly by high-affinity α4β2 and α6β2 receptors, often incorporating an accessory α5 subunit, which are enriched along the mesolimbic and nigrostriatal dopaminergic pathways. By contrast, affective (emotional) withdrawal signs are linked to α6β2 receptors within basal ganglia circuits, whereas somatic withdrawal signs are mediated predominantly by α3β4 receptors – frequently containing α5 or α7 subunits - localised in the medial habenula–interpeduncular nucleus pathway. 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 [22-24] (**Figure 3.**).

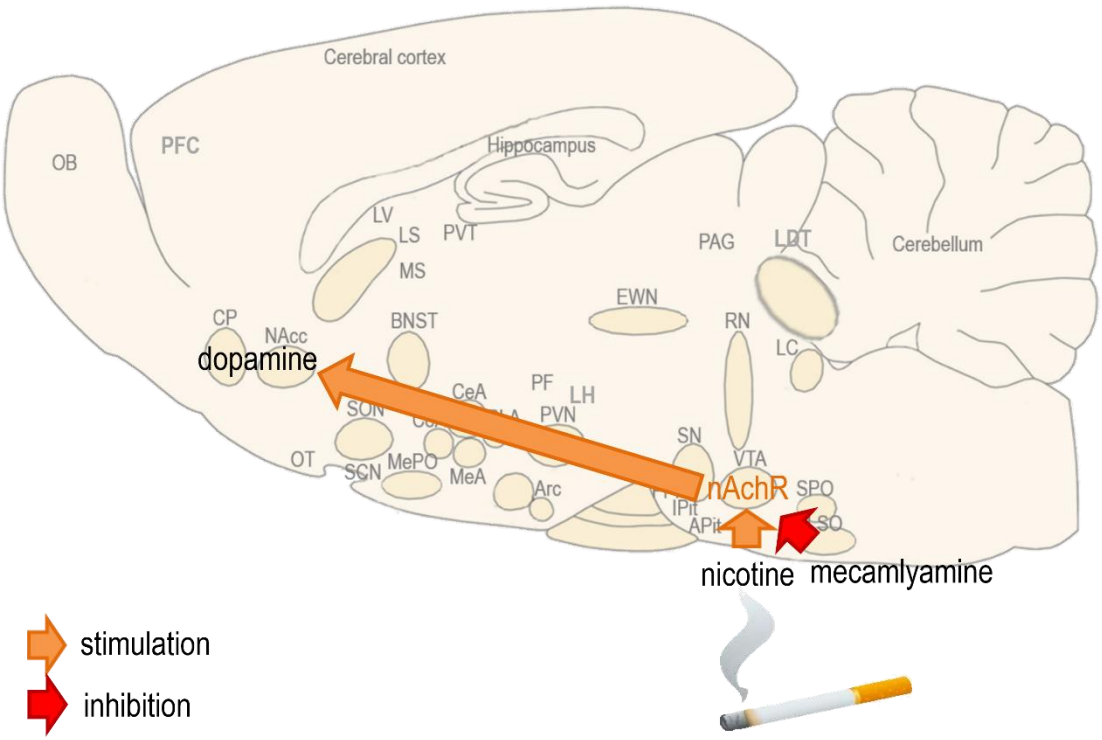


Figure 3. The sites and mechanism of action of nicotine and mecamylamine in the brain

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 [25]. 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" [26]. 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) [27] (**Figure 4**.). Ghrelin is derived from preproghrelin, a 117-amino-acid peptide that undergoes proteolytic processing to yield two functionally different peptides with highly conserved sequences among mammals, namely ghrelin and obestatin [28]. Native ghrelin undergoes further modification (e.g., acylation by the enzyme ghrelin-O-acyl-transferase). This acylated ghrelin is the biologically active peptide [28]. The other form, unacylated ghrelin, constitutes over 90% of plasma ghrelin; however, its biological role remains unclear [28]. 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 [29, 30]. 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 [29, 30]. Moreover, simultaneous administration of GHRP-6 and insulin increases the GH response [31]. However, consuming carbohydrates, dietary fats, or both around the time of GHRP-6 administration significantly blunts GH release [31]. In addition, administration of GHRP-6 in rodents induces significant changes in body composition, muscle growth, glucose metabolism, memory, and cardiac function [32].

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 [33]. 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 [34]. It can be found both in the CNS and the periphery [34]. In the CNS, the ghrelin receptor is expressed in regions where fenestrated capillaries are present, such as the arcuate nucleus (Arc) 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 [35] (**Figure 5.**).

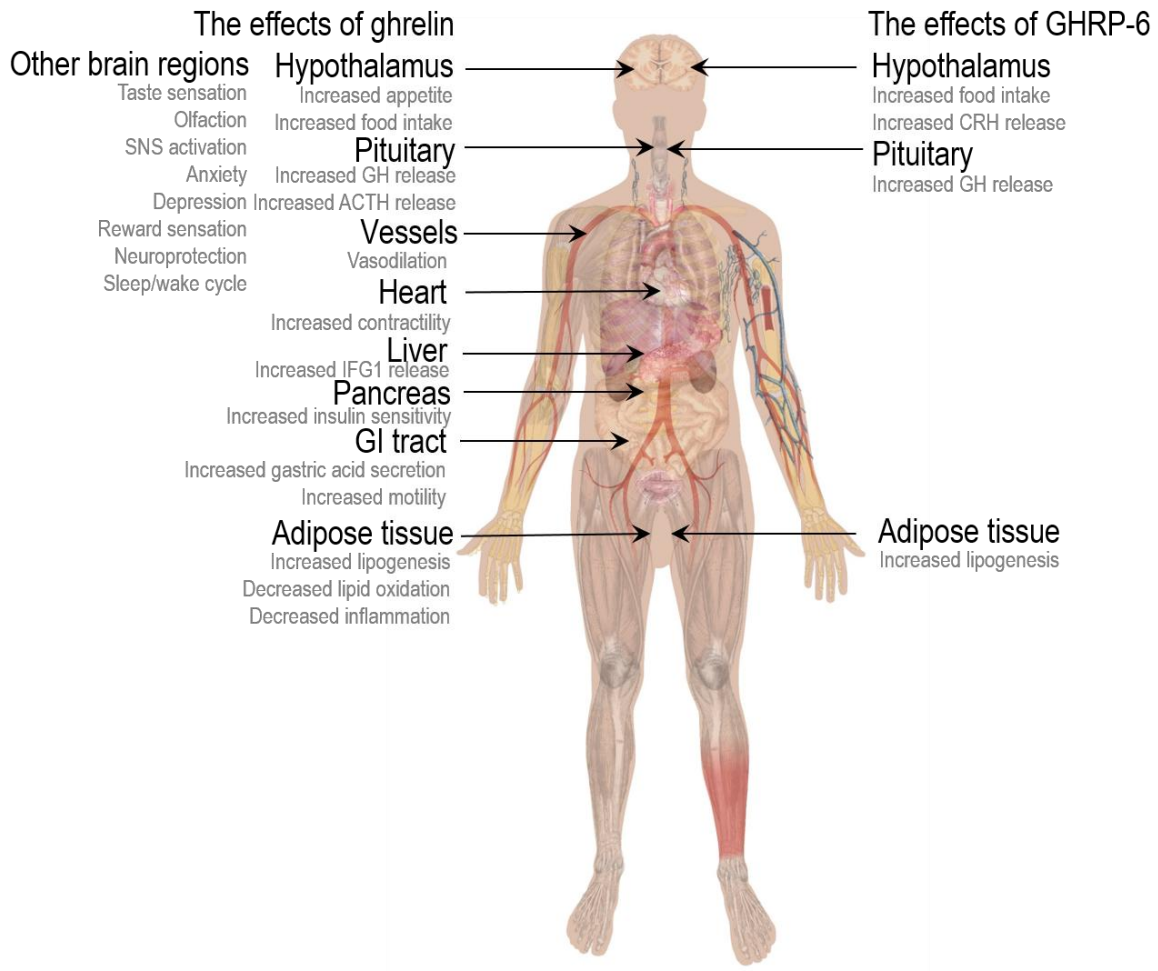


Figure 4. The effects of ghrelin and GHRP-6 on the human body

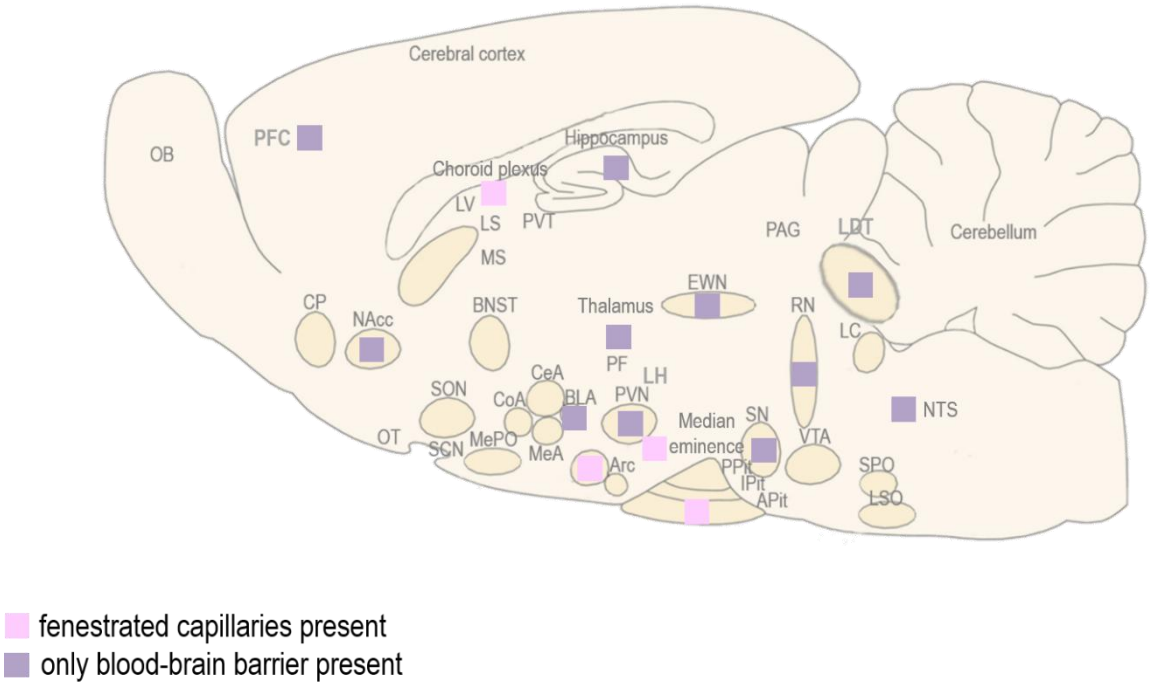


Figure 5. The sites of ghrelin receptor expression in the rat brain

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 [27]. Ghrelin, released from the empty stomach, enters the circulation and crosses the BBB to reach the Arc of the hypothalamus. There, it activates orexigenic mediators such as neuropeptide Y (NPY) and agouti-related peptide (AgRP), which subsequently stimulate downstream regulators of appetite, including orexin and melanin-concentrating hormone (MCH). Notably, exogenous GHRP-6 can further augment or inhibit this food intake signalling [35]. Several reports suggest that ghrelin may also be produced in the brain [36]. It is well established that peripherally produced ghrelin communicates with the CNS both directly, by crossing the BBB, and indirectly, by stimulating the vagus nerve [37-40] (**Figure 6.**).

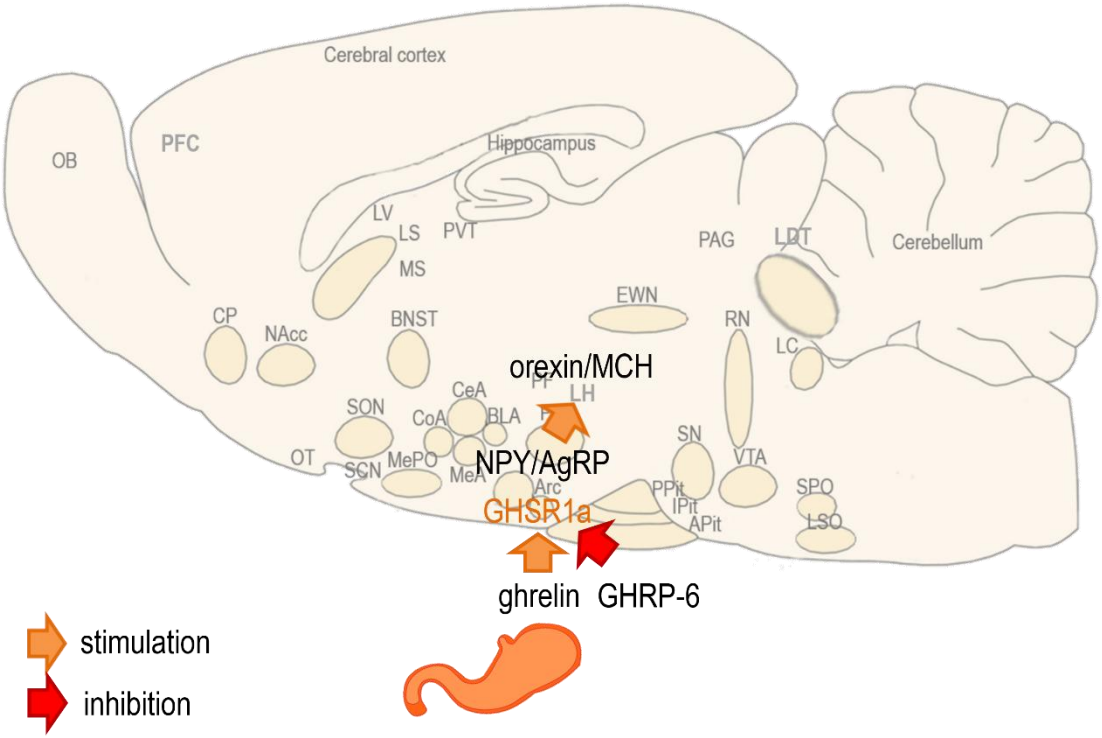


Figure 6. The sites and mechanism of action of ghrelin and GHRP-6 in the brain

Besides its well-known orexigenic role, animal studies have shown that ghrelin modulates neuroendocrine and behavioural responses to stress, and stress-induced disorders such as anxiety and depression [41, 42]. Central administration of ghrelin induced anxiety-like behaviour in mice tested in the elevated plus-maze [43] and in rats examined in the open-field test [44]. This anxiety-like effect was inhibited by the administration of a non-selective CRF antagonist [43, 44]. The anxiogenic response appears to result from CRF release within the

paraventricular nucleus (PVN), which activates the hypothalamic-pituitary-adrenal (HPA) axis, as evidenced by increased hypothalamic CRF expression and elevated plasma corticosterone concentrations [43]. This was also supported by the observation that administration of ghrelin directly into different hypothalamic areas, including the Arc and PVN, induced anxiogenic behaviour in mice tested in the elevated plus-maze [45]. A direct pituitary action of ghrelin that stimulates adrenocorticotropic hormone (ACTH) release, and thus corticosterone secretion, cannot however be ruled out [46-48]. Therefore, ghrelin may stimulate the activity of the HPA axis through the hypothalamus or pituitary, but probably independently of the orexigenic signals regulating appetite and food intake [43, 44, 46-48]. Regarding the role of ghrelin in anxiety, administration of ghrelin or knockout/overexpression of the ghrelin receptor led to contradictory results [43, 44, 49-51], but taken together, human and animal studies suggest that during acute stress ghrelin produces an anxiolytic effect, while in unstressed or chronically stressed animals it induces an anxiogenic effect [52]. Concerning ghrelin's role in depressive disorders, human studies are also somewhat inconsistent, because decreased, normal, and elevated ghrelin levels have been found in the blood of patients treated for depression [53]. However, ghrelin has been shown to exert an antidepressant effect in animal models of depression, including rats and mice [54-56].

Animal studies with GHRP-6 suggest that this compound acts primarily on the pituitary gland and is highly specific for GH release [57]. However, in conscious animals, responses to GH-releasing peptides are complex and can best be explained by effects exerted at both pituitary and hypothalamic sites, interacting with both GRF and somatostatin [58-60]. Furthermore, when more recently developed GHRP analogues were tested in humans, small but consistent elevations in ACTH and cortisol were observed [61, 62]. The clinical significance of these modest cortisol rises is unclear, although they are consistently seen with a variety of ghrelin analogues and in different species [63]. Overall, human data demonstrate that GHRP-6 is capable of modulating GH and ACTH secretion, as well as sleep, but the effects depend on dosage and duration of treatment [64, 65]. GHRP-6 probably acts within the hypothalamus to release an ACTH secretagogue, such as CRF. It is now well established that a key action of GHRPs is mediated by the hypothalamic release of CRF, which then potentiates GHRP signalling at the level of the pituitary somatotroph to promote GH secretion. [57, 59]. The ACTH-releasing activity has been demonstrated for all classes of GH-releasing peptides reported to date, and these activities are probably mediated via the ghrelin receptor, which could be expressed in the parvocellular region of the PVN, the site of the cell bodies expressing CRH and arginine vasopressin (AVP) [66]. Nevertheless, the effect of GHRP-6 may not be exerted

directly on CRH cell bodies, because GHRP-6-induced Fos protein expression was detected in the Arc but not in the PVN. [67]. However, many of the arcuate cells activated by GHRP-6 are NPY-positive, and the PVN receives a major projection from arcuate NPY neurons [68, 69]. It is, therefore, conceivable that the action of GHRP-6 on NPY cells in the Arc could activate the paraventricular CRH neurons.

Beyond its effects on the HPA axis, GHRP-6 may also affect mood disorders such as anxiety and depression. Previous studies reported that acute ghrelin administration alleviated depressive symptoms associated with chronic stress [70]. In one of these studies, chronic peripheral administration of ghrelin (5 nmol/kg/day for 2 weeks, i.p.) attenuated anxiety- and depression-like behaviours induced by chronic unpredictable mild stress in mice [70]. Anxiety-like behaviour of mice was assessed with the open-field and elevated plus-maze tests, whereas depression-like behaviour was evaluated with the forced swim test and sucrose-preference test [70]. Central administration of ghrelin (10 μg/rat/day for 2 weeks, icv) or GHRP-6 (a GHSR agonist, 10 μg/rat/day for 2 weeks, icv) significantly alleviated depression-like behaviour induced by chronic unpredictable mild stress in both depression-related tests [70]. Based on these animal data, both ghrelin and GHRP-6 appear to have antidepressant effects.

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 [71]. Indeed, several lines of evidence demonstrate an interaction between ghrelin and nicotine [72]. 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 [73-80]. 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 [81-83]. Moreover, studies in habitual smokers suggest that endogenous ghrelin levels correlate positively with years of smoking and may predict the risk of smoking relapse [84-86]. However, no clear association has been found between ghrelin levels and the severity of nicotine dependence, withdrawal, or craving [84, 86, 87]. Nicotine addiction usually develops through a combination of positive reinforcement from nicotine's pleasurable effects and negative reinforcement from the relief of the aversive effects of stress induced by nicotine withdrawal. Therefore, these studies suggest that ghrelin and GHRP-6 may contribute to the development and maintenance of nicotine addiction. Ghrelin's interaction with nicotine is well documented in the VTA and striatum, yet its effects within the BNST and on withdrawal-related behaviours are uncertain. This thesis investigates these unexplored aspects.

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. Below, we describe the methods for each in detail.

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 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. [88]. In previous experiments, we used only male Wistar rats because at that time we believed that the behaviour of females would be affected by too many variables, including hormonal fluctuations associated with the female reproductive cycle [89, 90]. However, a meta-analysis demonstrated that female rats were not more variable than male rats in terms of behavioural, electrophysiological, neurochemical, and histological measures at any stage of the oestrous cycle [91]. Therefore, in the present experiments, both male and female rats were included, and the stage of the female reproductive cycle was not considered. Nevertheless, the effects of chronic nicotine treatment and acute nicotine withdrawal on locomotion could be influenced by many factors, including sex, strain, age, and housing conditions [92-96]. In general, female animals are less sensitive to the effects of nicotine but more sensitive to the impacts of acute nicotine withdrawal than males [89, 91, 92, 94, 97]. Furthermore, different strains of rats react differently to nicotine: when Long-Evans and Sprague-Dawley rats were compared, horizontal activity increased more in Long-Evans females, whereas vertical activity remained unchanged in Sprague-Dawley males [92]. In addition, adolescent rats exhibit increased sensitivity to the positive, rewarding effects of nicotine and decreased sensitivity to the negative, aversive effects of nicotine withdrawal, which may contribute to the higher risk of developing nicotine addiction in adolescents than in adults [96, 98]. Moreover, the effects of nicotine on locomotion also appear to be influenced by housing conditions [93]. For instance, in male rats exposed to chronic nicotine, horizontal and vertical activity increased when they were housed together, but decreased when housed individually [93]. In contrast, chronic nicotine treatment did not induce any change in the horizontal or vertical activity of female rats relative to controls [93]. The influence of housing conditions was even more pronounced during acute nicotine withdrawal, at least in males [93].

The second aim of this work was to investigate the effects of ghrelin and GHRP-6 on DA release in the BNST, which could mediate the changes in locomotor activity observed during chronic nicotine treatment and acute nicotine withdrawal. Regarding the interaction between ghrelin and nicotine, previous studies have mainly focused on their actions in typical dopaminergic areas, such as the VTA, SN, striatum, and amygdala, where ghrelin and nicotine have similar stimulatory effects on the extracellular DA output [75, 76, 99-105]. For example, 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 [75, 76]. However, less attention has been paid to the actions of ghrelin and nicotine in the BNST [106-109]. The BNST was first described a century ago as a band or ridge of gray matter lying medial to the caudate nucleus [109]. Anatomically, it is located ventral to the septum, above and below the anterior commissure, and anterior to the hypothalamus. It is enclosed by the globus pallidus of the basal ganglia on both sides. The BNST can be divided into anterior and posterior divisions, comprising up to 18 subregions [110]. The anterior division includes the anterolateral, anteromedial, oval, fusiform, juxtacapsular, rhomboid, dorsomedial, ventral, and magnocellular nuclei [110]. The posterior division of the BNST comprises the principal, the interfascicular, and the transverse nuclei. Despite its small size in anatomical preparations, the BNST has been postulated to play a key role in physiological and pathological processes, including stress, anxiety, and depression [110]. Functionally, the BNST is an important relay station in the extended amygdala circuit that consists of the CeA, the BNST, and the shell of the NAcc; the extended amygdala, including the BNST, is considered an interface between reward and stress systems, in which DA signalling could be implicated [111]. 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. The results of our previous *in vitro* superfusion experiments investigating DA release in the rat amygdala and striatum will also be presented and discussed in the thesis.

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 [112]. 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.

Rats received intraperitoneal (ip) injections of 2 mg/kg nicotine (Sigma-Aldrich Inc., St. Louis, USA) or 0.9% saline (B. Braun Inc., Melsungen, Germany) twice daily at 08:00 and 20:00 h for 7 days. 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 [16]. In parallel, 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 [113, 114]. The main apparatus is a square open-field black box with a side length of 60 cm, surrounded by a 40-cm-high wall and illuminated by a 60 W light bulb positioned 1 m above the arena floor of the box. The arena

floor is divided into 36 (6×6) small squares. Each animal was carried to the experimental room in its home cage and placed in the centre of the arena, where it was allowed to acclimatise for 5 min. Then, 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 box was cleaned between sessions with 96% ethanol (Reanal Ltd., Budapest, Hungary).

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. They were housed together and kept in their home cages 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*. All rats were decapitated at the end of the experiments. Every effort was made to minimise the number of animals used and to reduce suffering.

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 [115].

After the rats were decapitated, their brains were rapidly removed and dissected in a Petri dish filled with ice-cold Krebs solution. The BNST was isolated according to the stereotaxic atlas of the rat brain [116]. The stereotaxic coordinates were anteroposterior = + 7.8

mm from the interaural line, lateral = 4 mm from the medial suture, and dorsoventral = − 5.8 mm from the skull. After extraction, the BNST was cut into 300 μm slices with a McIlwain tissue chopper.

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% CO_2 and 95% O_2 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. The doses of the agonists (nicotine and ghrelin) and antagonists (mecamylamine and GHRP-6) were based on previous *in vitro* superfusion studies [75, 76]. 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 Stata 13 (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 (**Figures 7. and 8.**). When the rats were separated into 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 in males, but not in females (**Figure 7.**).

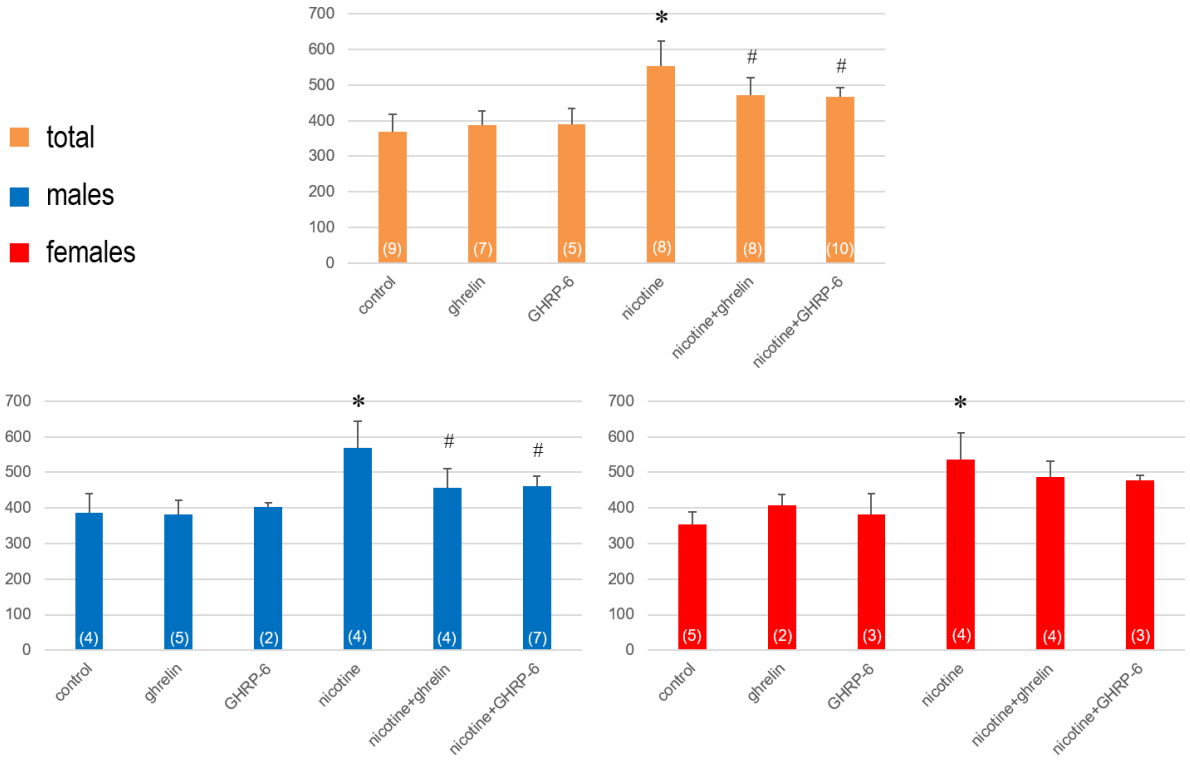


Figure 7. The horizontal activity determined on the 8th day in rats exposed to 7 days of nicotine treatment. Values are presented as means ± SEM. The numbers in parentheses represent the number of animals in each group. A difference was considered statistically significant at $p < 0.05$ and indicated with * for nicotine ip + saline icv versus (vs.) saline ip + saline icv and with # for nicotine ip + ghrelin or GHRP-6 icv vs. nicotine ip + saline icv.

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 (**Figure 8**.).

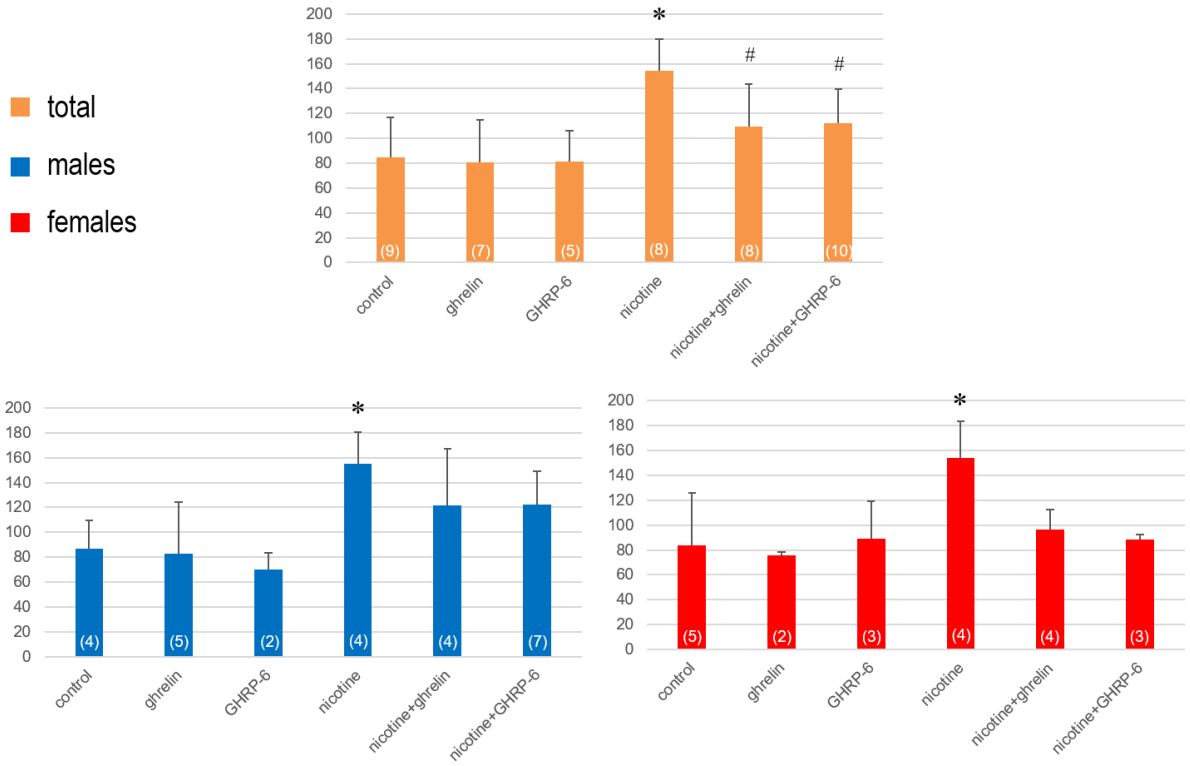


Figure 8. The vertical activity determined on the 8th day in rats exposed to 7 days of nicotine treatment. Values are presented as means ± SEM. The numbers in parentheses represent the number of animals in each group. Statistically significant difference was accepted for $p < 0.05$ and indicated with * for nicotine ip + saline icv vs. saline ip + saline icv and with # for nicotine ip + ghrelin or GHRP-6 icv vs. nicotine ip + saline icv.

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 (**Figures 9. and 10.**). 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 (**Figure 9.**).

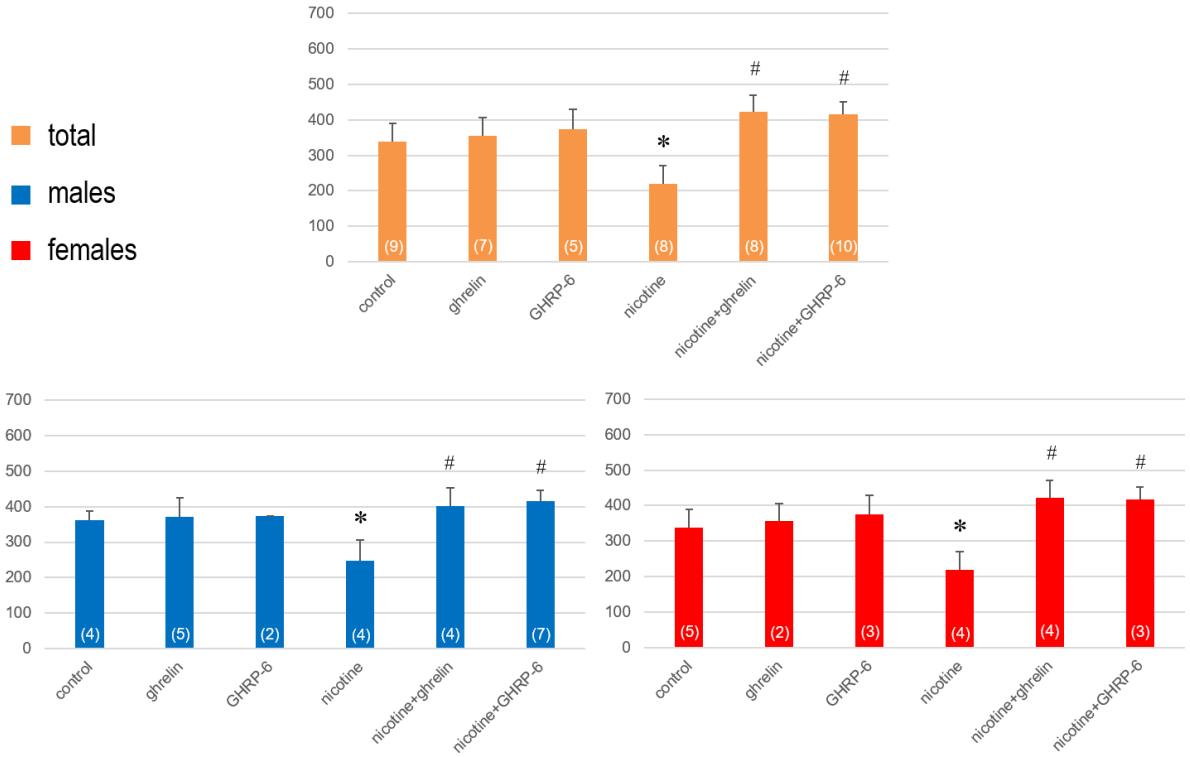


Figure 9. The horizontal activity determined on the 9th day in rats exposed to 7 days of nicotine treatment and 1 day of withdrawal. Values are presented as means ± SEM. The numbers in parentheses represent the number of animals in each group. A difference was considered statistically significant at $p < 0.05$ and indicated with * for nicotine ip + saline icv vs. saline ip + saline icv and with # for nicotine ip + ghrelin or GHRP-6 icv vs. nicotine ip + saline icv.

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 (**Figure 10.**).

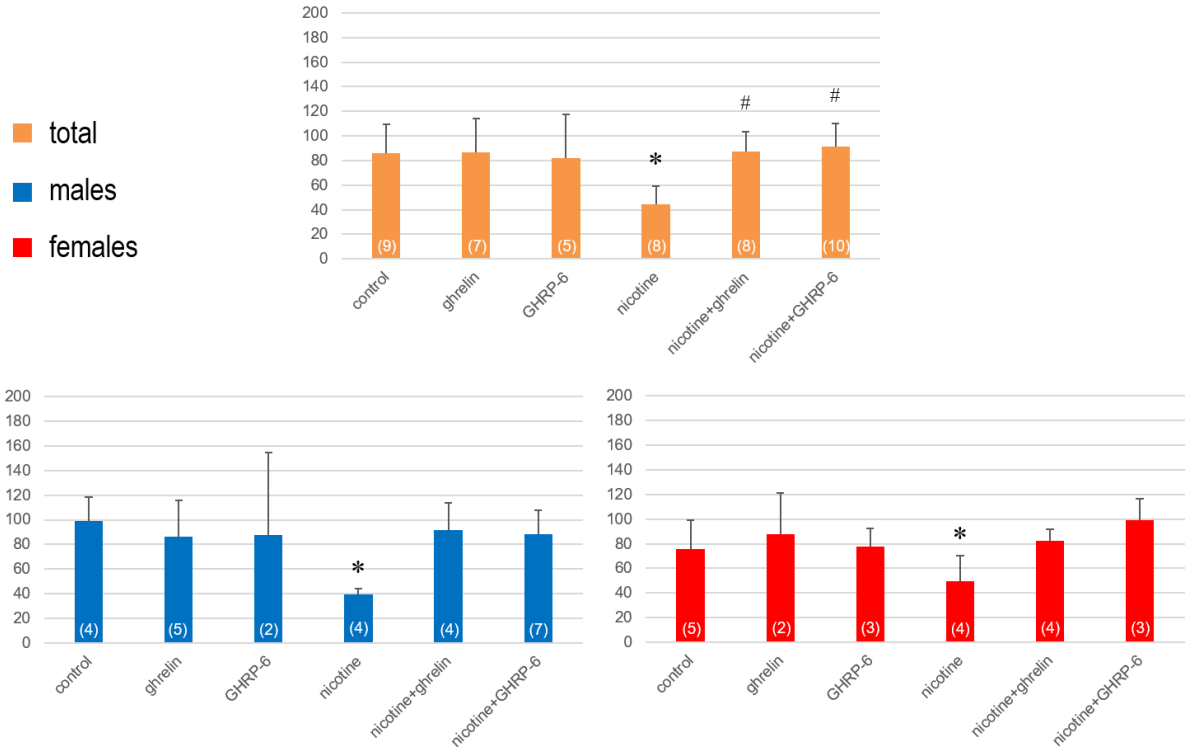


Figure 10. The vertical activity determined on the 9th day in rats exposed to 7 days of nicotine treatment and 1 day of withdrawal. Values are presented as means ± SEM. The numbers in parentheses represent the number of animals in each group. Statistically significant difference was accepted for $p < 0.05$ and indicated with * for nicotine ip + saline icv vs. saline ip + saline icv and with # for nicotine ip + ghrelin or GHRP-6 icv vs. nicotine ip + saline icv.

The statistical analysis is summarised in **Table 1**.

Parameter	**p for nicotine vs. control**	**p for nicotine + ghrelin vs. nicotine**	**p for nicotine + GHRP-6 vs. nicotine**
Horizontal	< 0.001 (T)	0.016 (T)	0.006 (T)
activity	< 0.001 (M)	0.031 (M)	0.019 (M)
after 12 hours	0.001 (F)	0.742 (F)	0.679 (F)
Vertical	0.001 (T)	0.019 (T)	0.026 (T)
activity	0.007 (M)	0.133 (M)	0.476 (M)
after 12 hours	0.002 (F)	0.062 (F)	0.342 (F)
Horizontal	< 0.001 (T)	< 0.001 (T)	< 0.001 (T)
activity	0.016 (M)	0.001 (M)	< 0.001 (M)
after 24 hours	0.002 (F)	< 0.001 (F)	< 0.001 (F)
Vertical	0.006 (T)	0.006 (T)	0.001 (T)
activity	0.036 (M)	0.084 (M)	0.075 (M)
after 24 hours	0.038 (F)	0.231 (F)	0.876 (F)

Table 1. Summary of statistical analysis of the *in vivo* results (F, female; M, male; p, probability value; T, total)

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 ($F(3,31) = 19.78$; $p < 0.001$) (**Figure 11.**).

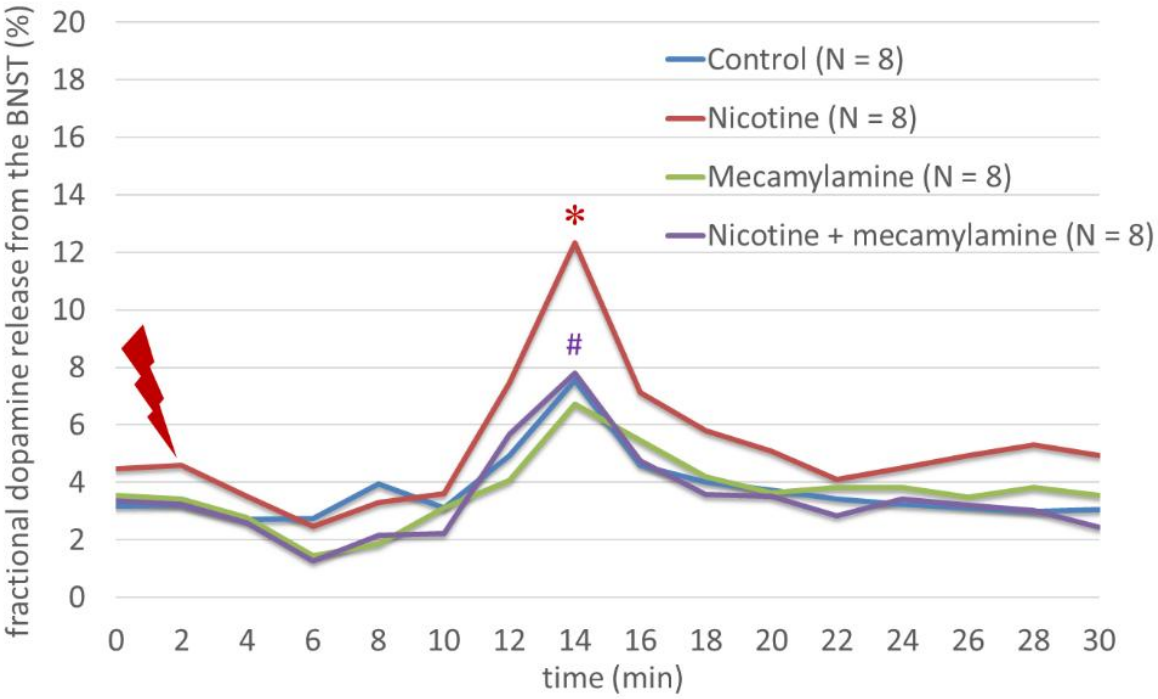


Figure 11. The effects of nicotine and mecamylamine on DA release from the BNST. Nicotine significantly increased the fractional [^{3}H]DA release from rat BNST after electrical stimulation, an effect that was significantly inhibited by mecamylamine. * indicates a statistically significant difference for agonist *vs.* control, whereas # indicates a statistically significant difference for agonist + antagonist *vs.* agonist alone.

Ghrelin increased the fractional [^{3}H]DA release even more than nicotine did ($F(3,31) = 16.58$; $p < 0.001$), and this effect was significantly inhibited by GHRP-6 ($F(3,31) = 16.58$; $p < 0.001$) (**Figure 12.**).

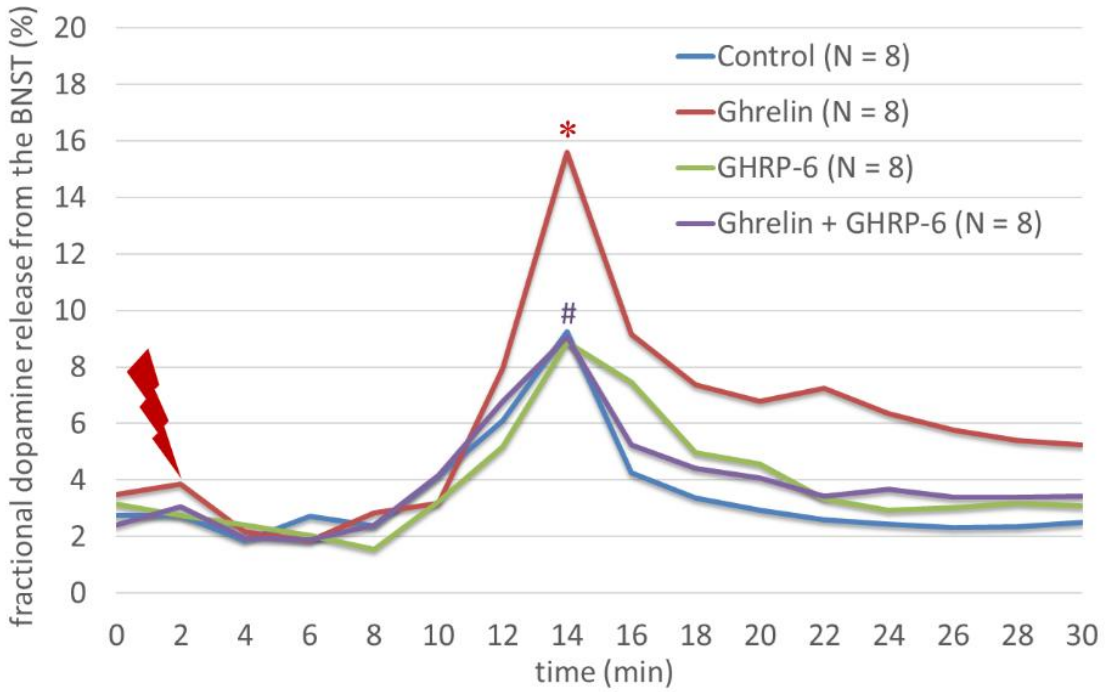


Figure 12. The effects of ghrelin and GHRP-6 on DA release from the BNST. Ghrelin significantly increased the fractional [^{3}H]DA release from rat BNST after electrical stimulation, an effect that was significantly inhibited by GHRP-6. * indicates a statistically significant difference for agonist *vs.* control, whereas # indicates a statistically significant difference for agonist + antagonist *vs.* agonist alone.

Moreover, when ghrelin and nicotine were administered together, ghrelin significantly amplified the nicotine-induced fractional [^{3}H]DA release from rat BNST after electrical stimulation ($F(3,31) = 13.19$; $p < 0.001$) (**Figure 13.**).

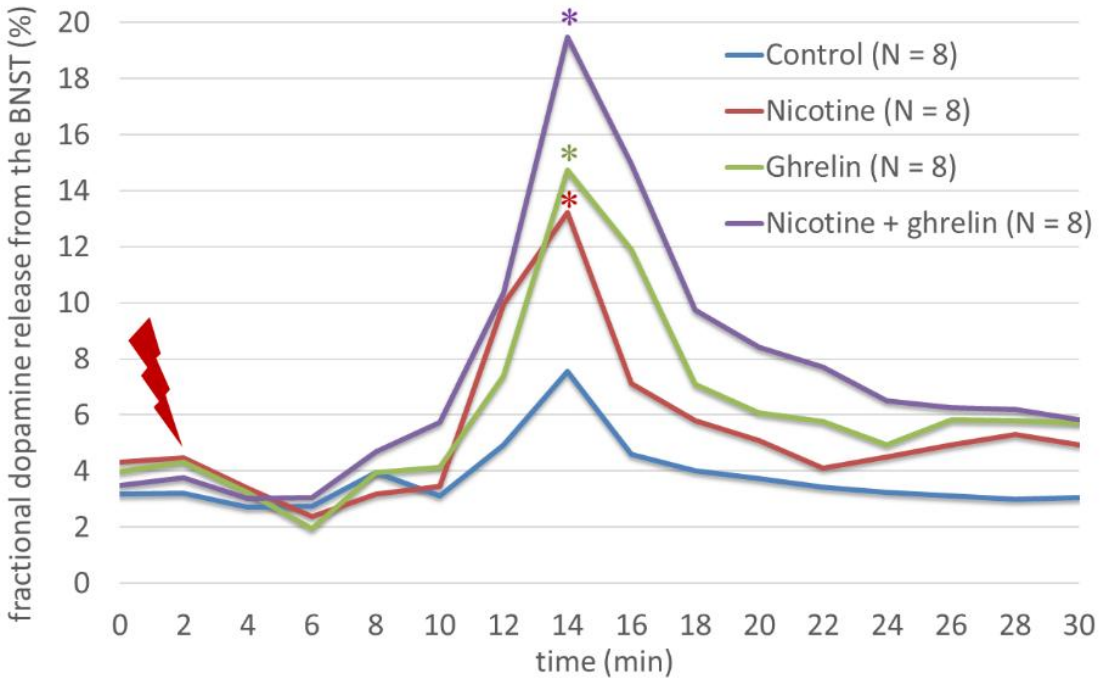


Figure 13. The effects of nicotine and ghrelin on DA release from the BNST. Ghrelin significantly amplified the nicotine-induced fractional [^{3}H]DA release from rat BNST after electrical stimulation. * indicates a statistically significant difference for agonist *vs.* control.

The additive effect of nicotine and ghrelin was partially reversed by mecamylamine ($F(3,31) = 16.58$; $p < 0.001$) and by GHRP-6 ($F(3,31) = 13.5$; $p < 0.05$) (**Figure 14.**).

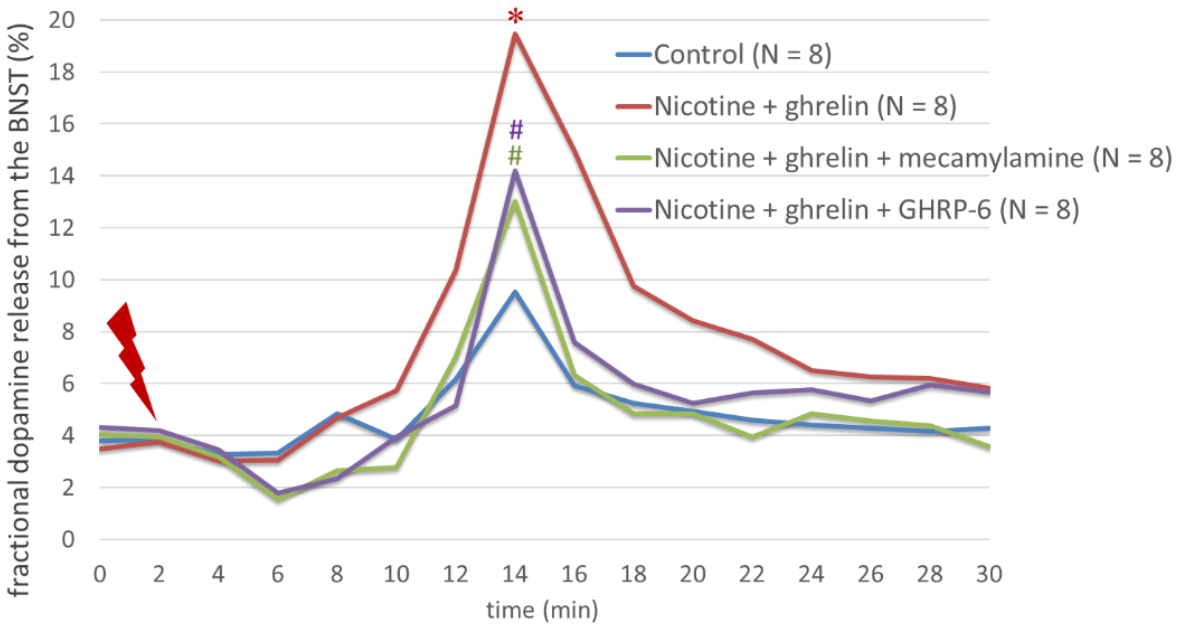


Figure 14. The effects of nicotine, ghrelin, mecamylamine, and GHRP-6 on DA release from the BNST. The additive effect of nicotine and ghrelin on the fractional [^{3}H]DA release from rat BNST after electrical stimulation was partially reversed by mecamylamine and partially by GHRP-6. * indicates a statistically significant difference for agonist *vs.* control, whereas # indicates a statistically significant difference for agonists + antagonist *vs.* agonists.

For comparison, DA release data from the striatum are also presented in Figures 15–17., summarising our previous findings that ghrelin amplifies nicotine's effects in this region.

Ghrelin significantly increased the fractional [^{3}H]DA release from rat striatum slices following electrical stimulation ($F(1,10) = 24.6409$, $p < 0.05$ for ghrelin *vs.* control). This increase was abolished by both mecamylamine ($F(1,10) = 22.0428$, $p < 0.05$ for ghrelin + mecamylamine *vs.* ghrelin) and GHRP-6 ($F(1,10) = 21.0244$, $p < 0.05$ for ghrelin + GHRP-6 *vs.* ghrelin) (**Figure 15.**).

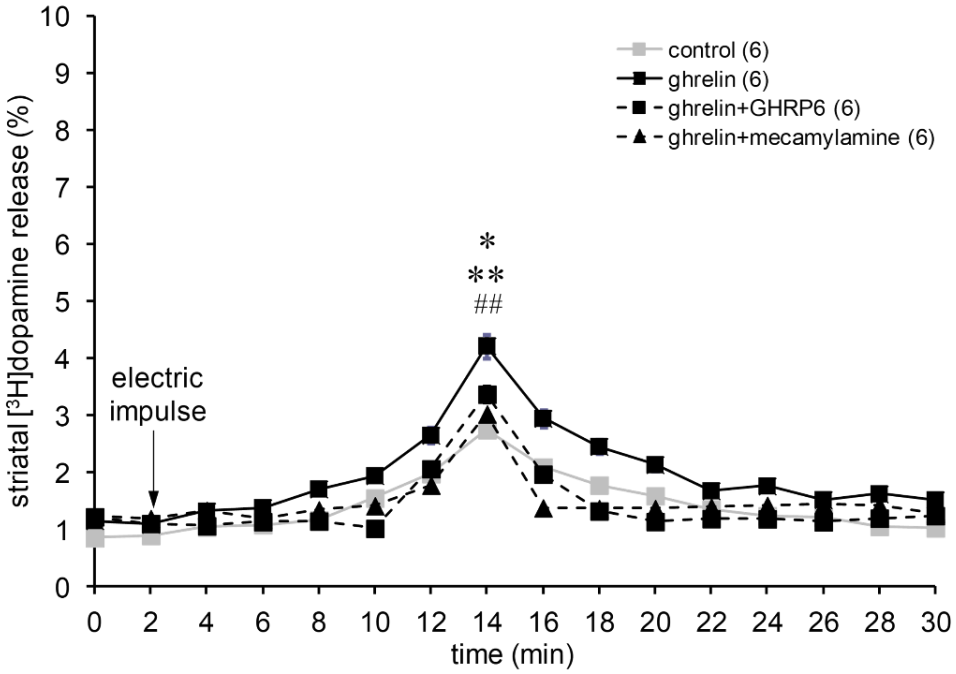


Figure 15. The effects of mecamylamine and GHRP6 on ghrelin-induced DA release in rat striatum. Ghrelin significantly increased the fractional [^{3}H]DA release from rat striatum slices following electrical stimulation. This effect was abolished by both mecamylamine and GHRP-6. Values are presented as CPM ± SEM for each 2 min sample; the numbers in brackets indicate the number of slices. *$p < 0.05$ ghrelin *vs.* control; $^{\#\#}p < 0.05$ ghrelin + mecamylamine *vs.* ghrelin alone; **$p < 0.05$ ghrelin + GHRP-6 *vs.* ghrelin alone.

Nicotine significantly enhanced the fractional [^{3}H]DA release from rat striatum slices following electrical stimulation ($F(1,10 = 36.9874$, $p < 0.05$ for nicotine *vs.* control). This effect was completely reversed by mecamylamine ($F(1,10) = 54.4473$, $p < 0.05$ for nicotine + mecamylamine *vs.* nicotine), but was not significantly affected by GHRP-6 ($F(1,10) = 10.1076$, $p = 0.3384$ for nicotine + GHRP-6 *vs.* nicotine) (**Figure 16.**).

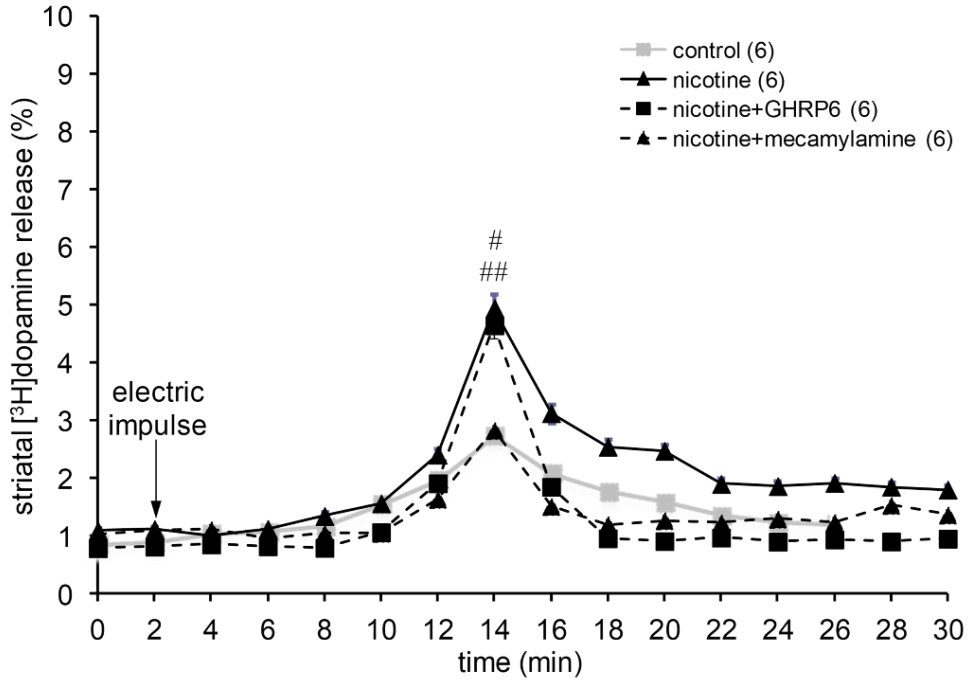


Figure 16. The effects of mecamylamine and GHRP-6 on nicotine-induced DA release in rat striatum. Nicotine significantly elevated the fractional [3^H]DA release from rat striatum slices following electrical stimulation. This effect was completely reversed by mecamylamine, whereas GHRP-6 was ineffective. Values are presented as CPM ± SEM for each 2 min sample; the numbers in brackets indicate the number of slices. $^{\#}p < 0.05$ nicotine *vs.* control; $^{\#\#}p < 0.05$ nicotine + mecamylamine *vs.* nicotine alone.

Ghrelin combined with nicotine significantly elevated the fractional [3H]DA release from rat striatum slices following electrical stimulation ($F(1,10) = 105.8094$, $p < 0.05$ for ghrelin + nicotine *vs.* control). The effect of ghrelin and nicotine in combination appeared additive compared to ghrelin or nicotine alone. The elevation of striatal DA release with combined treatment was almost completely blocked by pretreatment with mecamylamine ($F(1,10) = 63.8740$, $p < 0.05$ for ghrelin + nicotine + mecamylamine *vs.* ghrelin + nicotine) and was partially inhibited by pretreatment with GHRP-6 ($F(1,10) = 22.9018$, $p < 0.05$ for ghrelin + nicotine + GHRP-6 *vs.* ghrelin + nicotine) (**Figure 17.**).

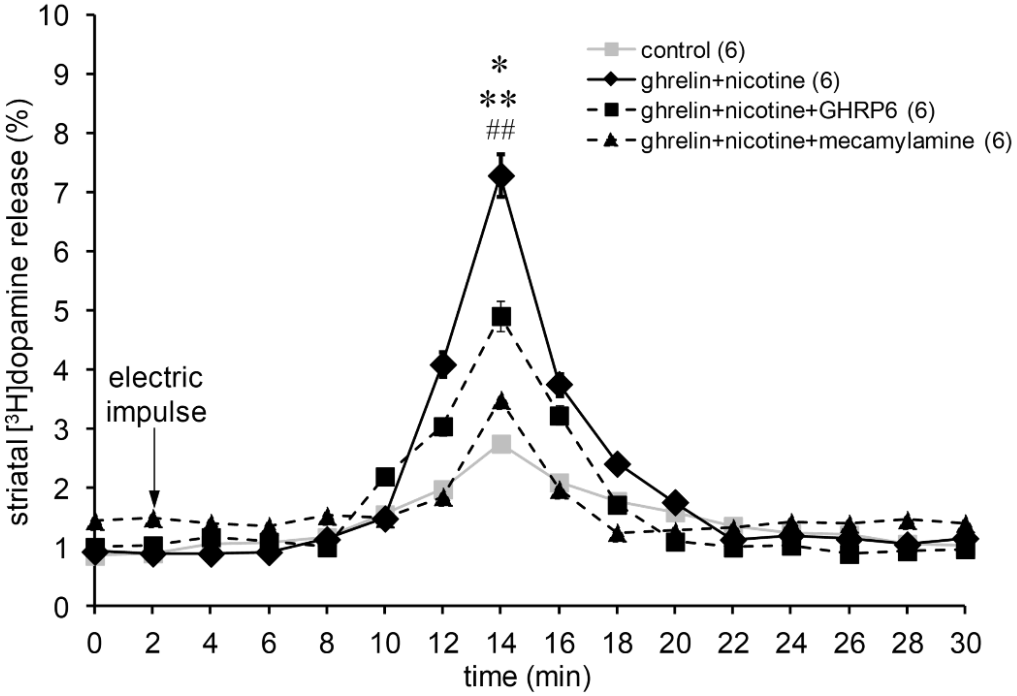


Figure 17. The effects of mecamylamine and GHRP-6 on combined ghrelin and nicotine-induced DA release in rat striatum. Co-administered ghrelin and nicotine significantly enhanced the fractional [^{3}H]DA release from rat striatum slices following electrical stimulation. This effect was almost completely inhibited by mecamylamine and was partially reversed by GHRP-6. Values are presented as CPM ± SEM for each 2 min sample; the numbers in brackets indicate the number of slices. *$p < 0.05$ ghrelin + nicotine *vs.* control; $^{\#\#}p < 0.05$ ghrelin + nicotine + mecamylamine *vs.* ghrelin + nicotine; **$p < 0.05$ ghrelin + nicotine + GHRP-6 *vs.* ghrelin + nicotine.

Ghrelin significantly increased the fractional [^{3}H]DA release from rat amygdala slices following electrical stimulation ($F(1,10) = 82.4272$, $p < 0.05$ for ghrelin *vs.* control). This effect was completely reversed by GHRP-6 ($F(1,10) = 62.1864$, $p < 0.05$ for ghrelin + GHRP-6 *vs.* ghrelin), and was partially reversed by mecamylamine ($F(1,10) = 16.0746$, $p < 0.05$ for ghrelin + mecamylamine *vs.* ghrelin) (**Figure 18.**).

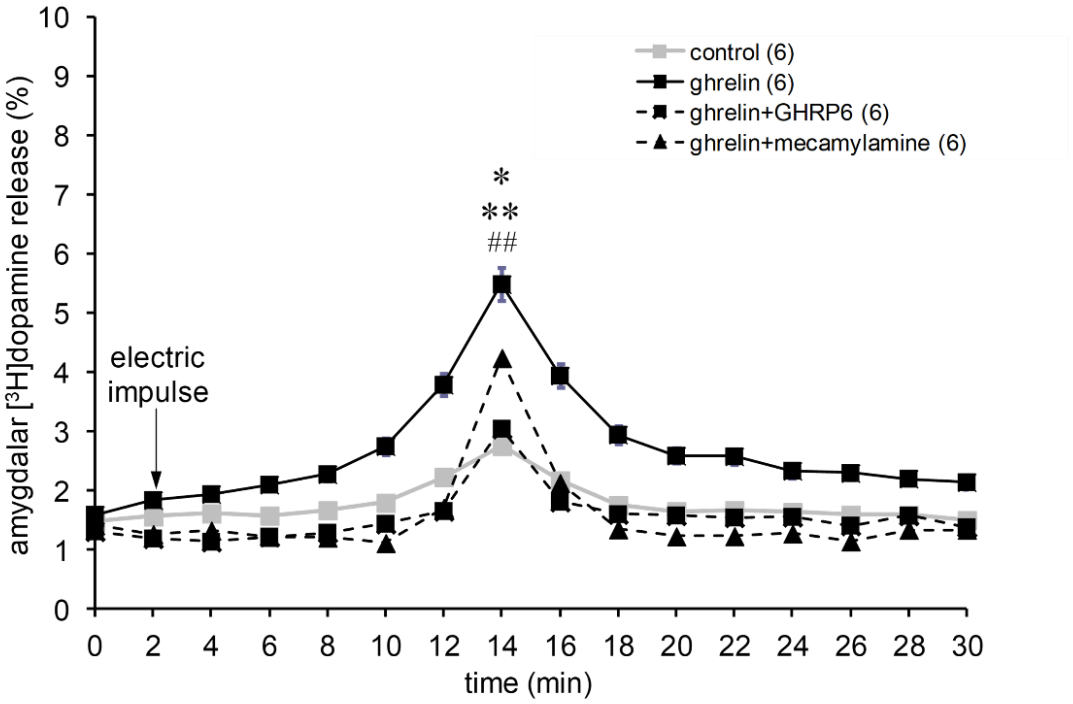


Figure 18. The effects of mecamylamine and GHRP-6 on ghrelin-induced DA release in rat amygdala. 1 μM ghrelin significantly increased the fractional [^{3}H]DA release from rat amygdala slices following electrical stimulation. This effect was completely reversed by 1 μM GHRP-6, and partially reversed by 100 μM mecamylamine. Values are presented as mean percentages ± SEM for each 2 min sample; the numbers in brackets indicate the number of slices. *$p < 0.05$ ghrelin *vs.* control; $^{\#\#}p < 0.05$ ghrelin + mecamylamine *vs.* ghrelin alone; **$p < 0.05$ ghrelin + GHRP-6 *vs.* ghrelin alone.

Nicotine also significantly increased the fractional [^{3}H]DA release from rat amygdala slices following electrical stimulation ($F(1,10) = 40.5000$, $p < 0.05$ for nicotine *vs.* control). This effect was significantly inhibited by mecamylamine ($F(1,10) = 28.5480$, $p<0.05$ for nicotine + mecamylamine *vs.* nicotine), but was not significantly affected by GHRP-6 ($F(1,10) = 1.1732$, $p = 0.3041$ for nicotine + GHRP-6 *vs.* nicotine) (**Figure 19.**).

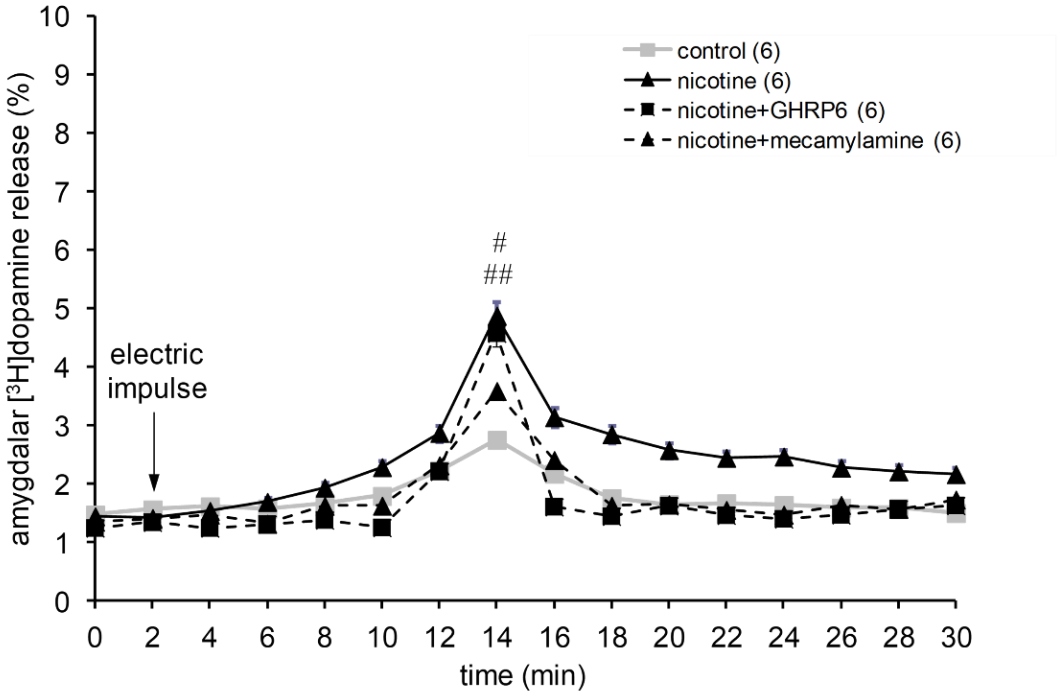


Figure 19. The effects of mecamylamine and GHRP-6 on nicotine-induced DA release in rat amygdala. 100 μM nicotine significantly enhanced the fractional [^{3}H]DA release from rat amygdala slices following electrical stimulation. This effect was inhibited by 100 μM mecamylamine, but was not significantly affected by 1 μM GHRP-6. Values are presented as mean percentages ± SEM for each 2 min sample; the numbers in brackets indicate the number of slices $^{\#}p < 0.05$ nicotine *vs.* control; $^{\#\#}p < 0.05$ nicotine + mecamylamine *vs.* nicotine alone.

Ghrelin combined with nicotine significantly elevated the fractional [^{3}H]DA release from rat amygdala slices following electrical stimulation ($F(1,10) = 54.6615$, $p<0.05$ for ghrelin + nicotine *vs.* control). Co-administration of ghrelin and nicotine produced a similar increase in amygdalar DA release to that induced by ghrelin or nicotine alone. The elevation of amygdalar DA release under combined treatment was reduced by pretreatment with either mecamylamine ($F(1,10) = 25.6499$, $p < 0.05$ for ghrelin + nicotine + mecamylamine *vs.* ghrelin + nicotine) or GHRP-6 ($F(1,10) = 20.2275$, $p < 0.05$ for ghrelin + nicotine + GHRP-6 *vs.* ghrelin+nicotine) (**Figure 20.**).

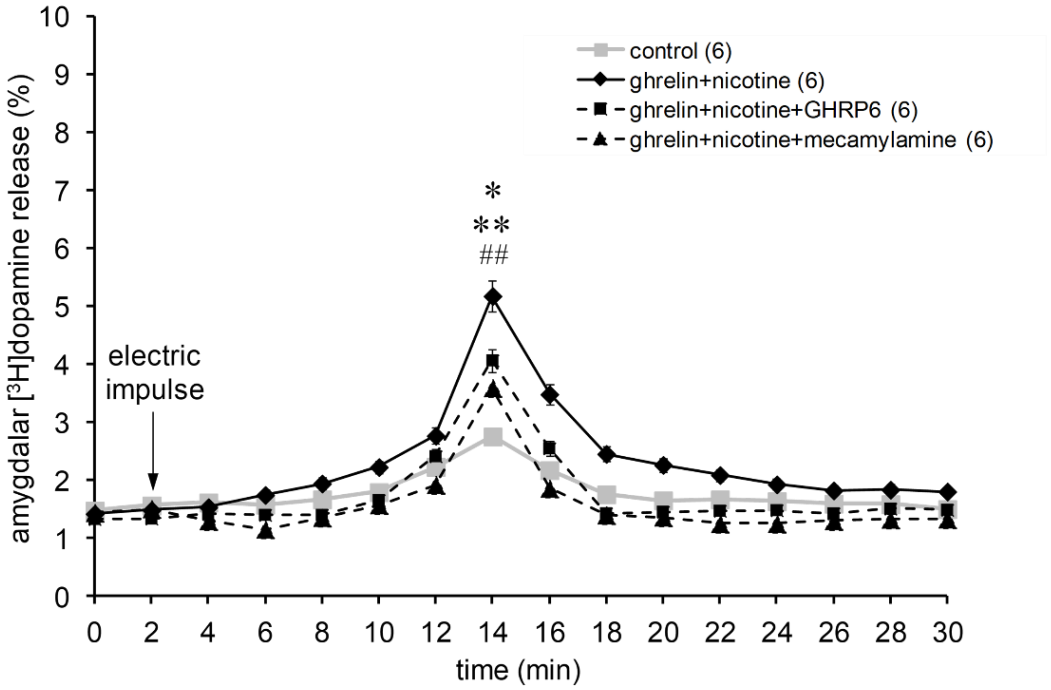


Figure 20. The effects of mecamylamine and GHRP-6 on co-administered ghrelin and nicotine-induced DA release in rat amygdala. Combined treatment with 1 μM ghrelin and 100 μM nicotine significantly elevated the fractional [^{3}H]DA release from rat amygdala slices following electrical stimulation. This effect was partially reduced by pretreatment with 100 μM mecamylamine and 1 μM GHRP-6. Values are presented as mean percentages ± SEM for each 2 min sample; the numbers in brackets indicate the number of slices. *$p < 0.05$ ghrelin + nicotine *vs.* control; $^{\#\#}p<0.05$ ghrelin + nicotine + mecamylamine *vs.* ghrelin + nicotine; **$p < 0.05$ ghrelin + nicotine + GHRP-6 *vs.* ghrelin + nicotine.

The statistical analysis is summarised in **Table 2**.

Parameter	**p for nicotine vs. control**	**p for ghrelin vs. control**	**p for nicotine + ghrelin vs. control**
DA release in the rat BNST	< 0.001	< 0.001	< 0.001
DA release in the rat striatum	< 0.05	< 0.05	< 0.05
DA release in the rat amygdala	< 0.05	< 0.05	< 0.05

Table 2. Summary of statistical analysis of the *in vitro* results (p, probability value)

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 [117]. This finding is concordant with previous studies in which locomotor hyperactivity was reported on the 4th, the 8th, and the 10th days of chronic nicotine exposure [12, 13, 88]. 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 [88, 118]. Acute administration of nicotine stimulates the release of DA in both the dorsal and ventral striatum, and this can cause reward sensation and locomotor hyperactivity in rats, as indicated by both *in vivo* and *in vitro* studies [9, 10]. For example, nicotine infused into the striatum increased DA output and locomotor activity in freely moving rats ; this increase was reduced by administration of mecamylamine [88, 119]. In addition, superfusion of nicotine to the striatum increased DA release in rats [120, 121], and this effect was reduced by administration of mecamylamine [119, 122]. During acute nicotine exposure, both the mesolimbic and nigrostriatal dopaminergic pathways are activated, but typically there is a higher DA release in the NAcc than in the CP [123-126]. This can be explained by the different expression of DA receptors (D1-type *vs.* D2-type) in the two subdivisions of the striatum, which exert distinct stimulatory and inhibitory effects on DA release [12, 127]. During chronic nicotine exposure, DA output can either increase or decrease in the striatum, leading to different behavioural outcomes depending on whether tolerance or sensitisation to nicotine develops [14, 15]. Tolerance is more likely to occur with continuous infusion of nicotine, whereas behavioural sensitisation typically develops with intermittent nicotine injection. The net effect of these competing phenomena can manifest as locomotor hyperactivity or hypoactivity [14, 15]. Furthermore, chronic stimulation of nAChRs can influence the release of other neurotransmitters, including acetylcholine, glutamate, and GABA, with diverse impacts on locomotor activity [4].

On the 9th day, horizontal and vertical activities declined in rats undergoing one day of acute nicotine withdrawal [117]. 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 [19]. The physical signs start promptly within a few hours, and peak around 24 hours following nicotine cessation [19]. The affective symptoms may start early, but can persist from days to months, resulting in chronic nicotine withdrawal characterised by craving and increased risk of relapse

[19]. Nicotine addiction develops due to a combination of the rewarding, positive actions produced by nicotine, and the avoidance of the aversive, negative effects induced by nicotine withdrawal [4]. This is reflected in our previous and current findings, which show that reward deficit and locomotor hypoactivity appear after 24 hours – but not after 12 hours – of nicotine withdrawal, along with signs of anxiety and depression. [18, 88]. Nevertheless, in the present study, we observed general hypoactivity, whereas in our previous study only vertical activity and ventral striatal DA release were decreased. In contrast, horizontal activity and dorsal striatal DA release remained elevated following one day of nicotine withdrawal [88]. This can be explained by the different abilities of the two major subtypes of nAChRs expressed in the dorsal and ventral striatum (α5 *vs.* α6 subunits) to undergo tolerance or behavioural sensitisation to nicotine, which can also be affected by daily peptide injection [123, 124].

The locomotor hyperactivity observed in nicotine-treated rats on the 8th day was significantly reduced by ghrelin and GHRP-6 [117]. 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 [29, 30]; 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 [128, 129]. Therefore, the interaction among ghrelin, nicotine, and GHRP-6 may occur in various brain regions, including the hypothalamus and cerebellum [130-133], 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 [1, 99, 100, 105, 134-136]. This observation is supported by *in vivo* studies in which ghrelin, injected peripherally or directly into the VTA, increased DA release in the NAcc, locomotor activity, and food consumption in rats [44, 137-139], and by *in vitro* studies in which ghrelin, administered locally, produced a concomitant release of acetylcholine in the LDT and DA in the NAcc [99, 100, 102-105]. The interaction between ghrelin and nicotine may also take place in the extended amygdala, a functional unit that includes anatomical regions such as the CeA, the BNST, and the NAcc shell. This speculation is based on *in vivo* studies in which ghrelin and nicotine stimulated DA release in various brain regions (including the amygdala and striatum) [88, 140-142], and on *in vitro* studies in which ghrelin and nicotine stimulated DA release in the amygdala, BNST, and striatum [74-76]. Moreover, when administered together, ghrelin amplified the nicotine-induced release of DA in the BNST and striatum, and

this effect was partially reversed by mecamylamine and partially by GHRP-6 [74, 76]. Interestingly, our BNST findings parallel our previous striatal results in that ghrelin amplifies nicotine's dopamine release, but differ in that blocking ghrelin receptors (with GHRP-6) partially reduced the nicotine and ghrelin effect in BNST yet has little effect on nicotine alone in striatum. This might indicate region-specific interactions, perhaps in the BNST, endogenous ghrelin tone is more involved in nicotine's action than in the striatum. We presume that excess DA in the ventral and dorsal striatum promotes the positive, rewarding actions of chronic nicotine administration, whereas DA deficiency in the CeA, BNST, and NAcc shell mediates the negative, aversive symptoms induced by acute nicotine withdrawal [6, 11].

In the present study, the rats were of the same strain and age, and were housed under identical conditions, so the only factor that could influence horizontal and vertical activity was sex [117]. Regarding horizontal activity, female rats were less sensitive to the locomotor effects of nicotine, and this finding has been reported by previous studies [89-91, 143-145]. Regarding vertical activity, female rats were less sensitive to the locomotor effects of nicotine, and this finding has also been reported by previous studies [92-94, 96, 146-148]. When the rats were separated into male and female groups, only horizontal activity decreased significantly during nicotine withdrawal and was reversed significantly by ghrelin and GHRP-6 treatment in both sexes. The lack of a statistically significant effect for the other locomotor parameters could be related to the relatively small sample size after dividing the rats into two groups. In humans, women exhibit a more rapid escalation from casual drug use to drug addiction, express a wider range of drug-withdrawal symptoms when drug use stops, and tend to be more vulnerable than men in terms of treatment outcome [91, 143]. In rodents, short-term oestradiol intake in female rats enhances acquisition and escalation of drug taking, motivation for addictive drugs, and relapse-like behaviours. This can be explained by sex differences in DA responses of the ventral and dorsal striatum [91, 143]. For instance, oestradiol treatment of ovariectomised female rats enhances DA release in the dorsolateral striatum but not in the NAcc, and when drug taking becomes habitual, DA release increases in the dorsolateral striatum and decreases in the NAcc [91, 143]. Therefore, the sex difference in the balance between the two dopaminergic pathways projecting to the CP and NAcc may underlie the sex differences in addiction [91, 143]. Sex differences in the CRF system regulating the HPA axis and the locus coeruleus-norepinephrine (LC-NE) arousal system may also explain why female rats are more sensitive to the effects of nicotine withdrawal, resulting in activation of both systems [89, 90]. For example, there are significant sex differences in CRF function, ranging from its presynaptic regulation to its postsynaptic efficacy, as well as differences in CRF receptor expression, distribution,

trafficking, and signalling that lead to increased reactivity to stress in females during drug withdrawal [89, 90]. In addition, there are important sex differences in the structure and function of the LC-NE system and its projections. Oestradiol administration increases the capacity for NE synthesis and decreases NE degradation, potentially increasing arousal in females during drug withdrawal [89, 90].

In addition to the relatively small sample size following separation by sex, our study may have other limitations [149]. For example, instead of repeated ip or sc injections, other animal models of nicotine dependence and withdrawal – such as continuous nicotine infusion via osmotic minipumps, oral nicotine intake (drinking), nicotine vapour exposure, or tobacco smoke exposure – could be considered more appropriate with regard to construct, face, and predictive validity [149]. When using ip injections, at least 4 days of repeated nicotine injections are required to induce dependence in mice and rats [149]. The advantage of this method is that the dose and timing of nicotine administration are well controlled and produce fluctuating plasma nicotine levels, similar to those observed in smokers who smoke at certain intervals [149]. The disadvantage of this method is that the rate of nicotine absorption from ip or sc injections is slower than when nicotine is inhaled; therefore, nicotine injections might be less rewarding than inhalation of nicotine [149]. In addition, repeated ip or icv injections of nicotine lead to accumulation of nicotine in the system and can produce toxicity [149]. Rats are less sensitive to the toxic effects of nicotine, but repeated injections with high doses of nicotine (6 mg/kg, sc; 3 injections per day for 7 days) can lead to a relatively high mortality rate, which is not observed when lower doses of nicotine (1–3 mg/kg sc, 2 injections per day for 7 days) are administered [149]. Repeated ip injections may also be a source of stress, although this was demonstrated to be mitigated by the daily handling of the animals [150]. The surgical implantation of an icv cannula and repeated icv injections may also be stressful for the animals; however, this stress was partly ameliorated by allowing a 7-day recovery period before the experiments started. To accurately model human smoking, the blood nicotine and cotinine levels in rodents should be similar to those in smokers; however, in the present study, these were not measured. We were also unable to determine the exact states of euphoria and dysphoria that occurred in rats after 7 days of nicotine treatment and 1 day of acute nicotine withdrawal. Furthermore, we assessed the effects of ghrelin and GHRP-6 only during spontaneous nicotine withdrawal; we did not examine their ability to modulate mecamylamine-precipitated withdrawal or compare them with drugs of therapeutic potential, such as bupropion and varenicline. Future studies are required to address these issues. Since in the present study, the rats were of the same strain, age, and housing conditions, the impact of these factors on

horizontal and vertical activity – and the ultimate impact of ghrelin and GHRP-6 under various conditions – were not investigated either.

Nevertheless, one of our previous studies – which included both *in vivo* and *in vitro* experiments – indicated that different doses (0.5–5 μg) of ghrelin administered icv caused significant increases in both horizontal and vertical activity monitored by the Conducta apparatus. However, only the dose of 5 μg evoked a significant increase in spontaneous locomotor activity recorded by telemetry, and this was associated with dose-dependent increases in plasma corticosterone concentration, reflecting activation of the HPA axis. The locomotor hyperactivity observed was diminished by the non-selective CRF antagonist α-helical CRF(9–41) and the non-selective DA antagonist haloperidol (which has higher affinity for D_2 receptors), suggesting that both CRF release and dopaminergic neurotransmission are involved in ghrelin-evoked locomotor responses [44]. Administration of GHRP-6 at 10 μg intravenously can also activate the HPA axis, resulting in small but significant increases in plasma concentrations of ACTH and corticosterone in rats [63]. As these secretagogues do not release ACTH directly, they probably interact with hypothalamic neurohormones regulating ACTH release (such as CRF and AVP). However, the ability of GHRP-6 to modulate dopaminergic neurotransmission, just as ghrelin does, cannot be excluded [63]. Taken together, the present and previous studies suggest that ghrelin and GHRP-6 may modulate acetylcholine release in the LDT, and consequently, the release of DA in the NAcc and CP, compensating for the excess or deficiency of DA during periods of chronic nicotine treatment and acute nicotine withdrawal, respectively (**Figure 21.**). Therefore, the ghrelin receptor may represent a new therapeutic target for nicotine dependence.

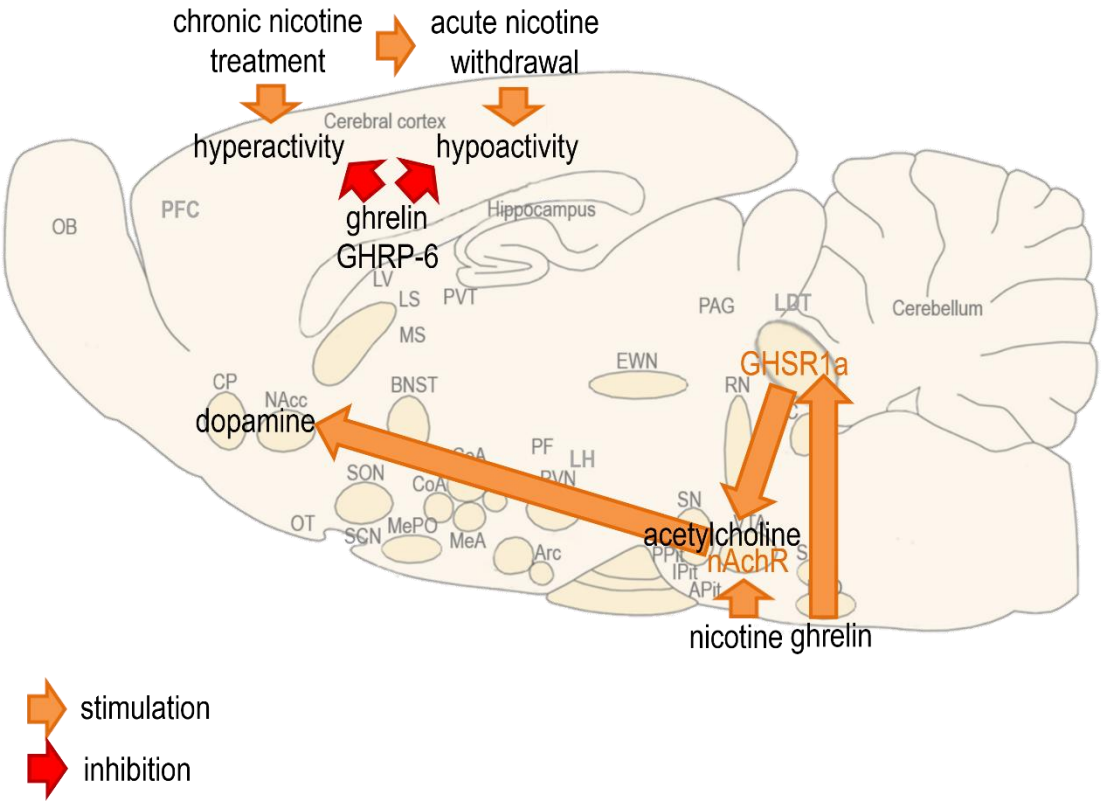


Figure 21. The possible sites and mechanism of interaction of ghrelin and nicotine in the brain

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

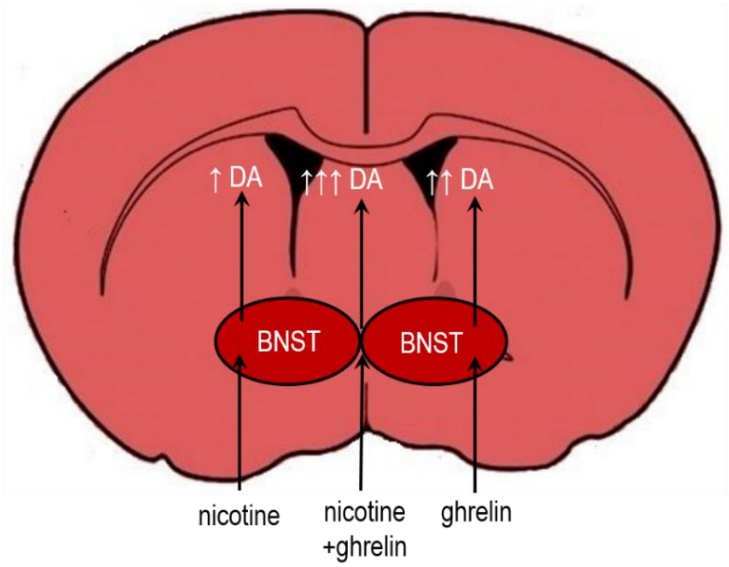
Nicotine significantly amplified the release of DA from the rat BNST after electrical stimulation, and this effect was inhibited significantly by mecamylamine, a non-selective nAChR antagonist [74]. This finding is concordant with previous *in vivo* and *in vitro* studies, which indicated that nicotine stimulates DA release in several subcortical and cortical brain regions [88, 140-142]. This stimulatory effect of nicotine on DA is mediated by different nAChR subtypes expressed on dopaminergic terminals in the BNST [1], and may contribute to the reward sensation produced by nicotine [151, 152]. As mentioned earlier, the BNST is a heterogeneous brain region that can be divided into anterior and posterior divisions, which can be further divided into 18 subregions [110]. Both the anterior division (which includes the anterolateral, anteromedial, oval, fusiform, juxtacapsular, rhomboid, dorsomedial, ventral, and magnocellular nuclei) and the posterior division (which comprises the principal, interfascicular, and transverse nuclei) receive and send distinct cholinergic, noradrenergic, dopaminergic, GABAergic, and glutamatergic projections [110]. Dopaminergic inputs that may underlie the rewarding action of nicotine originate from the VTA and periaqueductal gray (PAG), projecting

primarily into the dorsolateral subdivision of the BNST and synapsing directly onto CRF neurons [153]. However, which of the two major dopaminergic inputs (originating in the VTA or PAG) to the BNST drives this behaviour is not known [153]. 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 [154]. Likewise, increased release of DA in the BNST was described following exposure to natural rewarding substances, such as sucrose [155, 156]. Furthermore, blocking DA D_1 receptors in the BNST reduced ethanol and sucrose self-administration [157]. Nevertheless, several studies suggest that DA signalling in the BNST may also play a role in the negative affect induced by nicotine withdrawal [158, 159]. Consistently, neurons within various nuclei and subnuclei of the BNST have been shown to co-express a variety of neuropeptides, such as CRF and NPY [160], and dysfunction of these neuropeptides may contribute to stress reactions, anxiety, and depression observed during nicotine withdrawal [110].

Ghrelin increased the release of DA from the rat BNST after electrical stimulation even more significantly than nicotine did, and this effect was significantly inhibited by GHRP-6, a selective GHSR1a antagonist. This finding is supported by *in vivo* studies, which reported that administration of ghrelin induces concomitant release of ventral tegmental acetylcholine and accumbal DA, an effect associated with the locomotor hyperactivity induced by ghrelin [101-103, 105]. 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 NAcc [99, 100, 134-136]. Activation of the cholinergic-dopaminergic reward link by ghrelin may lead to stimulation of DA release from dopaminergic terminals in the BNST [1], and may induce a similar reward sensation to that produced by nicotine [5,50]. One of the most important DA inputs to the BNST arises from the VTA and projects into the dorsolateral subdivision of the BNST [57]. However, most BNST neurons are GABAergic (~97%) and a small minority (~3%) are glutamatergic [110]. Therefore, ghrelin may locally activate GABAergic and glutamatergic neurons, which may impact dopaminergic terminals within the BNST [134, 155-157, 161]. Generally, changes in the firing rate of dopaminergic, GABAergic, and glutamatergic neurons in the BNST can evoke distinct affective states and motivated behaviours that may contribute to both positive and negative reinforcement from drugs, including nicotine [162, 163]. Continuous seeking of the

positive reinforcement produced by nicotine, and avoidance of the negative reinforcement induced by nicotine withdrawal, can result in relapse to smoking – especially in periods of stress – and lead to a spiral into nicotine addiction [1, 163]. Specifically, GABAergic and glutamatergic inputs to the anterior BNST emerge from several cortical and limbic regions (such as the prefrontal cortex and basolateral amygdala) and innervate different nuclei of the BNST (e.g., the oval nucleus), projecting to other limbic regions such as the VTA and CeA [44]. The posterior BNST receives GABAergic input from the lateral septum and various amygdala nuclei, and glutamatergic input from the paraventricular region of the thalamus and different regions of the hippocampus. These inputs innervate the dorsolateral subdivision of the BNST and send projections to the ventral striatum, dorsal striatum, and PVN of the hypothalamus, through which they modulate the activity of the HPA axis, also known as the stress axis [110]. Ghrelin is believed to activate the HPA axis directly by increasing the release of CRF at the hypothalamic level, but an interaction of ghrelin with CRF at the level of the BNST cannot be excluded [44, 164]. In addition, the anterior division of the BNST is rich in CRFR1, whereas the posterior division is richer in CRFR2 [110]. An imbalance between these receptors may contribute to hyperactivity of the HPA axis, anxiety, and depression, which can be observed during nicotine withdrawal [158].

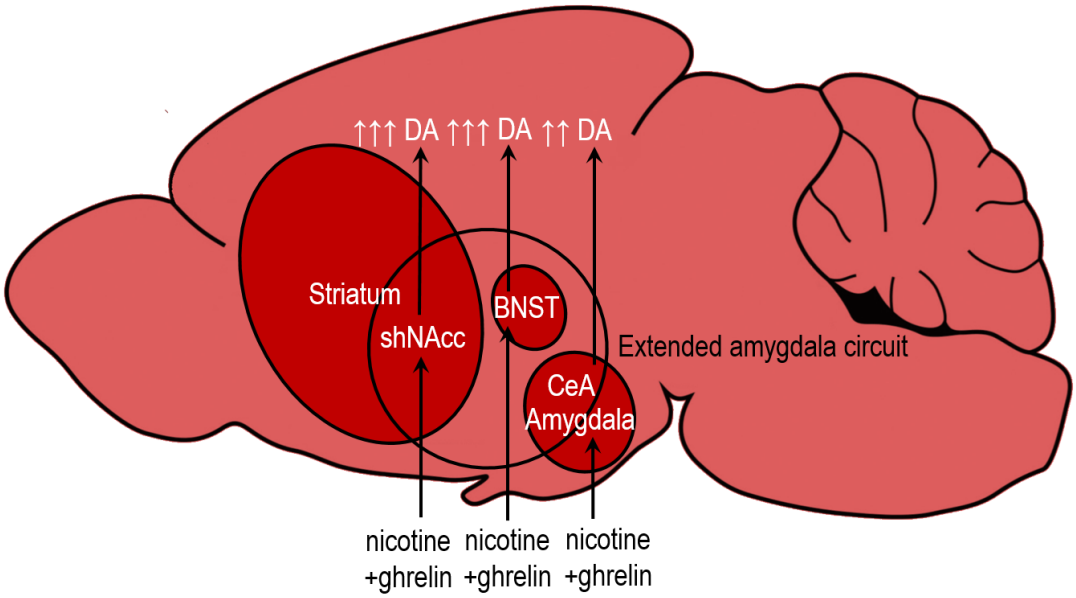
Moreover, when administered together, ghrelin significantly amplified the nicotine-induced release of DA from the rat BNST after electrical stimulation, and this effect was partially reversed by mecamylamine and partially by GHRP-6 [74] (**Figure 22.**).



BNST = bed nucleus of stria terminalis
DA = dopamine

Figure 22. Coronal section of the rat brain

Our previous in vitro superfusion studies have yielded similar results. Ghrelin and nicotine stimulated DA release equally in the rat amygdala, and ghrelin amplified the nicotine-induced DA release even further in the rat striatum [30,31] (**Figure 23.**).



BNST = bed nucleus of stria terminalis
CeA = central nucleus of amygdala
DA = dopamine
shNAcc = shell of nucleus accumbens

Figure 23. Sagittal section of the rat brain

Despite the different anatomical and physiological functions attributed to the BNST, amygdala, and striatum, there is a strong functional correlation between these brain regions. On the one hand, the BNST is an important relay station within both the cholinergic–dopaminergic reward link and the extended amygdala circuit. On the other hand, dopaminergic neurons of the VTA send projections to each part of the extended amygdala, including the NAcc shell, the BNST, and the CeA [10]. Therefore, in addition to the amygdala and striatum, a possible site of interaction between ghrelin and nicotine could be the BNST, possibly via activation of cholinergic, GABAergic, or glutamatergic neurons that influence dopaminergic terminals in the BNST. Both animal experiments and human studies indicate the existence of an interaction between ghrelin and addictive drugs such as nicotine [71, 165]. Most notably, ghrelin has been implicated in reward sensation and drug-seeking behaviour induced by alcohol, and the ghrelin receptor has been suggested to be a possible pharmacological target in the treatment of alcohol addiction [166-175]. Nevertheless, there is also clear evidence of a connection between ghrelin and nicotine. For instance, higher plasma ghrelin levels have been found in smokers compared with non-smokers [176, 177]. However, salivary ghrelin levels were found to decrease acutely after smoking a cigarette, and plasma ghrelin was also decreased after a period of abstinence from tobacco – this was considered a rebound effect after smoking [178-180]. Some authors have even suggested that ghrelin levels could be used as a biomarker for an increased risk of relapse to smoking [84, 85, 181, 182]. Consequently, these studies suggest that ghrelin levels have prognostic value that is independent of the severity of negative affects (withdrawal or craving) [84, 85, 181, 182]. Overall, these studies indicate a positive association between smoking and ghrelin levels – an association reversed during abstinence, particularly among individuals likely to maintain cessation. Accordingly, ghrelin may be used as a prognostic tool, and the ghrelin receptor may serve as a pharmacological target in the treatment of nicotine dependence.

Superfusion is a classic method for delivering water-soluble electrolytes or other compounds to small tissue preparations by diffusion from the outside to the inside of the tissue along a concentration gradient. It has its advantages and disadvantages. The main advantage of this technique is the ability to study brain tissue isolated from practically any region of the brain, as long as the tissue is large enough to be dissected, even if intact neural circuitry is not present [183, 184]. Another advantage is the ability to quickly switch superfusion solutions (for example, switching between Krebs buffer and other solutions) to explore the tissue's response to various physical and chemical manipulations (e.g., electrical stimulation, increased K^+ content) [183, 184]. However, the intensity of the electrical current applied or the concentration

of K^+ in the solution must be carefully controlled to avoid damaging the tissue or causing cell death [183, 184]. The major drawback of superfusion is that it relies on diffusion; therefore, the tissue sample must be small enough to allow for adequate and homogeneous penetration of the superfusate. Another disadvantage is that the preparation must not be very dense and should have a large surface area relative to a small cross-section [183, 184]. This technique is also less effective if the superfusate contains very large or highly hydrophilic molecules that need to penetrate the tissue [183, 184]. A major concern when using superfusion is the viability of the preparation over time. Specifically, the superfusate flow rate must be high enough to ensure that the tissue is continually bathed with fresh, oxygenated, buffered, warmed solution, but low enough to avoid excessive washout of substances and tissue damage. Nonetheless, when used properly, superfusion is a valid experimental technique that allows for the study of a variety of brain regions, including the striatum, amygdala, hippocampus, hypothalamus, and even the BNST [185-189]. It is important to mention that no *in vitro* technique can truly replace *in vivo* models under all physiological and pathophysiological conditions. Isolated tissue inherently loses the natural environment in which it normally functions. However, because it is extremely difficult to control all experimental parameters *in vivo*, isolated tissue models are an acceptable experimental tool to investigate physiological and pharmacological effects of chemical agents, including agonists such as nicotine and ghrelin, and their synthetic antagonists such as mecamylamine and GHRP-6 [74-76, 88, 158].

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. The interaction between nicotine and ghrelin is likely mediated by the cholinergic–dopaminergic reward link, which includes an afferent cholinergic projection from the LDT to the VTA and a mesolimbic dopaminergic pathway originating in the VTA that projects to the NAcc and sends additional projections to the CeA.

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.

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