Study of the effects of the ghrelin-associated peptide obestatin on stress-related behaviors

Ph.D. Thesis

Candidate: Júlia Szakács MD

Department of Pathophysiology

Faculty of Medicine

University of Szeged

Supervisor: Prof. Dr. Gyula Szabó, M.D., Ph.D., D.Sc.

Doctoral School of Theoretical Medicine

University of Szeged

2017

TABLE OF CONTENTS

PUBLICATIONS RELATED TO THE SUBJECT OF THESIS
In extenso publications

I. Lipták N, Dochnál R, Csabafi K, Szakács J, Szabó G.
Obestatin prevents analgesic tolerance to morphine and reverses the effects of mild morphine withdrawal in mice. Regul Pept. 186:77-82, 2013
IF: 2.014

II. Szakács J., Csabafi K., Lipták N., Szabó G.
The effect of obestatin on anxiety-like behaviour in mice
Behavioural Brain Research 293 ,41–45, 2015
IF: 3.002

III. Szakács J., Csabafi K., Pataki I., Szabó G.
Obestatin induces depressive-like effects in the FST
In preparation; 2017

List of citable abstracts related to the subject of thesis

1. Manczinger M., Dochnal R., Babits A., Szakács J., Szabó G.
     The influence of ghrelin on the acute and chronic effects of nicotine
     Acta Phys Hung, 97 (S4), 455, 2010

2. Szakács J, Mácsai M, Dochnál R, Babits A, Pál Á, Szabó G.
     The effect of the neuropeptide PACAP on morphine-induced locomotor activity
     Acta Phys Hung, 97 (4), 475 , 2010

3. Szakács J., Dochnál R., Babits A., Lipták N., Csabafi K., Szabó G.
     Role of Obestatin in morphine-induced behavioral responses
     ActaPhys, 202, (S684), 112, 2011

4. Lipták N., Szakács J., Babits A., Csabafi K., Tóth G., Szabó G.,
     The role of Pituitary Adenylate Cyclase-Activating Polypeptide in morphine withdrawal-induced anxiety and locomotor activity in mice
     ActaPhys, 202, (S684),   71, 2011

5. Liptak N., Dochnal R., Csabafi K., Szakacs J., Szabo G.
     The effects of obestatin on morphine withdrawal-induced behavioural changes in mice
     Ideggyogy Sz; 65(S1), 42, 2012

6. Szakacs J., Csabafi K., Liptak N., Szabó Gy.
     Analysis of behaviour activity of obestatin in mice
     Period Biol,115, S(2), 47, 2013

7. Szakács J., Csabafi K., Kincses B., Bene K., Bagosi Zs., Szabó G.
    The effect of obestatin on corticosterone secretion and anxiety behaviourActa
    Physiol Scand, 211, (S697), 132, 2014

8. Csabafi K., Szakács J., Kincses B., Bene K., Bagosi Zs., Telegdy Gy., Szabó G.
    The effect of kisspeptin on cocaine-evoked behavioral changes
    Acta Physiol Scand, 211, (S697), 150, 2014

List of full papers not related to the subject of thesis

1. Lázár Gy., Lázár Gy. Ifj., Husztik E., Szakács J., Duda E., Agarwal M.K.
    Tolerancia és túlérzékenység kísérletes modellekben.
    Leg Art Med 7, 546-550, 1997.

2. Lázár G., Husztik E., Lázár G. Jr., Kiss I., Oláh J., Szakács J.:
    Immunomodulation by gadolinium chloride-induced Kupffer cell phagocytosis
    blockade.
    J. Alloys and Compounds 275-277, 908-910, 1998.

3. Lázár Gy., Husztik E., Ifj. Lázár Gy., Szakács J.
    A makrofágok szerepe a szervezet védelmében.
    Orvostudományi Értesítő 74, 67-69, 1999.

4. Lázár Gy., Husztik E., Ifj. Lázár Gy., Szakács J., Duda E.
    A Kupffer sejtek szerepe az elzáródásos icterusban kifejlődő rezisztenciacsökkenés
    pathomechanizmusában.
    Orvostudományi Értesítő, 73, 161-165, 2000.

5. Szakács J., Lázár G. Jr.,Lázár G., Husztik E.
    The effect of the glucocorticoid Oradexon on endotoxin-induced peritoneal cell
     response.
    Acta Physiol. Hung. 87, 161-166, 2000

6. Lázár Gy., Husztik E., Szakács J., Ifj. Lázár Gy., Kaszaki J.:
    Kupffer sejt blokád hatása az anaphylaxiás shockra
    Orvostudományi Értesítõ 74, 275-278, 2001.

7. Lázár G. Jr, Paszt A., Kaszaki J., Duda E., Szakács J., Tiszlavicz L., Boros M.,
    Balogh Á., Lázár G.
    Kupffer cell phagocytosis blockade decreases morbidity in endotoxemic rats with
    obstructive jaundice
    Inflammation Res. 51, 1-8, 2002.

8. Lázár Gy, Husztik E, Szakács J, Lázár G.Jr.:
     Makrofágok szerepe a normális és kóros immunválaszban
     Transzfúzió 35, 205-208, 2002.

9. Lázár Gy., Husztik E., Ifj. Lázár Gy., Lázár I., Szakács J.:
    Sport hatása az immunfunkciókra és a daganatos megbetegedésekre.
    Orvostudományi Értesítõ, 76, 100-107,2003

Book chapters:

1. Lázár G., Kiss I., Lázár G. Jr., Oláh J., Szakács, J., Husztik E.
    Humoral immune response modulation induced by Kupffer cell phagocytosis
    blockade. in "Cell of the Hepatic Sinusoid",
    Vol. 6. (E. Wisse, D.L. Knook, C, Balabaud, Eds.)
    Kupffer Cell Foundation, Leiden,. 391-392, 1997.

2. Lázár G.Jr., Duda E., Szakács J., Oláh J., Balogh Á., Lázár G
    Effect of Kupffer cell phagocytosis blockade on endotoxin sensitivity and
    endotoxin-induced cytokine production in obstructive jaundice, in Cell of Hepatic
    Sinusoids, (Eds. E. Wisse, D:L: Knook, de Zanger R., Fraser R.)
    Kupffer Cell Foundation, Leiden, The Netherlands, vol. 7, 237-238, 1999.

List of citable abstracts not related to the subject of thesis

1. Lázár G., Husztik E., Lázár G. Jr., Kiss I., Oláh J., Szakács J.
    Effect of Kupffer cell phagocytosis blockade on humoral immune response.
    Immunology Letters 25, 120, 1997.

2. Lázár G., Husztik E., Lázár G. Jr., Kiss I., Oláh J., Szakács J.
    Immunomodulation by Kupffer cell phagocytosis blockade.
    Shock 88 (S8), 1997.

3. Lázár G., Husztik E., Szakács J., Lázár G. Jr., Duda E.
    Effects of parenchymal and nonparenchymal cell interactions on endotoxin
    sensitivity and endotoxin-induced cytokine production in obstructive jaundice.
    Shock 10, 15-16, 1998

4. Lázár G., Husztik E., Szakács J., Lázár G. Jr., Duda E.
    Effect of cellular interaction on endotoxin sensitivity and endotoxin-induced
    cytokine production in obstructive jaundice.
    Eur Cytokine Netw, 9, 490, 1998.

5. Lázár G., Husztik E., Szakács J., Lázár G., Jr., Duda E.
    Effects of Kupffer cell phagocytosis blockade induced by gadolinium chloride on
    endotoxin sensitivity and endotoxin-induced cytokine production in obstructive
    jaundice.
    Shock S12, , 38-39, 1999.

6. Lázár G. Jr., Duda E., Szakács J., Oláh J., Balogh Á., Lázár G.
    Role of Kupffer cells in LPS sensitivity and cytokine release in obstructive
    jaundice in the rat.
    Eur Surg Res, 31, S1, 178-179, 1999.

7. Lázár G. JR., Duda E., Szakács J., Paszt A., Balogh Á., Lázár G.
    Role of Kupffer cell in LPS sensitivity, LPS-induced cytotoxicity and cytokine release in experimental rats with obstructive jaundice.
    Shock, 13 (S1), 38-39, , 2000.

8. Lázár G., Lázár G. Jr., Szakács J., Husztik E. Duda E.
    Effects of Kupffer cell phagocytosis blockade on endotoxin sensitivity, tissue localization of endotoxin and TNF-a production.
    Shock, 13 (S1), 38-39, 2000.

9. Lázár G. Jr., Husztik E., Szakács J., Duda E.
    Alteration of local and systemic effect of bacterial endotoxin by Kupffer cell blockade in rodent model of obstructive jaundice.
    Eur. Surg. Res. 32, S1, 14, 2000

10 .Lázár G. Jr., Husztik E., Szakács J., Duda E.
    Role of Kupffer cells in mouse anaphylaxis.
    Eur. Surg. Res. 32, S1, 100, 2000.

11. Szakács J., Lázár Gy. Jr., Lázár Gy., Husztik E.
    Glucocorticoid-mediated mechanisms in endotoxin-induced peritoneal cell response.
    J. Physiology 526, 187, 2000.

12. Szakács J., Lázár G. Jr., Husztik E., Lázár G.
    The influence of glucocorticoid and it's antagonist on endotoxin-induced peritoneal cell response
    Scand J Immunol. 54 (S.1). 111, 2001

13.Szakács J, Lázár G, Lázár G. Jr, Husztik E
    Modification of endotoxin-induced inflammatory cell response by glucocorticoids
    Eur. Surg. Res., 34, S (1), 99, 2002.

14. Szakács J., G., Lázár G., Husztik E.
    Study of the inflammatory cell response induced by bacterial LPS
    Acta Physiologica , 186 (S1), 210, 2006

15.Lázár G., Husztik E., Hegedűs H., Lázár S., Szakács J.,
    Effect of Kupffer cell blockade induced by gadolinium chloride on the development of biliary cirrhosis
    Acta Phys Hung 93, 202, 2006

16. Szakács J, Lázár G, Szabó G, Lázár G Jr
    Study of the inflammatory cell response induced by $GdCl_3$ and bacterial LPS
    Acta Phys Hung, 94 (4), 392, 2007

17. Szakács J., Lázár G., Lázár G.Jr., Szabó G
    The effect of steroids in Gram-negative sepsis
    Acta Phys Hung, 96, (1), 130, 2009

ABBREVIATONS

[D-Lys3]-GHRP6	[D-Lys3]- Growth Hormone Releasing Peptide-6
aCSF	artificial cerebrospinal fluid
ACN	Arcuate Nucleus
ACTH	Adrenocorticotropic Hormone
AgRP	Agouti-Related Peptide
AN-R	Restrictive Type of Anorexia Nervosa
AVP	arginine-vasopressin
cAMP	Cyclic Adenosine Monophosphate
CCK8	Cholecystokinin Octapeptide
CREB	cAMP Response Element Binding Protein
CRF	Corticotropin-Releasing Factor
CRH	Corticotropin-Releasing Hormone
CRHR1	Corticotropin-Releasing Hormone Receptor 1
DRN	Dorsal Raphe Nucleus
EPM	Elevated Plus Maze
ERK 1/2	Extracellular Signal Related Kinase
FST	Forced Swimming Test
GABA	γ-Aminobutyric Acid
GH	Growth Hormone
GHSR	Growth Hormone Secretagogue Receptor
GHSR1a	Growth Hormone Secretagogue Receptor 1a
GI	gastro-intestinal
GLP-1	Glucagon-Like Peptide 1
GLP-1R	Glucagon-Like Peptide 1 Receptor
GPR39	G-Protein Coupled Receptor
HPA axis	Hypothalamic-Pituitary-Adrenal Axis
icv	intra-cerebroventricular
KO	knock-out
MAPK	Mitogen-Activated Protein Kinases
NAcc	Nucleus Accumbens
non-REM	Non-Rapid-Eye-Movement
NPY	Neuropeptide Y
NTS	Nucleus of the Solitary Tract
OAE%	Open Arms/Total Number of Entries %
OAT%	Open Arm Time/Total Time %
OF	Open Field
PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PVN	Paraventricular Nucleus
SAH	subarachnoidal haemorrhage
sc	subcutaneous
type 2 DM	Type 2 Diabetes Mellitus
TST	Tail Suspension Test
VTA	Ventral Tegmental Area
WHO	World Health Organization

1. INTRODUCTION

1.1. The preproghrelin-derived peptides

The preproghrelin gene encodes several peptides with different structure and function, acyl ghrelin, desacyl ghrelin and obestatin *(fig.1)*. Ghrelin is a 28 amino acid peptide, was named after the word root "ghre", meaning "grow" in Proto-Indo-European languages *(1)*, and originally identified from the rat stomach to stimulate growth hormone (GH) secretion via the growth hormone secretagogue receptor 1a (GHSR1a), *(2, 3)*. The endogenous ligand for this receptor was purified from the stomach and later it was pointed out as the first peripheral hormone with potent orexigenic activity to regulate food intake, body weight and a long term regulator of energy homeostasis *[(4) and fig. 2]*. Research during the past almost two decades has demonstrated, that the ghrelin system involves many interrelated peptides and receptors distributed in different tissues, forming a complex network, which exerts autocrine, paracrine and endocrine actions in order to tightly regulate different physiological processes.

The dysegulation of these circuits can generate multiple pathologies of the endocrine, metabolic, cardiovascular and central nervous system. Therefore, the different ghrelin-associated peptides have been suggested as diagnostic, prognostic, or therapeutic targets by a high number of recent studies *(5)*. From a molecular point of view, all these peptides originate from the same preproghrelin precursor, encoded by a single-copy gene located on the short arm of chromosome 3 in humans. The originally identified preproghrelin mRNA transcript encodes a 117-amino acid long peptide, which, by multiple proteolytic processing yields two functionally different peptides with highly conserved sequences among mammals, named ghrelin and obestatin, respectively *(5)*. Native ghrelin undergoes further modification e.g. acylation by the enzyme ghrelin-*O*-acyl-transferase, this acylated ghrelin being the biologically active peptide, identified originally by Kojima et al. *(2)*. The other form, unacylated ghrelin, though constituting over 90% of plasma ghrelin, the biological role of it has not been identified yet *[(5-7) and fig. 1]*.
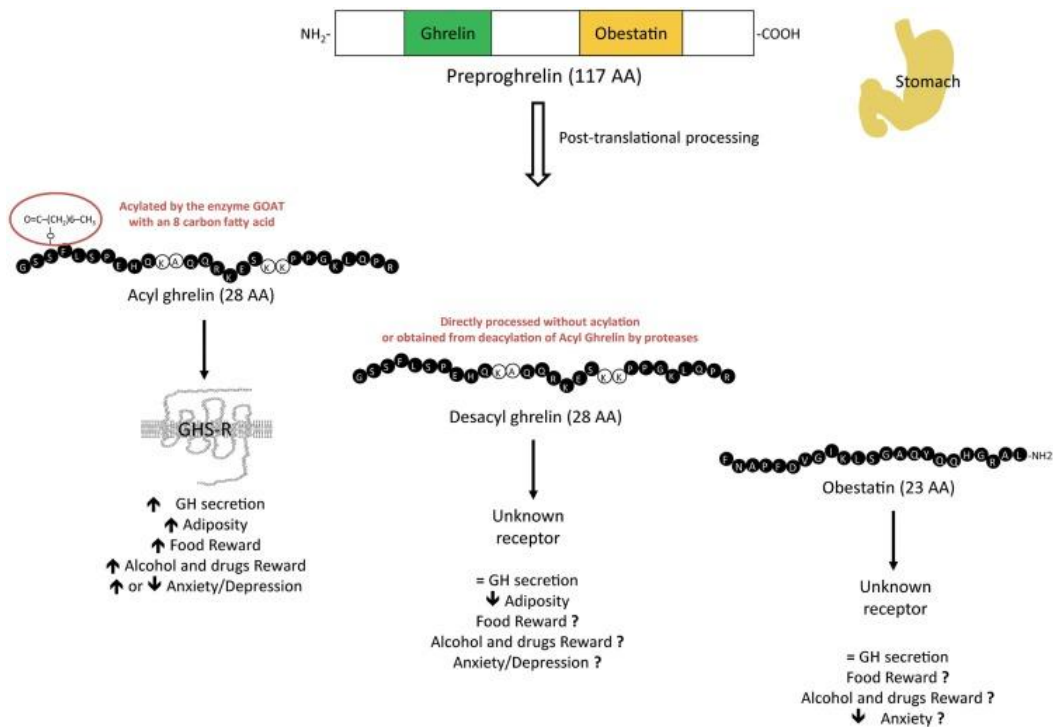
Figure 1. The synthesis and function of preproghrelin derived peptides (8)

1.2. Ghrelin, the brain-gut peptide

Metabolic signals from the stomach are transmitted mainly via afferent vagal nerves through the nucleus of the solitary tract (NTS) to the hypothalamus, where ghrelin enhances food intake by activating the orexigenic neurons expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP) in the arcuate nucleus (ACN) and leading to peptide release via Y1 and Y5 receptors in paraventricular nucleus (PVN) *(9-13)*. At the same time the anorexogenic neurons expressing pro-opiomelanocortin (POMC) are suppressed via activation of the inhibitory γ-aminobutyric acid (GABA)-ergic inputs and AgRP antagonizes melanocortin 4 receptor (MC4R)-containing neurons and prevents the anorectic actions of αMSH *(9, 14-16)*.

Figure 2. The effect of ghrelin on hypothalamic control of food intake (10)

Ghrelin is a peripheral orexigenic peptide with unique central actions, which acts primarly as a hunger signal to increase appetite and caloric intake. In rats, ghrelin dose-dependently increased feeding when adm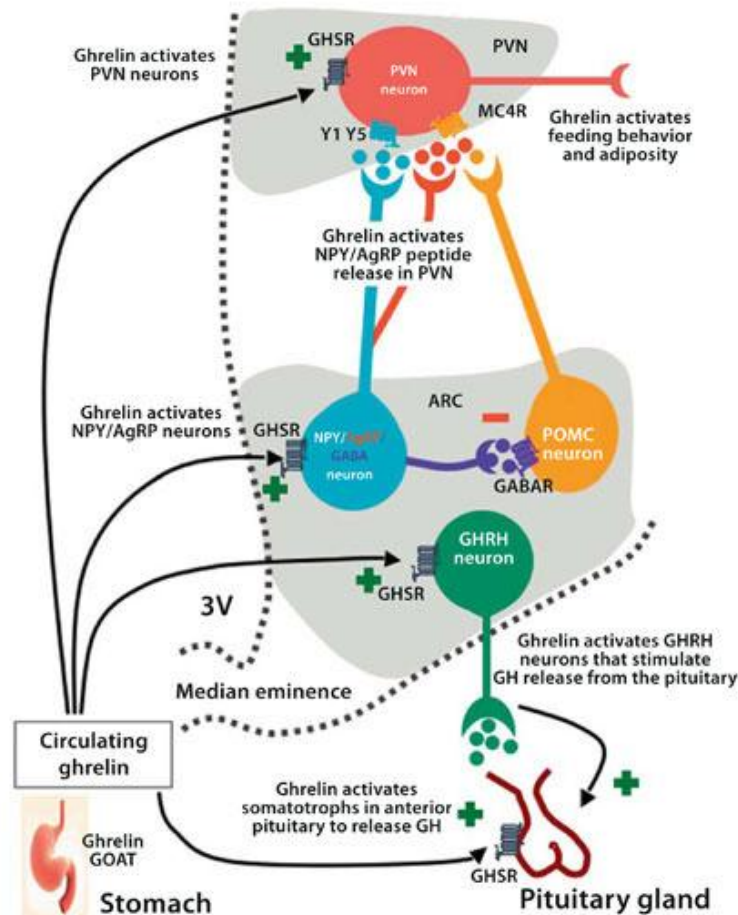inistered into the hippocampus and dorsal raphe nucleus *(17)*. In another rodent study peripheral daily administration of ghrelin caused weight gain, while both food intake and body weight were dose-dependently increased by intra-cerebroventricular (icv) ghrelin injection *(4)*.

The very first randomised study in man also showed increased appetite and food intake in healthy volunteers after intravenous injection of ghrelin *(18)*. Ghrelin levels rise during fasting and before meals, and return to normal postprandially. Moreover, compensatory responses of ghrelin levels were observed related to changes of body weight: since weight gain is accompanied by decreased, while weight loss by increased ghrelin levels.

Furthermore, ghrelin acts on both components of energy balance (caloric intake as mentioned previously and energy consumption) by promoting fat storage and lipogenesis to maintain homeostasis in the face of environmental challenges such as food restriction. Ghrelin, not only increases energy intake but also decreases energy expenditure, which

effect was blunted by the administration of anti-ghrelin antibodies in a rodent study *(19)*.

Furthermore, blockage of ghrelin receptor increased energy expenditure by stimulating the non-shivering thermogenesis in brown adipose tissue *(20)*. These findings may have important therapeutical implications since GHSR1a antagonists may represent attractive drug candidates to treat obesity without the need of exercise and dietary restrictions *(21)*. However, it should be taken in consideration that ghrelinergic compounds can not effectively target centrally-controlled food intake without affecting the delicate balance of metabolic and neuroendocrine pathways regulated by ghrelin *(6, 12, 22)*.

Studies in animals and humans have also demonstrated that ghrelin not only induces food intake and a positive energy balance, but also stimulates food-seeking behavior and the hedonic aspects of eating by promoting the preference for highly palatable, calorie-dense food. To note, in one study conducted in healthy volunteers ghrelin levels were increased by just visual presentation of hedonic food *(23)*. The above mentioned data are extremely important in the current circumstances of abundant food and sedentary lifestyle, when the physiological effects of ghrelin might become pathological and contribute to the global epidemic of overweight and obesity *(24)*.

The normal ghrelin signaling is disturbed in eating disorders such as obesity, anorexia and bulimia nervosa. In a variety of obesity syndromes (monogenic obesity, metabolic syndrome, Prader-Willi syndrome) with different etiology the values of the preproghrelin products (total ghrelin, acyl ghrelin and obestatin) were found either reduced, unchanged or increased [*(8) and fig.3*]. Some studies in obese people have shown a compensatory reduction of ghrelin secretion, however a lack of postprandial ghrelin suppression was also observed, which could contribute to the maintanance of the positive energy balance in these people *(25)*.

In the undernourished restrictive type of anorexia nervosa, acyl ghrelin and obestatin levels are elevated, while the ghrelin/obestatin ratio is low, suggesting a resistance to the orexigenic action of acyl ghrelin. In anorexia nervosa patients with binge-purging the lower obestatin levels probably favor motivation to bingeing-related behavior [*(26, 27) and fig. 3*]. The present data regarding the role of ghrelin/obestatin balance in nutrition are still coflicting, however merit further thorough investigation.
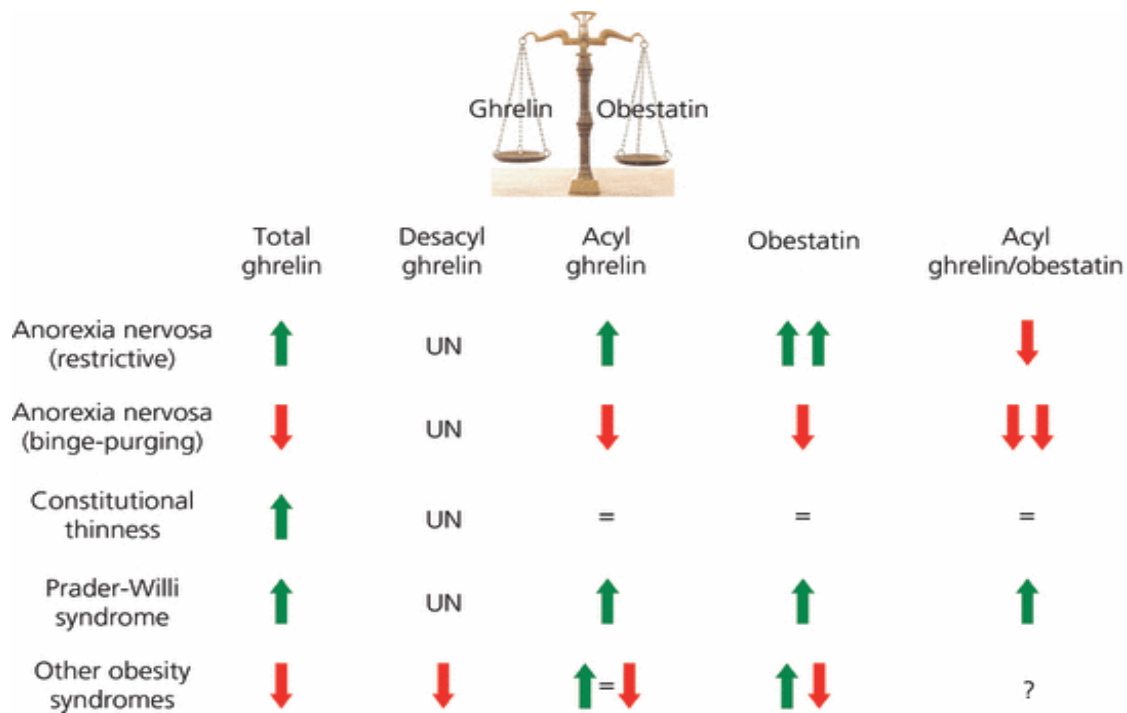
	Total ghrelin	Desacyl ghrelin	Acyl ghrelin	Obestatin	Acyl ghrelin/obestatin
Anorexia nervosa (restrictive)	↑	UN	↑	↑↑	↓
Anorexia nervosa (binge-purging)	↓	UN	↓	↓	↓↓
Constitutional thinness	↑	UN	=	=	=
Prader-Willi syndrome	↑	UN	↑	↑	↑
Other obesity syndromes	↓	↓	↑=↓	↑↓	?

Figure 3. The changes of ghrelin, obestatin and ghrelin/obestatin ratio in ifferent eating disorders (Hassouna R. 2010) UN – unchanged

1.2.1. The role of ghrelin in neuroprotection, anxiety and depression

In the central nervous system ghrelin was shown to have antiapoptotic, and antiinflammatory effect, protecting also the neurons against oxidative stress and hypoxia. Furthermore, it is involved in neurogenesis, promoting the proliferation of neural stem/progenitor cells *(13).* Ghrelin was shown to induce hippocampal neurogenesis both *in vivo* and *vitro* and to enhance memory performance and spatial learning in rodents *(28).* The neuroprotective effect was blunted in GHSR1a-knock-out (KO) mice exposed to chronic stress *(29).* These effects position ghrelin as a possible therapeutic candidate in the treatment of traumatic and ischaemic brain injury and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease *(13, 30).*

Identified as a hunger hormone initially, in the past years ghrelin has received an unique role as a gut-brain peptide, at the interface of appetite and metabolic control and behaviors related to psychological stress, mood, anxiety and depression *(31).* The first study completed on rats identified ghrelin az an anxiogenic agent, since the animals have shown decreased activity in the open field (OF) and elevated plus maze (EPM) tests, respectively *(17, 32).* GHSR1a, the receptor for ghrelin is widely expressed in distinct brain areas (hypothalamus, pituitary, amygdala, hippocampus, ventral tegmental area – VTA). Consequently, ghrelin can also control different neuroendocrine functions as GH

secretion, regulation of energy homeostsis, mood, anxiety, depression, learning and memory processes, reward-related behaviors and neuroprotection *(7, 9, 13)*. The injection of ghrelin into various brain regions (hippocampus, amygdala, hypothalamus, dorsal raphe nucleus – DRN) has shown, that the DRN is the primary site of ghelin's anxiogenic action where it acts through the serotonergic sytem *(17)*. Similarly both central and peripheral injection of ghrelin induced anxiogenesis in mice in the EPM test which was blunted by the administration of a CRH antagonist, furthermore elevated corticosterone levels were also detected after ghrelin administration. This effect was attributed to the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the release of corticotrophin-releasing hormone (CRH) in the PVN *(33)*, indicating that GHSR1a acts directly on CRH-containing neurons *(9)*. This correlation was further proved in a rat model of acute stress, which increased both ACTH and ghrelin levels *(34)*.

Other studies have also reported that ghrelin induces anxiogenic-like effects in the EPM when administered acutely to different hypothalamic areas (eg. ACN and PVN) *(35)*. Additionally, chronic central administration of ghrelin was shown anxiogenic in the OF and EPM tests, and induced depression in the forced swim test (FST) in rats *(36)*.

The stimulation of HPA axis by ghrelin occurs via hypothalamic, yet not completely elucidated pathways, though independently from the orexigenic signals regulating food intake *(37)*. In contrast with the above mentioned studies, elevating ghrelin levels by 10 days of calorie restriction or subcutaneous ghrelin injection resulted in anxiolytic- and antidepressant-like responses in the EPM and FST. Similarly, Ghr-R KO mice showed greater social avoidance compared to wild-type litermates during chronic social defeat stress *(38)* and an increased anxiety-like behavior was detected in ghrelin KO mice after acute restraint stress *(39)*. Acute peripheral administration of ghrelin also induced anxiolytic-like effect in both the EPM and OF tests *(40)*. Central administration of ghrelin also produced anti-depressant effect in tail suspension test (TST) and blunted the depressive effects (hyperlocomotion) induced by bilateral olfactory bulbectomy, an accepted depression model in rodents *(41)*.

Exposure to chronic social defeat stress (a model of major depression and posttraumatic stress disorder) increased ghrelin (and corticosterone) levels, and behavioral deficits like social isolation were more pronounced in ghrelin-receptor-deficient mice *(42)*. The hippocampus is one of the primary site to mediate the cognitive aspects of depression like memory retention and ghrelin deficient mice have impaired behavioral memory, while injection of ghrelin into the hippocampus causes memory retention *(43)*.

The antidepressant properties of ghrelin could be related to its effect on promoting hippocampal neurogenesis, protecting mitochondrial function, similarly to the findings in different models of neurodegenerative disorders *(44)*. In this regard, ghrelin has shown proliferative, antiapoptotic and neurogenic activities in hippocampal progenitor cells, mediated by its receptor, the GHSR1a *(45, 46)*. Furthermore, it exerted a protective role in experimental rodent models of Alzheimer's disease by improving memory processes *(47)* and promoting the survival of rat hippocampal neurons treated with amyloid β *(48, 49)*.

Human findings related to ghrelin levels in depression are somewhat inconsistent: decreased, unchanged, but also higher levels have been described. These increase in ghrelin levels were normalized after treatment with antidepressants *(50)*. However, in one study ghrelin administration induced an improvement tendency of depressive symptoms in unmedicated men, but not women with major depression *(51)*. HPA axis and sleep patterns are disturbed in depression. Furthemore, gender differences were also observed related to the effect of exogenous ghrelin on cortisol levels and sleep in the same study. The non-REM sleep improved in men, and after an initial increase in cortisol levels in both sexes, a blunted hormonal response was observed in men, which suggests the involvement of the HPA and gonadal axes and deserves further investigation *(51)*. Non-REM sleep was also increased in healthy male volunteers receiving bolus injections of ghrelin in low doses, while EEG patterns in healthy women remained unchanged *(52)*. However, endogenous or high-doses exogenous ghrelin may disrupt sleep due to increased hunger. In a series of studies in rats, central injections of ghrelin in the areas involved in sleep and feeding control induced increased wakefulness, feeding and motor activity *(53, 54)*. These results underlie the possible role of ghrelin in night eating syndrome, characterized by disturbed sleep due to hunger followed by excessive eating *(30)*.

The conflicting data related to ghrelin's effect on anxiety and depression might be due to differences in study design (dosage, route and timing of injection, strain or species used) *(43)*, as well as the duration and type of stress exposure *(44, 55)*. After more than one decade of research a dual effect for ghrelin was suggested *(39, 44)*. Accordingly, in conditions of acute stress ghrelin would have an anxiolytic effect, while during basal, unstressed conditions or when exposed to chronic stress the animals would show an anxiogenic effect and enhanced fear memory after the injection of ghrelin *(17, 32, 33, 36, (56)*.

The anxiolytic- and antidepressant-like effect of ghrelin may be a critical counter regulatory mechanism to cope with different stress conditions, promoting food seeking, maintenance of energy homeostasis and survival advantage during evolution *(31, 56, 57)*.

However, the activation of HPA axis and the release of glucocorticoids may enhance ghrelin's effect on the consumption of highly palatable, rewarding food both in animals and humans, but at the expense of high caloric intake and development of obesity *(42)*. In one remarkable study in mice, exposure to chronic social defeat stress increased the preference for and the intake of rewarding, high-fat diet, which effect was mediated by ghrelin signaling, presumably to ameliorate the depressive-like symptoms, but also to increase body weight *(42)*. As a consequence, the development of obesity triggers a vicious circle, with altered central ghrelin signaling, and increased susceptibility to develop anxiety, depression and addictive behaviors *(8)*.

1.2.2. Neuroendocrine mechanisms connecting obesity and stress-related behaviors

Obesity is a pathological condition, which results from an imbalance between caloric intake and expenditure, and is characterized by excessive body fat accumulation, that has severe impact on life quality and life expectance due to the burden of associated co-morbidities. Recent data from the World Health Organization suggest that 11% of the world population (more than half a billion people) is obese, while 35% is overweighed *(58)*. Furthermore, the prevalence of obesity is continously increasing worlwide, so revealing the pathomechanism and finding effective treatments have become urgent and essential *(59)*. During the past decades much research has highlighted that neurotransmitter systems controlling appetite and feeding behavior, cognitive function, stress, mood and reward behavior are strongly and reciprocally connected *(60)*. Food intake is normally regulated by a homeostatic drive to restore energy balance, while in certain conditions hedonic or reward-based regulation favors the consumption of highly palatable, energy-dense foods *(61), (62)*. Notably, exposure to stress influences dramatically food intake and energy homeostasis. In some individuals it leads to decreased appetite and weight loss, while many human and rodent studies have also demonstrated high preference of calorie-rich, tasty diet which reduces the discomfort of stress and provides relief and positive emotions in the short term *(63-65)*.

However, chronic overconsumption of such foods triggers the disruption of the HPA axis, and the vicious circle of increased vulnerability to stressors, central obesity, metabolic dysfunction and mood-related disorders *(63)*. Sustained exposure to stress and elevated glucocorticoids on the other hand promote the accumulation of visceral adiposity and pro-inflammatory responses leading to the development of insulin resistance and metabolic syndrome *(66)*.

1.2.3. The effect of ghrelin on reward-related behavior

The pleiotropic hormone ghrelin was shown not only orexigenic, but also highly involved in food-seeking and reward related behaviors induced by palatable food, alcohol, nicotin, amphetamine and cocaine by activating the mesolimbic-dopaminergic neurocircuits consisting of VTA, nucleus accumbens (NAcc), amygdala, hippocampus and medial prefrontal cortex (PFC). Indeed, central ghrelin infusion enhances the reward/reinforcement properties of food, drugs of abuse in different rodent models as measured by conditioned place preference (CPP), locomotor stimulation, and NAcc dopamine release. The effect of ghrelin on psychostimulant action can be blunted by genomic or pharmacological ablation of GHSR1a, indicating the involvement of GHSR1a signaling in the VTA *(6, 8, 67, 68)*. Regarding its influence on food-seeking and food-motivated behavior, ghrelin administered centrally, peripherally or directly into the VTA stimulates the consumption of highly palatable, rewarding food (e.g. sucrose solution) in rats, which effect was blunted by the administration of a ghrelin antagonist. Furthermore, acute ghrelin administration into the amygdala decreased anxiety in the EPM and OF tests, but only in fasting rats, in order to help the animals to find food *(31, 69)*.

Human studies indicate that high ghrelin levels are associated with abstinence, contributing to alcohol craving and GHSR gene polymorphism is presumably associated with heavy alcohol consumption *(8)*. Concerning the other ghrelin-derived peptides, there are no data available yet on their role in drug addiction and reward related behaviors.

1.3. Peripheral effects of ghrelin

The GHSR1a was detected in many different peripheral organs (e.g. GI tract, pancreas, heart, lung, vasculature, kidney, gonads), and its endogenous ligand, ghrelin is involved in the regulation of different organ systems, e.g. the digestive, reproductive and cardiovascular system [*(6, 15, 16, 70) and fig. 4)*]. Multiple beneficial cardiovascular effects were attributed to ghrelin such as vasodilation, inotropic effect, attenuation of

ventricular remodeling and protection against cardiac cachexia *(16, 71)*. In patients and animals with heart failure ghrelin improved myocardial function and reduce post MI mortality *(72)*. Furthermore, in patients with metabolic syndrome ghrelin reversed endothelial dysfunction by the activation of nitric oxide-mediated and antiinflammatory mechanisms *(73)*.

1.3.1. Ghrelin and the glucose metabolism

Ghrelin is considered essential in maintaining glucose homeostasis during undernutrition, by activating gluconeogenic and/or growth hormone-regulated pathways *(21)*. Howewer, it was also reported to negatively influence glucose homeostasis by inhibiting insulin secretion in the pancreatic β cells and increasing hepatic glucose production in normal feeding states or obesity *(16, 21, 70)*. Furthermore, reduced plasma ghrelin levels were found in insulin–resistant conditions such as obesity, type 2 diabetes and hypertension *(74)*. These data suggest, that ghrelin might play a role in atherosclerosis, hypertension and diabetes.
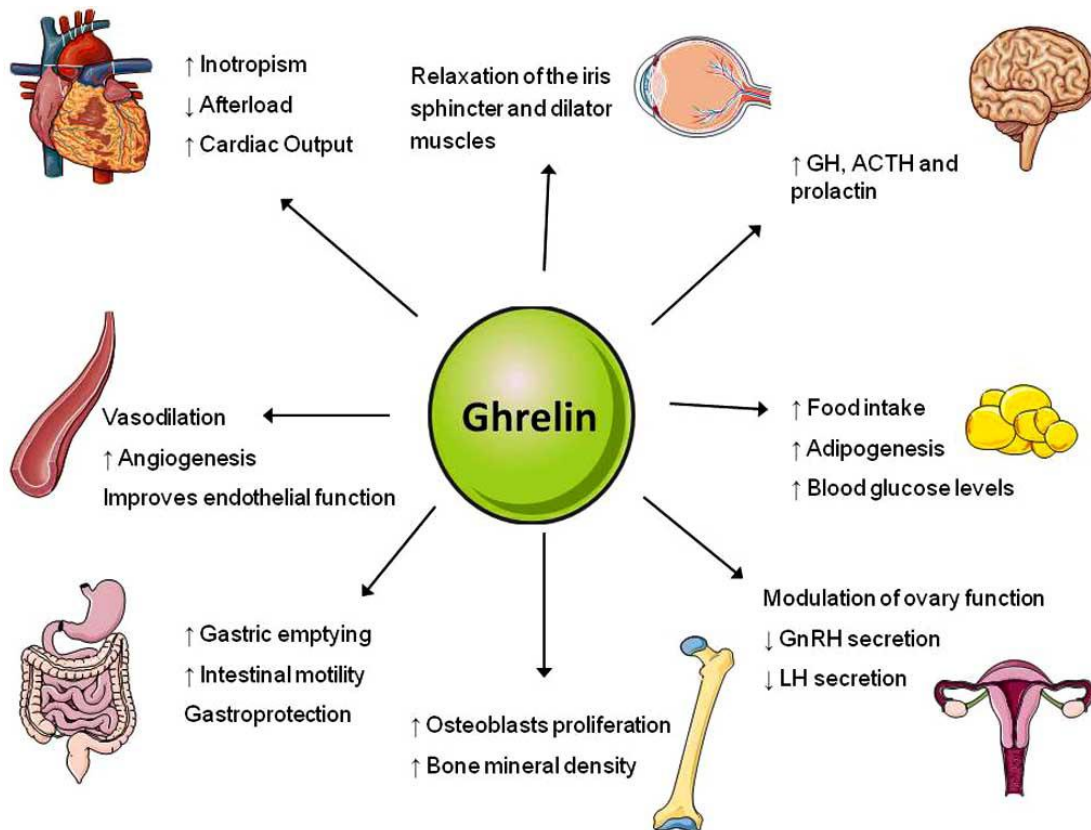

Figure 4. Peripheral effects of ghrelin (70)

2. OBESTATIN, THE SIBLING HORMONE

Obestatin is a 23-acid metabolic peptide (named from the Latin words "obedere" to devour and "statin" to suppress), derived from the preproghrelin gene *(fig. 1),* which was isolated first from the rat stomach in 2005 *(75).* However, obestatin is also expressed in other GI organs (pancreas, liver), adipose tissue, skeletal muscle, lungs, thyroid and mammary glands and testes, suggesting a multifunctional role of it, which can act both centrally and peripherally *(76).* It was originally described as a direct antagonist of ghrelin with anorexigenic effect. Both central and peripheral injection decreased food intake in a time- and dose-dependent manner *(75, 77, 78)*, body weight gain *(78),* and intestinal motility via the G-protein coupled receptor 39 (GPR39) – a member of the GHSR family *(75)* which was rapidly refuted as a receptor for obestatin by several studies *(79, 80).* To note, recent data suggest that obestatin may act through the GPR39 receptor in an autocrine/paracrine manner peripherally, namely as mitogenic factor in myoblasts *(81)* and GPR39 could mediate the metabolic effects of obestatin in the adipose tissue and GI system *(82, 83).*

However, the anorexigenic effect of obestatin where re-investigated and rejected under a variety of conditions *(84, 85)*, and only its acute food-intake inhibiting effect was reproducible *(86).* Furthermore, the initial effects on gastrointestinal motility were also questioned *(87).* This lack in experimental reproducibility may be attributable to the short biological half-life of the peptide in circulation *(88)* and in the central nervous system *(87)* and to the fact that, compared to other feeding hormones, obestatin barely passes the blood brain barrier *(89).* Despite these controversies, it is important to highlight that obestatin antagonizes acyl ghrelin's effect on GH secretion and food intake in rodents and fish *(90, 91)* while fasting resulted in elevated ghrelin- and reduced obestatin levels *(90).*

The ability of obestatin and its naturally occuring variant preproghrelin polymorphism Gln90Leu (Q90L) to inhibit acyl ghrelin's action on food intake and GH secretion may occur by targeting NPY and GHRH neurons *(92).* As it is shown on the figure *(fig. 5),* obestatin antagonizes the acyl ghrelin-induced inhibition of GABA neurotransmission and NPY neuronal activation, through a GHS-R antagonism or via other receptors still unidentified *(26).* The other mechanisms of the anorectic role of obestatin could be related to the inhibition of dopamin release (reversed by ghrelin), and antagonism of the hypothalamic serotonin inhibitory effects of ghrelin, when these two peptides were co-perfused in hypothalamic synaptosomes *(78).* Other anorectic hormones such as leptin, amylin and peptide YY (3-36) also inhibited the release of dopamine from rat

hypothalamic synaptosomes *(93, 94)*. On the other hand, serotonin also plays a well established, yet inhibitory role in feeding control, and the orexigenic ghrelin was shown to inhibit hypothalamic serotonin release. Obestatin reversed the effects of ghrelin on serotonin release, which can be partly explained by its anorectic role in the control of feeding *(78)*.
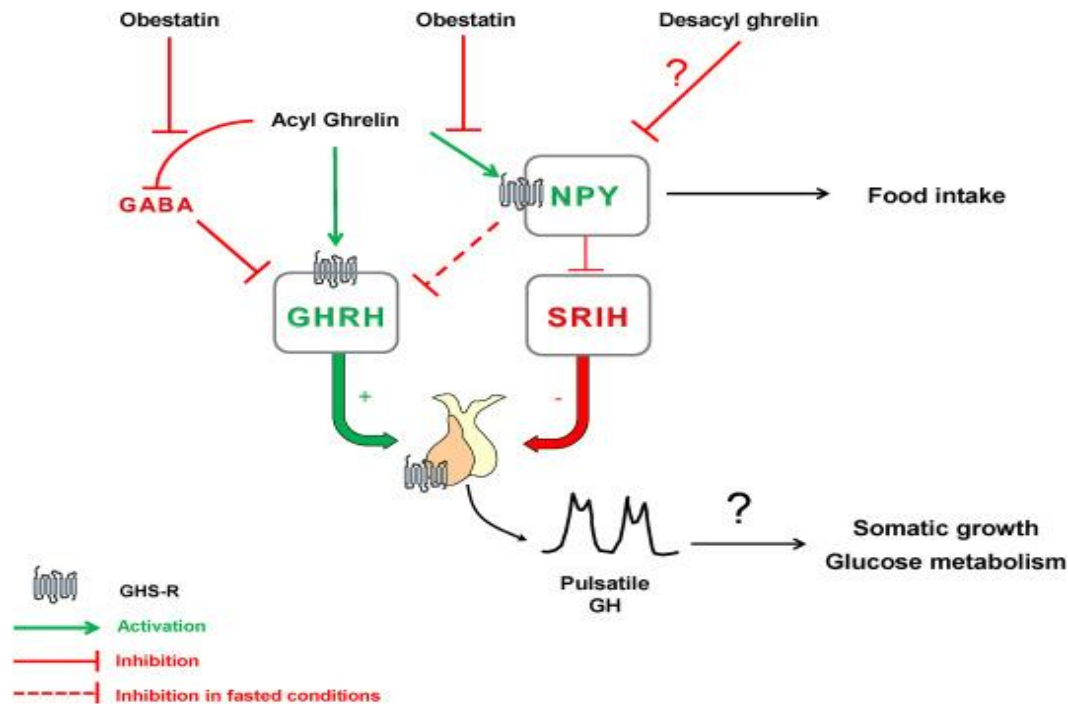

Figure 5. Regulation GH secretion and appetite by ghrelin-derived peptides in the hypothalamus (26)

In a recent remarkable study in mice, co-administration of obestatin and CCK8, a known satiety peptide, was more effective in inhibiting appetite and to decrease weight gain than CCK8 or obestatin alone, which indicate that it may modulate the function of other gastrointestinal peptides *(95)*.

2.1. Peripheral effects of obestatin
2.1.1. Obestatin and glucose metabolism

In the past years much of the scientific interest has focused on obestatin's effect on glucose metabolism *(96, 97)*. Obestatin was shown as an important regulator of pancreatic endocrine function and survival factor for pancreatic islet cells. It promotes proliferation and inhibits apoptosis in pancreatic β-cells by modulating the expression of adipogenic and glucoregulatory genes *(96)*.

The adipose tissue has multiple physiological functions, such as the control of energy balance, glucose and lipid metabolism, and the failure of these processes may induce

obesity, insulin resistance and diabetes. Obestatin can also influence insulin secretion and glucose uptake of different tissues, for instance by reducing insulin resistance and inflammation in mice on high fat diet *(98)*. Obestatin was shown protective in streptozotocin-induced experimental diabetes protecting the islet cells and increasing insulin secretion and reducing blood glucose levels *(99)*. Furthermore, because many similarities exist with the function of glucagon-like peptide 1 (GLP-1), obestatin was suggested as a ligand for the GLP-1 receptor in pancreatic beta cells and adipocytes *(100)*.

Obestatin level was found altered in diabetes and obesity which was documented in obese/overweight patients and those with abnormal glucose homeostasis (type 2 DM, insulin resistance and metabolic syndrome), *(101-103)*. Another study showed that basal secretions of obestatin and ghrelin were decreased in obese patients with insulin resistance reflecting both hormones as potential markers for adiposity and diabetes *(104)*. Obestatin levels also increased after gastric surgery for body weight reduction in obese and type 2 DM patients *(83)*.

Obestatin can also modulate the function of adipose tissue. Acting presumably in an autocrine-paracrine manner, it promotes adipocyte differentiation and survival, lipid accumulation, and it also regulates lipogenesis/lipolysis and circulating lipid levels *(fig.6, 7)*. Considering the crucial role of the adipose tissue dysregulation in obesity and diabetes, obestatin represents an attractive potential as a multitarget drug in these disorders *(83)*.

The effects of obestatin on glucose metabolism and adipose tissue are mediated through different signaling pathways, such as cAMP, phosphatidylinositol 3-kinase (PI3K)/Akt, and extracellular signal-related kinase 1/2 (ERK1/2)*,* which are known to be involved in cell survival and proliferation, inhibition of apoptosis, and cell differentiation [*(97), fig. 6*].
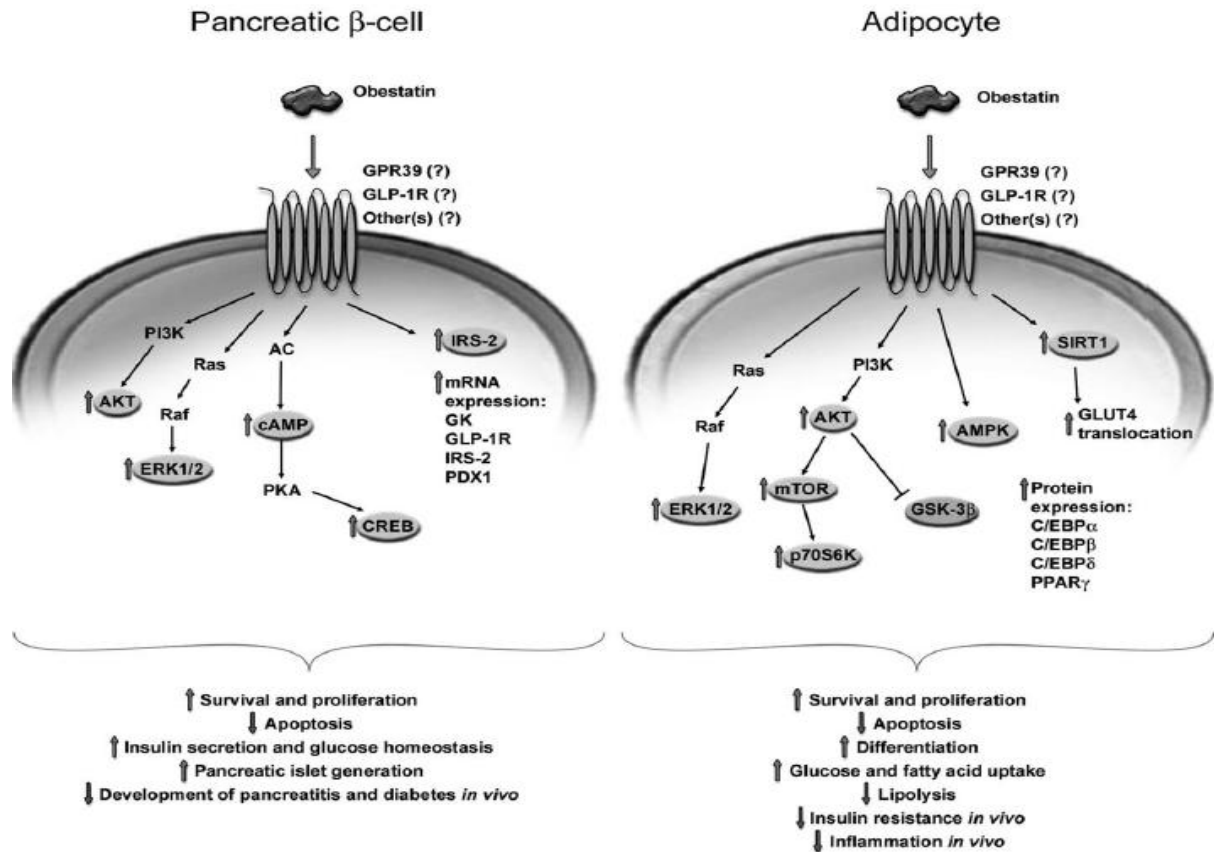
Figure 6. The mechanism of action of obestatin in endocrine pancreas and adipocytes (Gesmundo I. 2013)

GLP-1R, glucagon-like peptide-1 receptor; GPR39, G protein-coupled receptor 39; PI3K/Akt, phosphatidylinositol 3-kinase/Akt; ERK1/2, extracellular signal-regulated kinase 1/2; AC, adenylyl cyclase; cAMP, cyclic AMP; PKA, protein kinase A; CREB, cAMP response element-binding protein; IRS-2, insulin receptor substrate-2; GK, glucokinase; PDX1, pancreatic and duodenal homeobox-1; mTOR, mammalian target of rapamycin; p70S6K, p70 S6 kinase; AMPK, AMP-activated protein kinase; GSK-3β, glycogen synthase kinase-3β; SIRT1, sirtuin 1; GLUT4, glucose transporter 4; C/EBP, CCAAT/enhancer binding protein; PPARγ, peroxisome proliferator activated receptor-gamma

2.1.2. The role of obestatin in the GI system

The first study related to obestatin has demonstrated, that intraperitoneal injection in mice not only decreases food intake and body weight gain, but, opposite to ghrelin, it also inhibits gastric emptying and jejunal motility *(75)*. Since then, the inhibitory effects of obestatin on feeding and gastrointestinal motility have remained controversial, with some studies proving *(105, 106)*, while others failing to reproduce these effects *(84, 107, 108)*. To note, one study in rats has proven not only the GI motility-inhibiting effect of intravenous obestatin, but also, that its action might involve the activation of brain corticotropin-releasing hormone (CRH) type 1 and type 2 receptors and partially of the vagal afferent pathways, all being involved in the regulation of gastrointestinal motility.

The administration of obestatin activated CRH- and urocortin-2-containing neurons in the paraventricular nucleus of the hypothalamus, in line with the previous findings, showing that obestatin may act through CRH type I and II receptors in the brain *(105)*.

Obestatin is widely distributed in the GI system, and it may have some beneficial effects in both experimental and human GI diseases, for example it attenuates the inflammation in experimental ulcerative colitis, while the ghrelin/obestatin ratio has increased in patients with inflammatory bowel diseases during exacerbation *(83)*.

On the other hand, obestatin has been connected to *Helicobacter pylori* infection and gastric ulcers by inhibiting the expression of inflammatory cytokines. Furthermore, the antioxidant effect of obestatin was shown in ischemia/reperfusion-induced inflammation of the rat ileum. In acute pancreatitis of humans obestatin levels rise paralelly with the disease' severity. Pretreatment with obestatin in rats attenuates inflammation, edema and the release of pancreatic enzymes *(109)*.
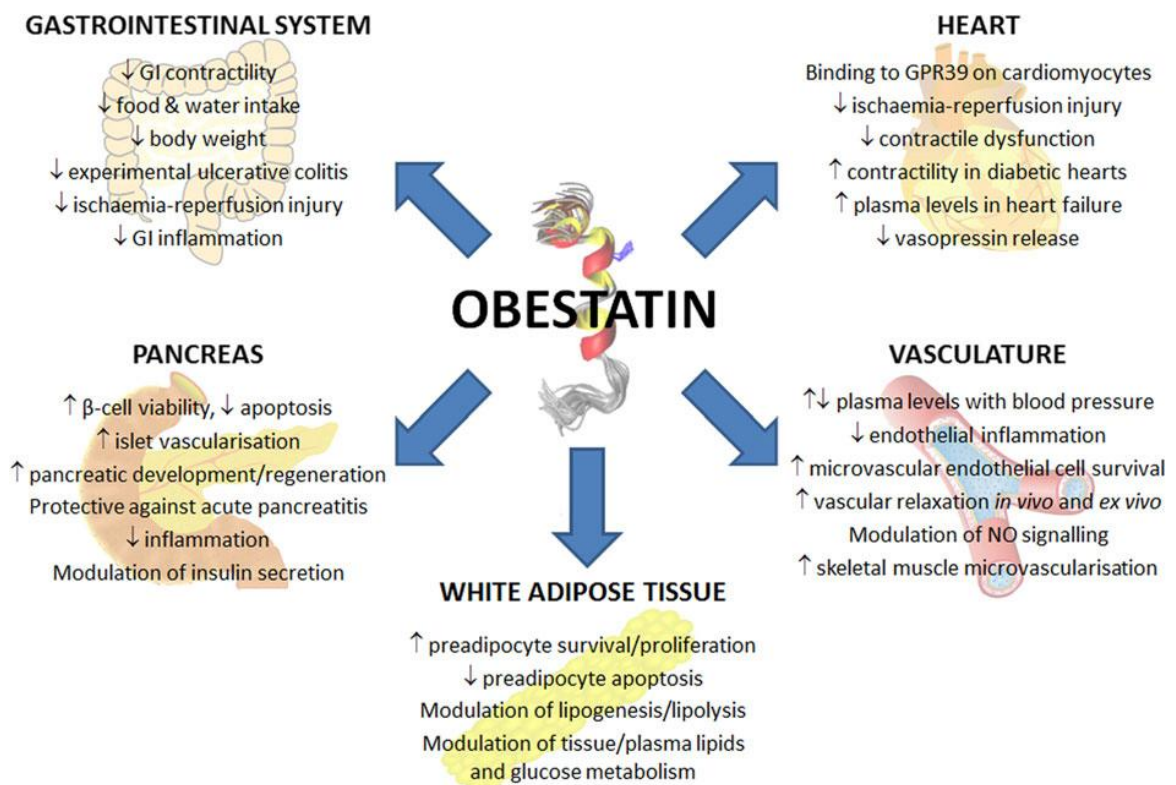

Figure 7. Metabolic, gastrointestinal and cardiovascular effects of obestatin (83)

2.1.3. The role of obestatin in the cardiovascular system

Metabolic syndrome includes comorbidities such as obesity, diabetes and hypertension, thus it has been plausible to suggest that obestatin may also exert important cardiovascular effects. In one remarkable study, fasting plasma obestatin levels were reduced in insulin resistant non-diabetics compared to insulin-sensitive subjects, and a negative correlation was found between plasma ghrelin and obestatin levels and abdominal fat accumulation, body mass index (BMI) and blood pressure *(101)*.

In another study hypertensive obese patients have shown significantly lower fasting plasma ghrelin and obestatin levels compared to normotensive obese subjects or controls. In addition, an inverse correlation was found between these hormone levels and fasting insulin as well as the homeostasis model assessment of insulin resistance (HOMA-IR), suggesting a role for both ghrelin and obestatin in metabolic syndrome-related conditions *(110)*. Furthemore, in patients with untreated hypertension low circulating plasma ghrelin and obestatin levels, and similarly a decreased ghrelin/obestatin ratio were observed *(111)*. In contrast to these data found in humans the same group has demonstrated higher hormone levels and an increased ghrelin to obestatin ratio in spontaneously hypertensive rats. The reason for these conflicting results is not known yet, but may be related to differences between species, however they indicate that the ghrelin/obestatin system participates in blood pressure regulation *(111)*, hypertension and obesity *(110)*.

Obestatin was also shown to protect the vascular endothelium by inducing NO-dependent vasodilation in both *ex vivo* and *in vivo* experiments in the mouse cerebral artery *(112)* as well as in obese or non-obese patients, which further suggest a role for obestatin in both normal cardiovascular function and in diabetes-related alterations *(83)*.

Regarding the direct effects of obestatin on the heart, in a rat model of ischaemia-reperfusion, obestatin was able to dose-dependently reduce infarct size, contractile dysfunction and to protect the cardiomyocytes from cell death, by triggering the activation of PI3K, Protein kinase C delta/-epsilon (PKC-ε, PKC-δ) and ERK1/2 intracellular pathways and a specific, yet unidentified receptor expressed in the heart *(113)*. Increased obestatin and ghrelin levels were reported in patients with chronic heart failure, especially those with cardiac cachexia *(114)* while in cardiorenal syndrome both obestatin and AVP levels were elevated *(115)*. The clinical significance of these findings requires further investigation.

2.2. Central effects of obestatin

2.2.1. Effects on thirst, sleep and thermoregulation

Hormones and neuropeptides control and integrate the neurocircuits of metabolism, thirst, thermoregulation, and sleep overlaping in the hypothalamus. Accordingly, besides its peripheral effects, central actions of obestatin were also identified.

To note first, when administered icv this peptide inhibited thirst in fed and fasted male rats, and pretreatment with obestatin also neutralized the dipsogenic effect of angiotensin II. Furthermore, it was also suggested that the anorexigenic effect of this peptide is a consequence of the thirst inhibition, the so called dehydration anorexia *(116)*. The same authors extended their studies, to reveal the effect of obestatin on fluid homeostasis in *ad libitum* watered and water-deprived animals. In these conditions obestatin inhibited water intake, as well as the stimulated and pharmacological secretion of AVP, that were reversed by the administration of anti-obestatin antiserum. Peripherally administered obestatin has a very short half life and presumably does not cross the blood-brain barrier *(89)*. Thus, the mechanism of action might involve pathways transmitting information through vagal afferents of the brainstem to the appetite and thirst centers *(117)*. Alternatively, obestatin might be released centrally, from pre-proghrelin expressing neurons of different brain sites *(3)*, in order to influence fluid balance and other important neurophysiological mechanisms *(117)*.

Studies in rats have also shown that obestatin influences sleep in a manner opposite to ghrelin, namely when given icv, it exerts a sleep-promoting activity. These findings may be related to obestatin's function as a satiety signal since several other anorexigenic hormones (e.g. leptin, CCK) increase sleep, while the orexigenic peptides (ghrelin, orexin, neuropeptide Y) have opposite effects by facilitating wakefulness *(118)*.

The products of the preproghrelin gene like ghrelin and obestatin are also involved in the adaptation of metabolism and sleep to thermoregulatory and feeding challenges as demonstrated by a remarkable study *(119)*. In mice, exposure to cold and fasting induces increased sleep in order to conserve energy, similarly to the state of hibernation. This effect was impaired in preproghrelin gene KO mice showing reduced sleep and a marked drop in the body temperature. However, the administration of obestatin attenuated the abnormal hypothermic response. These findings are extremely important, showing that obestatin may have a role in the complex humoral network regulating energy hoemostasis and sleep, and

the consequent comorbidities such as sleep disturbances, metabolic syndrome *(118)* and depression.

2.2.2. The effects of obestatin on neuroprotection

The neurogenesis in the adult hippocampus involves the proliferation, migration and differentiation of progenitor cells. These processes are impaired by different conditions, such as hypoxia, addictive drugs, sustained exposure to stress among others, while certain hormones and growth factors promote the proliferation and survival of the hippocampal neurons *(120)*.

Alzheimer's disease is the most common cause of dementia, characterized by the accumulation of amyloid plaques, abnormal phosphorylation of tau protein and the formation of neurofibrillary tangles, chronic inflammation and neurodegeneration leading to severe memory and cognitive dysfunction *(49)*. Since the hippocampus is crucial for learning and memory processes, the early impairment of neurogenesis at this brain site is a characteristic feature of Alzheimer's disease.

The search for neuroprotective agents is emerging, therefore a recent study has examined the potential role of obestatin on adult rat hippocampal progenitor cells exposed to growth factor deprivation and amyloid β peptide toxicity *(49)*. According to these results obestatin was shown to inhibit apoptosis and tau hyperphosphorylation and to promote proliferation and survival of cell progenitors. These effects involved enhanced GLP-1R mRNA and protein levels, as well as signaling through specific proliferative and survival pathways such as cAMP/PKA/CREB, MAPK/ERK1/2, PI3K/Akt. To note, GLP-1R was indicated to also mediate the survival and proliferative effects of obestatin on pancreatic β-cells and adipocytes *(96, 100)*.

A possible neuroprotective role of endogenous obestatin was also suggested, based upon the peptide immunoreactivity and the expression of preproghrelin gene in the hippocampal progenitors *(97)*. Obestatin also caused memory retention in two different tests (inhibitory avoidance and spontaneous object recognition), indicating that it influences both learning and memory processes related to brain structures such as the amygdala and hippocampus *(77)*.
In vivo studies in the future will hopefully reveal the posibble therapeutical potential of obestatin in neurodegenerative disorders *(97)*.

The possible neuroprotective role of obestatin was also investigated in a rat model of subarachnoidal haemorrhage (SAH). SAH leads to vasospasm, cerebral ischemia and

severe neuronal damage and death due to the activation of inflammatory pathways and accumulation of reactive oxygen species. The intraperitoneal administration of obestatin ameliorated the deleterious effects of SAH, by inhibiting apoptosis and leukocyte infiltration, the release of inflammatory mediators and cytokines, and it also protected the endogenous antioxidants of the brain. The antiinflammatory and antioxidant effects of obestatin deserve further investigations, in order to evaluate its possible role in treating conditions related to ischemic brain injury *(121)*.

In an earlier study *in vitro* obestatin elevated intracellular calcium by promoting ion influx and internal release in a population of cultured rat cortical neurons *(122)*. Calcium has well identified multiple physiological functions, as a second messenger being involved in muscle contraction, neural synaptic transmission, cell growth and proliferation, neurogenesis, learning and memory*,* the activity of enzymes, ion channels and pumps *(123)*. The identification of neuronal responses related to the obestatin receptor activation needs further *in vitro* and *in vivo* studies, however these results underscore the diverse functions of obestatin as a neuropeptide.

2.2.3. The role of obestatin in anxiety

The very first and until present the single study to reveal the effect of obestatin on anxiety has shown that icv administration of the peptide induces anxyiolitic-like effects in the EPM in rats *(77)*.

2.3. The stress response and depression as a stress-related disorder

Environmental and homeostatic challenges require integrated autonomic, neuroendocrine and behavioral responses from the body and the brain to maintain the homeostasis and survival of species. The sympatho-adrenal medullary system and the HPA axis are primarily responsible for the adaptive and protective mechanisms during stress, acting in concert with somatosensory cortex areas, the raphe nucleus and loecus coeruleus (involved in attention and arousal), and limbic structures, which mediate the cognitive (e.g. learning and memory) and behavioral processes of stress response.

As an immediate physiological response to stressor exposure the autonomic nervous system and catecholamines induce the activation of cardiovascular system (increased heart rate and blood pressure) and metabolic changes, described as the 'fight or flight' reaction, more than 100 years ago by Walter Cannon and colleagues *(125-127)*.

During stress adaptation the HPA axis and the sympathetic nervous system act synergistically in order to mobilise the necessary energy sources and to adapt the metabolism, vessels and the heart to stress *(125-127).* The key process of HPA axis activation involves the release of CRH from the parvocellular neurosecretory neurons and AVP from the magnocellular cells in the PVN of the hypothalamus. CRH on its turn stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the systemic circulation *(128).* CRH neurons innnervate and affect much of the brain and limbic areas (the amygdala, hippocampus, prefrontal cortex and nucleus accumbens) and also the monoaminergic systems (noradrenergic in locus coeruleus, serotoninergic of the dorsal raphe nucleus, dopaminergic of the VTA) in order to release their cognated neurotransmitters *(126, 129, 130).* ACTH acts on the adrenal gland for synthesis and secretion of glucocorticoids (cortisol in humans and corticosterone in rodents), the downstream effectors of the HPA axis. Glucocorticoids have a crucial role in mobilizing the energy sources necessary to adapt the body's metabolism towards coping with stress and restoration of homeostasis *(131).* The HPA axis and the adaptive response to stress is regulated by highly overlapping neuroendocrine circuits including glucocorticoid negative feedback, sympathetic andrenomedullary circuits, parasympathetic systems and the above mentioned limbic structures and higher-control cortical regions *(126).* Circulating glucocorticoids suppress CRH neurons within the PVN via endocannabinoid signaling acting on the excitatory neurotransmitter glutamate, and via the activation of hippocampal neurons and the inhibitory GABAergic circuits *(132).*

Although, the biological effects of glucocorticoids are usually adaptive to mantain homeostasis, inadequate or excessive activation of the HPA axis in terms of both magnitude and duration [(called allostasis or cacostasis *(129)*] may contribute to the development of different structural abnormalities in the central nervous system [(hippocampal atrophy and memory loss *(133)],* mental illnesses, obesity, DM, and cardiovascular pathologies such as atherosclerosis and hypertension *(129, 134-136).* The cardinal manifestations include anhedonia (loss of interest, drive and pleasure), poor concentration and self-esteem, feelings of worthlessness and guilt, impaired memory, anxiety, irritability, disturbances in sleep and appetite, and sustained disability in carrying out everyday life activities and social functioning *(60, 137).*

Different brain regions forming tightly interacting neural circuits were suggested to be involved in the development of these clinical manifestations. For instance the cognitive aspects of depression could be mediated by the hippocampus and neocortex, the striatum

(primarly the ventral striatum or nucleus accumbens and amygdala) might be responsible for the emotional manifestations of anhedonia and anxiety, while the neurovegetative disturbances of sleep, appetite, energy metabolism, and sexual activity suggest a role for hypothalamus *(138).*

Importantly, depression increases the risk of developing high-morbidity conditions such as cardiovascular diseases, stroke, Alzheimer`s disease and cancer. Furthermore it has been suggested to classify depression as metabolic syndrome type II, because it is strongly and bidirectionally associated with obesity and diabetes-related condition *(60, 139).* Depression can increase substantially the risk of developing type 2 DM, and the onset of disease may be related to major stressful events *(66).* The pathogenetic link needs to be elucidated, probably involving poor diet quality and sedentary lifestyle *(63),* sleep and circadian cycle disturbances, early life trauma and sustained stress exposure *(140).* Interestingly, studies have shown that effective antidepressant-anxiolytic therapies and stress handling strategies contribute to a better glycemic control in diabetic patients *(66).*

3. THE AIM OF THE STUDY

- The disruption of the HPA axis is involved in different conditions such as mood and eating disorders, addiction to drug of abuse, obesity and metabolic syndrome.

- The neuropeptide obestatin was shown to influence food intake, glucose and lipid metabolism, neurogenesis, thirst and sleep.

- However, there has been a single publication related to it's effect on anxiety in rats, while no data have been released on obestatin's effect on the behavioral patterns related to depression or drugs of abuse.

- For these reasons, in our experiments conducted in male CFLP mice we first tested the anxiety-related effects of the acute central administration of different doses of obestatin in the EPM and OF tests.

- In order to reveal the depression-related responses, by using a similar treatment regimen, we also investigated the effects of obestatin in the FST.

- Considering the well-established impact of the HPA axis in anxiety- and mood-related disorders, prior to obestatin treatments we administered Corticotropin-Releasing Hormone (CRH) receptor blockage with antalarmin in two different sets of paradigm, the OF test and FST, respectively.

- In order to underscore our behavioral results we also measured plasma corticosterone levels by fluorescence assay in the animal groups treated with obestatin and antalarmin.
- Furthermore, to find out whether obestatin's effect on anxiety and depression are mediated through Growth Hormone Secretagogue Receptor (GHSR) signaling, we administered ghrelin receptor antagonist pretreatment followed by OF testing and FST.
- Next, we investigated the possible effects of the chronic, central obestatin treatment on naloxone-precipitated morphine withdrawal, by using graded doses of morphine and obestatin, on day four followed by testing the animals in the OF and EPM tests.

4. MATERIALS AND METHODS
4.1. Experimental animals

CFLP male, 6 weeks old mice (Animal Husbandry Services, Domaszék, Hungary), weighing 25-35 g [*30 ± 5 g (141)*] were used for the experiments. Five animals per cage were housed in a room at controlled temperature (22-24°C) and on a 12-h dark–light cycle (lights on at 06:00 and off at 18:00 h), with food and water available *ad libitum*. Testing occurred between 8.00 to 10.00 am *(142)* and 10.00 to 12.00 am *(141)* respectively. At least 30 min before, mice were carried to the experimental laboratory in their home cages for habituation *(142)*. Each animal was used only on a single occasion for the experiments. All procedures were conducted in accordance with the instructions of the Ethical Committee for the Protection of Animals in Scientific Research of the University of Szeged *(142)*.

4.2. Surgery

For icv cannulation the mice were anesthetised intraperitoneally with sodium pentobarbital [Euthasol® 35 mg/kg or Nembutal®, Phylaxia-Sanofi, Budapest, Hungary *(141)*] and a polyethylene cannula was inserted into the lateral cerebral ventricle, at stereotaxic coordinates: 0.5 mm posterior, 0.5 mm lateral to the bregma, and 3 mm deep from the dural surface according to the atlas of Paxinos et al. *(143),* and fixed to the skull with cyanoacrylate containing instant glue. The animals were then allowed to recover for 5 or 4 days *(141),* respectively. After the end of the experiments, 2 µl of methylene blue was injected via the cannula of decapitated animals to check the permeability and the right

position. Data from animals with improper cannula were excluded from statistical analysis *(142)*.

4.3. Behavioral tests

4.3.1. Elevated plus maze (EPM) test

EPM is a well-known assay to monitor anxiety-like behavior in rodents *(144)*. The method is based on a conflict characteristic for rodents, namely the natural aversion to open, illuminated spaces and heights, and their drive to explore a new environment. The EPM apparatus (Columbus Instruments, Columbus, Ohio, USA) consists of four arms (87-mm wide, 155-mm long), elevated 63.8 cm above the floor, with two arms enclosed by 16.3-cm-high walls and illuminated with 60 W light situated 1 m above the maze. Mice were placed in the center of the maze facing toward an open arm, and the following behavioral parameters were recorded for 5 and 10 min respectively *(141):* the overall activity, reflected by the total number of entries into the arms, the percentage of open arm entries (open arms/total number of entries % – OAE%) and open arm time (open arm time/total time % – OAT%). An entry into the arms was registered when all four legs have crossed the entrance line to the respective arm *(142)*. After each animal, the apparatus was thoroughly cleaned with ethanol (96%) and water (141). The decreased number of open arm entries and time are associated with anxiety-like behavior, whereas an anxiolytic effect is reflected by an increase in these parameters *(144)*.

4.3.2. Open field (OF) test

The open field test is a widely used and accepted technique to evaluate the exploratory behavior and general locomotor activity in rodents. The apparatus (conducta, Experimetria Ltd., Hungary) consists of a set of five black-painted wood boxes ($40 \times 50 \times 50$ cm^3) located in an isolated room, with open top and a 60-W light placed 1 m above the arena floor. The apparatus is able to test the activity of 5 mice simultaneously, but separately *(141, 142)*. Each animal was placed individually in the center of the open field and their activity was registered for 5 min, the floor of the box being washed with ethanol (96%) and water and dried prior to the next animal testing *((141)*. During the test period the following parameters were monitored: ambulation time (s) and distance (cm), number of rearings and jumpings, immobility time (s), the percentage of the central distance (central/total ambulation distance %) and of the time spent in the central area (central/total ambulation time %) *(142)*.

4.3.3. Forced swimming test (FST)

The FST is a well-established, reliable and widely used behavioral test in rodents to evaluate depression-like behavior and the effectiveness of antidepressant drugs *(145)*. The immobility in the FST has been originally considered a response to an inescapable situation reflecting the behavioral despair found in human depressed patients *(146-148)*. In our laboratory the modified mouse FST was performed *(149, 150)*. A glass cylinder (12 cm in diameter and 30 cm in height) was filled with water (25 ± 1 °C temperature) to a height of 20 cm, the water was changed between mice. The animals were placed individually in the cylinder, and a 15 min pretest session was performed, during which diving mice were excluded from the experiment. The test session occured 24 h later for 5 min, when the durations of swimming, climbing and immobility were registered with a time-sampling scoring technique (every 5 sec). The behavioral procedure and analysis was performed by using a video recording device and the FST files were transfered to a PC and analyzed by an independent observer *(151)*. Swimming time was recorded when the mouse was in horizontal motion on the surface of the water; climbing time was measured when the mouse was participating in an active vertical motion with its forelegs above the water level; and immobility time was registered when the mouse was in an upright position on the surface with its front paws together and making only those movements necessary to keep itself afloat *(149, 150)*.

4.4. Treatment protocols

4.4.1. The effect of acute obestatin administration in the OF and EPM tests

Three different treatment protocols were used *(142)*.

1. One group of mice received graded doses (0.5 µg, 1 µg or 1.5 µg) of icv obestatin (Anaspec, Inc. USA). Control groups received 2 µl artificial cerebrospinal fluid (aCSF), the vehicle for peptide treatments was also 2 µl aCSF. The behavioral tests (EPM or OF) were performed 30 min after each treatment.

2. The CRHR1 antagonist antalarmin (Bachem, Switzerland) was given in a dose of 0.1 µg/2 µl aCSF icv, which did not influence the behavioral parameters *per se* in previous studies *(152)*, followed 30 min later by the icv injection of 1.5 µg obestatin, the most effective dose from the previous dose-response study. The OF test was performed 30 min after the administration of obestatin.

3. The third group of animals, 15 min before the administration of obestatin (1.5 µg/2 µl aCSF), received pretreatment with a selective GHSR1a antagonist, [D-Lys3]-Growth Hormone Releasing Peptide-6 ([D-Lys3]-GHRP6; Sigma-Aldrich Inc., USA) in a dose of 1 µg/2 µl aCSF (was ineffective alone in previous behavioral testing) followed after 30 min by the testing in the OF.

4.4.2. The effect of acute obestatin administration on plasma corticosterone levels

In order to determine the plasma corticosterone level, trunk blood was collected in heparinised tubes. After the initial centrifugation, the plasma samples were stored in *-80 °C* freezer, and analysed one week later. A fluorescence-based assay *(153)* was used to measure the plasma corticosterone concentrations 30 min after the different treatments *(142)*.

4.4.3. The effects of obestatin and of naloxone-precipitated withdrawal on the behavioral changes induced by morphine

Chronic morphine treatment was administered as described earlier *(154)*. Mice received subcutaneous (sc), twice-daily (at 08.00 am and 04.00 pm) injections of ascending doses of morphine according to the following regimen: day 1:10 mg/kg, day 2: 20 mg/kg, day 3: 40 mg/kg. Mice were also treated daily (at 08.15 am) with obestatin (1.5 µg/2 µl, aCSF icv). On the test day (day 4) at 08.00 am, a single dose of morphine (20 mg/kg, sc) was given followed by icv injection of obestatin at 09.45 am. Two hours after the last morphine injection, naloxone (naloxone-HCl, Sigma-Aldrich) was administered in a dose of 0.2 mg/kg sc, followed after 5 min by the testing of mice in the EPM or OF. Control mice received sc saline or icv aCSF *(141)*.

4.4.4. The effect of obestatin treatment on the FST parameters

Three different treatment regimen were used, similarly to those in the OF and EPM tests. Control groups received 2 µl aCSF, and the vehicle for peptide treatments was also 2 µl aCSF.

1. In order to generate a dose-response curve, mice were treated with different doses of (0.5 µg, 1 µg or 1.5 µg/2 µl aCSF) of icv obestatin (Anaspec, Inc. USA). The most effective dose of obestatin (1 µg) was used in the following experiments.

2. Next, 30 min prior to the injection of obestatin, pretreatment with antalarmin was given in a dose of 0.1 µg/2 µl icv, which in previous studies did not influence the behavioral parameters *per se (152).* The FST test was performed 30 min after the administration of obestatin, as described previously.

3. The third group of animals, 15 min before the administration of obestatin received pretreatment with [D-Lys3]-GHRP6 in a dose of 1 µg/2 µl aCSF (was ineffective *per se* in previous behavioral testing) followed after 30 min by FST testing.

4.5. Statistical analysis

Statistical analysis of the results was performed by one-way analysis of variance (ANOVA), followed by the Holm-Sidak *post hoc* test for multiple comparisons when the test prerequisites were fulfilled. When the test of the homogeneity of variances was not met, nonparametric ANOVA on ranks (Kruskal-Wallis) was performed, followed by Dunn's test for multiple comparisons. Data with morphine treatment was analyzed by 2-way repeated measure. $P < 0.05$ was accepted as a significant statistical value.

5. RESULTS

Data are presented as means ± SEM. Number in bars on the graphs indicate the number of animals used.

5.1. The effect of acute obestatin treatment in the OF test

The different doses of obestatin did not influence the immobility time $[F(3,24) = 0.712, p = 0.555]$, the ambulation distance $[F(3,24) = 0,935, p = 0.441]$ and time $[F(3,24) = 0.827, p = 0.493]$, or the number of rearings $[H = 0.192, p = 0.979]$ and jumpings $[H = 0.827, p = 0.843]$, respectively. The percentage of central ambulation (central/total ambulation distance %) was decreased by 1.0 µg and 1.5 µg obestatin compared to the control groups $[F(3,24) = 4.799, p = 0.010]$ *(Fig. 8),* while the percentage of central time (central/total ambulation time %) showed a decreasing tendency $[F (3,24)=2.902, p= 0.058]$. The CRHR1 antagonist antalarmin alone did not affect the parameters tested in the OF. Pretreatment with antalarmin increased the percentage of central ambulation compared to the mice group treated with 1.5 µg obestatin $[H = 33.127, p < 0.001]$ *(Fig. 9).* The ghrelin receptor antagonist [D-Lys3]-GHRP6 also blunted the effect of 1.5 µg obestatin on central ambulation $[H = 33.127, p < 0.001]$ *(Fig. 9),* and had no effect on the OF parameters *per se*.
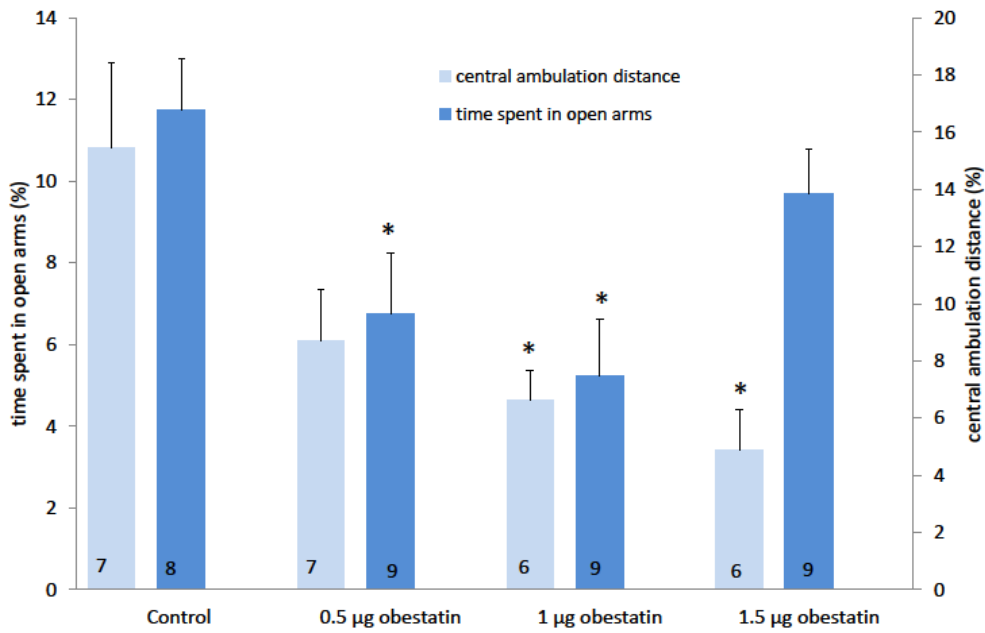
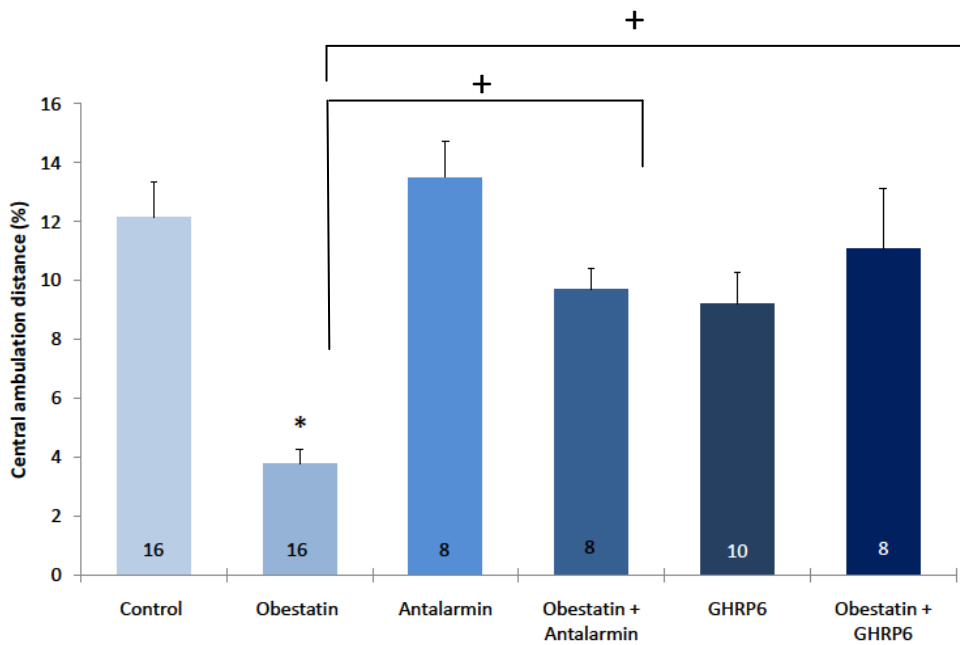*Figure 8. The effect of Obestatin on OF and EPM behavior. * p < 0.05 vs. Control*

*Figure 9. The effect of Antalarmin and GHRP6 on Obestatin -induced central ambulation distance in the OF test. * p < 0.05 vs. Control, + p < 0.05 vs Obestatin+Antalarmin, and Obestatin+GHRP6*

5.2. The effect of acute obestatin treatment in the EPM test

Treatment with different doses of obestatin did not influence the number of total entries, which indicates the total activity [$F_{(3,33)} = 2.107$, $p = 0.119$]. Doses of 0.5 µg and 1.0 µg obestatin decreased the OAT% [$F_{(3,33)} = 4.882$, $p = 0.007$] *(Fig. 8)*. Regarding the OAE% results a decreasing tendency was observed [$F_{(3,33)} = 2.002$; $p = 0.134$].

5.3. The effect of obestatin treatment on plasma corticosterone levels

Obestatin in doses of 1 µg and 1.5 µg increased corticosterone levels [$H = 22.560$, $p < 0.001$] *(Fig. 10)*. Antalarmin antagonised the effect of 1 µg obestatin (the most efficient dose) on corticosterone elevation [$F_{(3,34)} = 13.653$, $p < 0.001$] *(Fig. 11)*, while pretreatment with the ghrelin receptor antagonist [D-Lys3]-GHRP6 exerted no effect ($H = 13.728$; $p = 0.003$; data not shown).
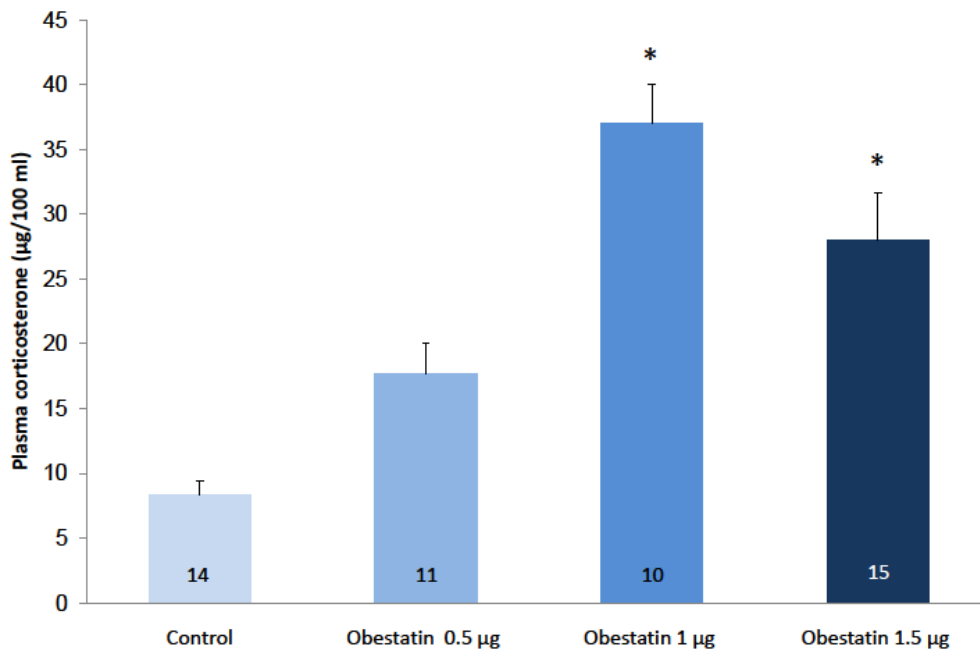

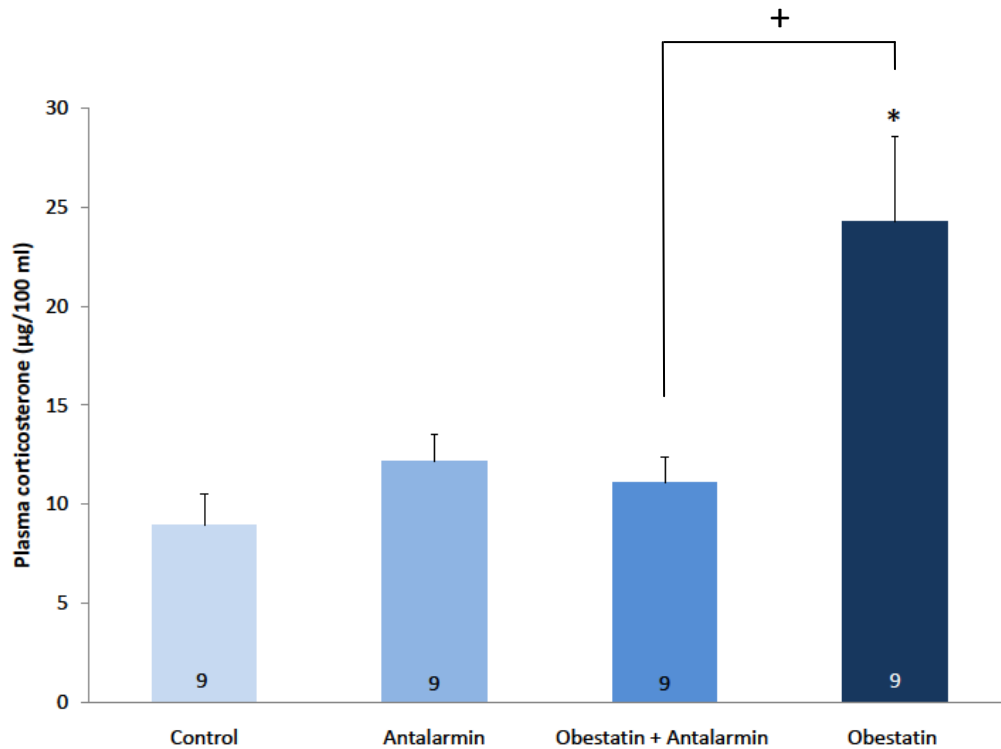*Figure 10. The effect of Obestatin on plasma corticosterone level. * p < 0.05 vs. Control*

*Figure 11. The effect of Antalarmin on Obestatin- evoked plasma corticosterone elevation .
* p < 0.05 vs. Control, $^{+}$ p < 0.05 vs. Obestatin+Antalarmin*

5.4. The effects of obestatin and of naloxone-precipitated withdrawal on the behavioral changes induced by morphine

5.4.1. EPM results

Treatment with the graded doses of morphine and obestatin did not influence significantly the parameters in the EPM. Obestatin treated mice undergoing withdrawal showed a decreased tendency in the OAT% and OAE% compared to the morphine withdrawal mice that did not receive obestatin. Morphine withdrawal mice receiving obestatin did not show significant changes in total activity compared to morphine withdrawal mice $[F_{(4,38)} = 9.243, \ p < 0.682]$. Naloxone-precipitated withdrawal induced a significant increase in both parameters compared to control mice and mice treated with morphine and obestatin (OAT%: $[F_{(4,35)} = 9.637, \ p < 0.0001]$; OAE%: $F_{(4,35)} = 7.12, \ p < 0.0003]$) *(Fig. 12, 13).*
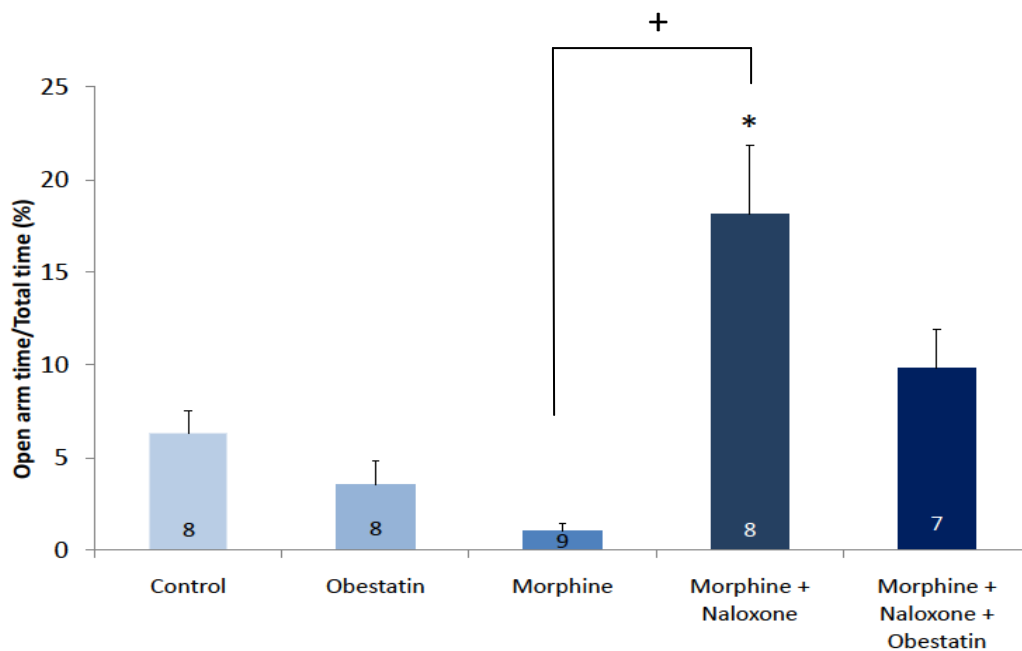
29

*Figure 12. The effect of Obestatin on Morphine withdrawal-induced OAT% in the EPM test. * p < 0.05 vs. Control, , + p < 0.05 vs Morphine*
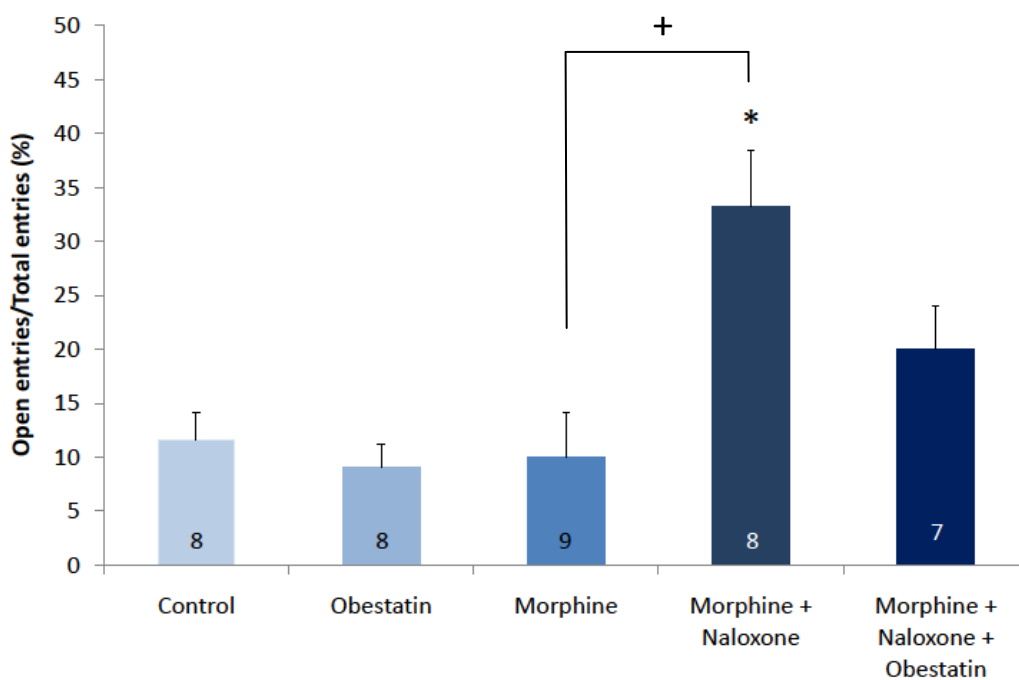

*Figure 13. The effect of Obestatin on Morphine withdrawal-induced OAE% in the EPM test. * p < 0.05 vs. Control, + p < 0.05 vs Morphine*

5.4.2. OF test results

Treatment with graded doses of morphine significantly decreased the percentage of center ambulation distance [$F_{(4,47)} = 16.13$, $p < 0.0001$], while the percentage of time spent in the central area showed a decreasing tendency. Chronic administration of obestatin alone had no significant effect on the OF parameters [$F_{(4,51)} = 13.149$, $p < 0.998$]. Naloxone precipitated morphine withdrawal caused a significant increase in the percentage of central ambulation distance [$F_{(4,47)} = 16.13$, $p < 0.0001$], and time [$F_{(4,47)} = 11.06$, $p < 0.0001$] *(Fig 14-15)*. Obestatin significantly decreased the percentage of central ambulation time [$F_{(4,47)} = 11.06$, $p < 0.0001$] and caused a decreasing tendency in central ambulation distance [$F_{(4,47)}=16.13$, $p< 0.0001$] in mice undergoing naloxone-precipitated morphine withdrawal *(Fig14-15)*.
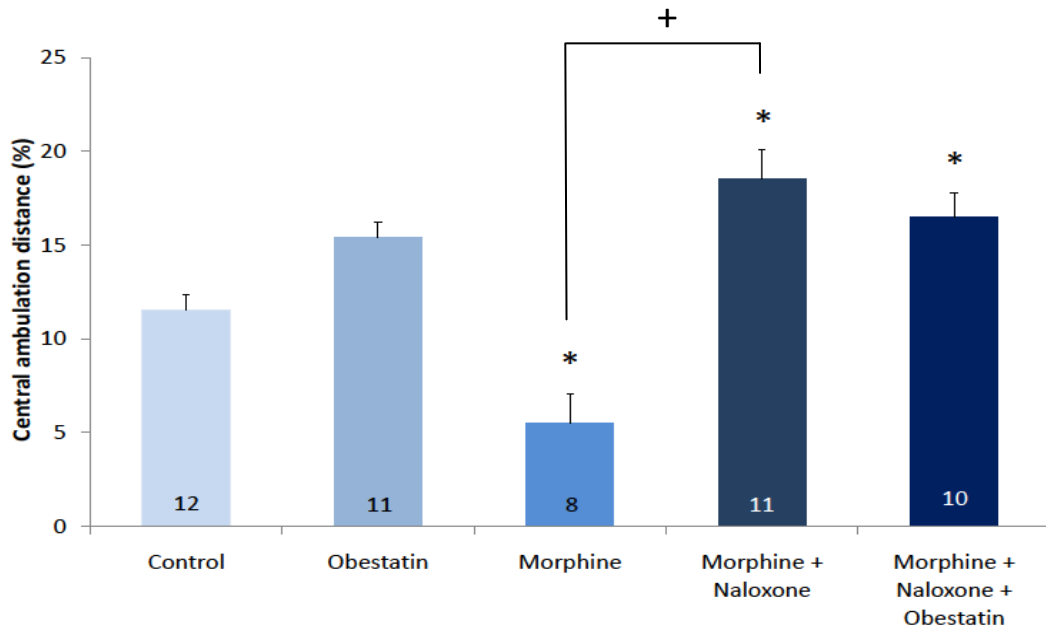

*Figure 14. The effect of Obestatin on Morphine withdrawal-induced central ambulation distance in the OF test. * p < 0.05 vs. Control,+ p < 0.05 vs Morphine*
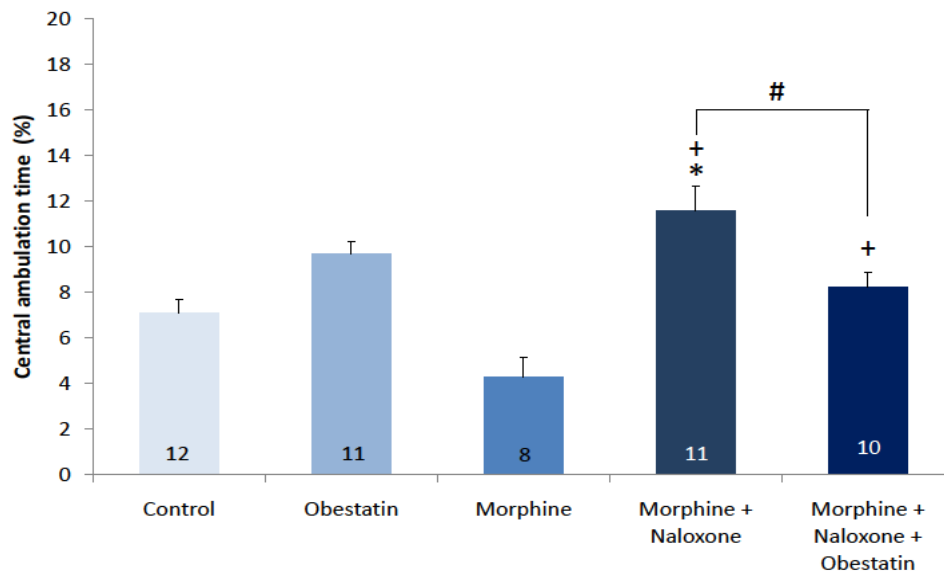
*Figure 15. The effect of Obestatin on Morphine withdrawal-induced central ambulation time in the OF test. * p < 0.05 vs. Control, [+] p < 0.05 vs. Morphine, # p < 0.05 Morphine+Naloxone*

5.5. Results in the FST

Immobility score was significantly increased [$F(3,59 = 4.557)$, $p < 0.006$] *(Fig. 16),* and the swimming score was decreased [$F(3,59 = 2.648$, $p < 0.05$] by 1 µg of obestatin *(fig. 17).* A decreasing tendency in the climbing score was also observed after different doses of obestatin [$F(3,59) = 1.146$], $p < 0.338$] *(fig. 18).* Pretreatment with antalarmin antagonized the effect of 1 µg obestatin on both immobility [$F(3,46) = 8.653$, $p < 0.0001$] *(fig. 19),* and swimming score [$F(3,46) = 5.515$, $p < 0.0025$] *(Fig. 20).* Combined treatment with obestatin and the ghrelin receptor antagonist [D-Lys3]-GHRP6 decreased the immobility score [$F(3,42) = 10.73$, $p < 0.0001$] *(Fig. 22),* also increased the swimming [$F(3,42) = 6.290$, $p = 0.0013$] *(fig. 23)* and the climbing scores [$F(3,42) = 3728$, $p < 0.0183$] *(fig. 24).*
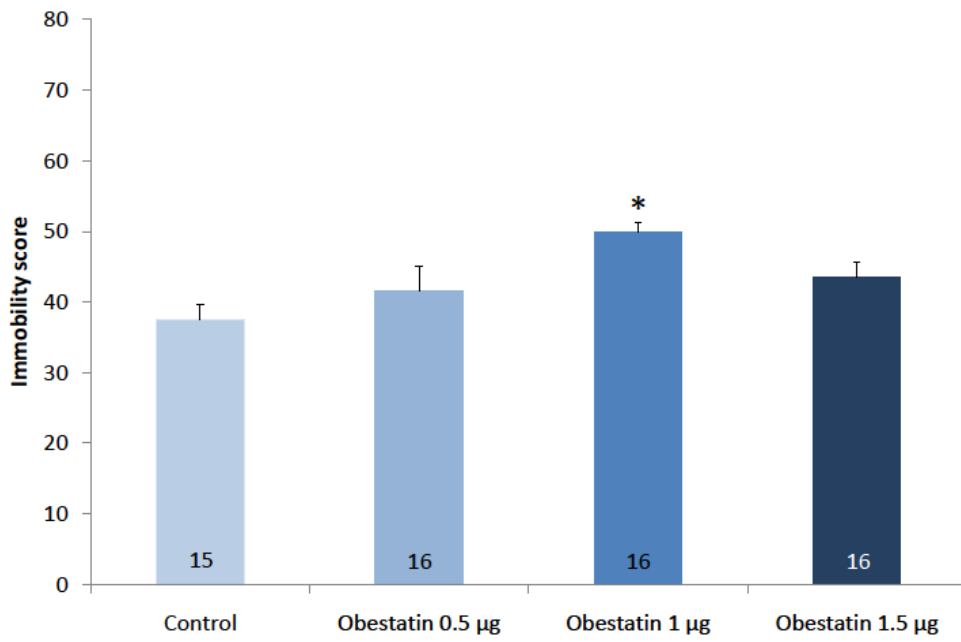
*Figure 16. The effect of Obestatin on immobility behavior in the FST test. * p < 0.05 vs. Control*
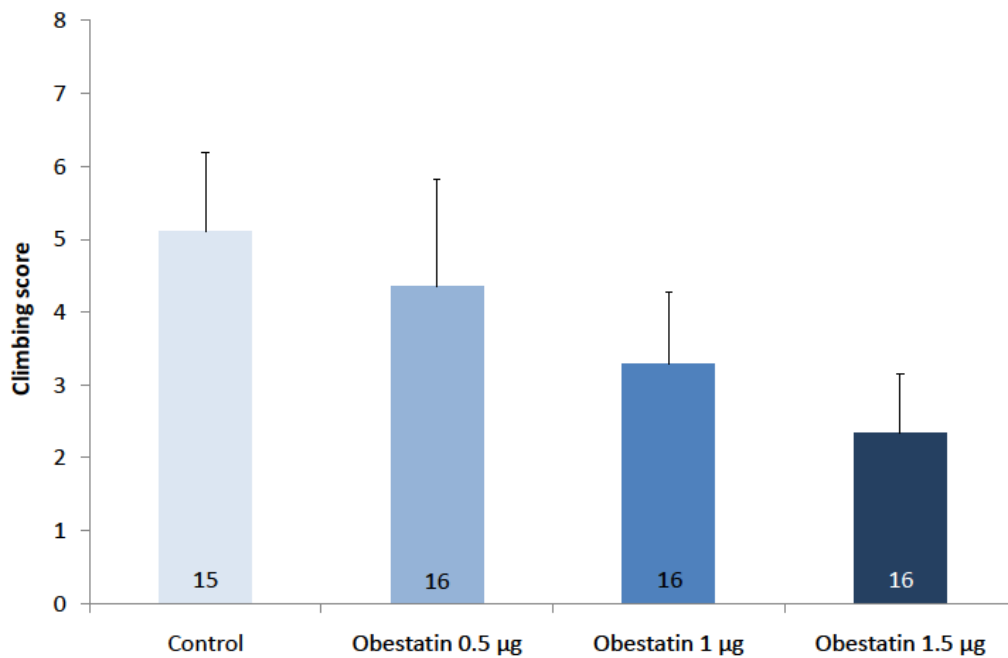
*Figure 17. The effect of Obestatin on swimming behavior in the FST test. * p < 0.05 vs. Control*
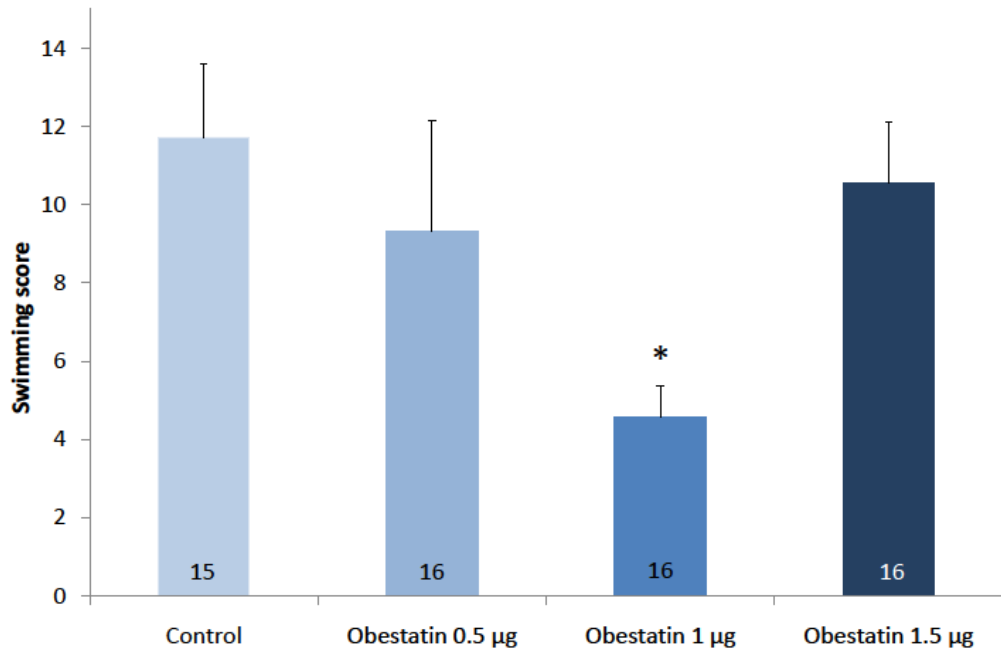
Figure 18. The effect of Obestatin on climbing behavior in the FST test. * p < 0.05 vs. Control
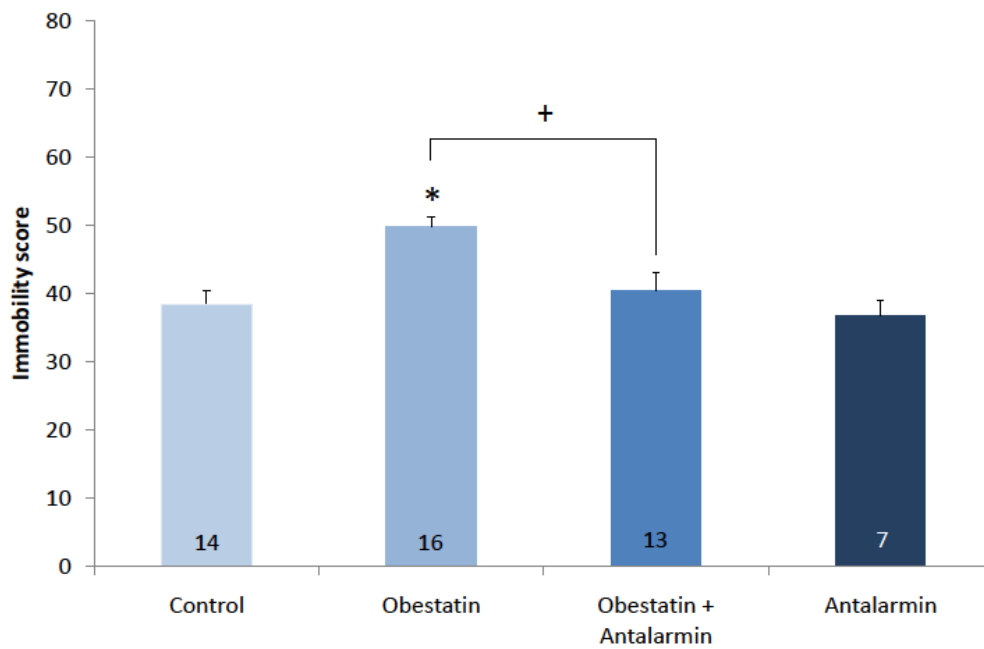
Figure 19. The effect of Antalarmin on Obestatin induced immobility in the FST test. * p < 0.05 vs. Control, + p < 0.05
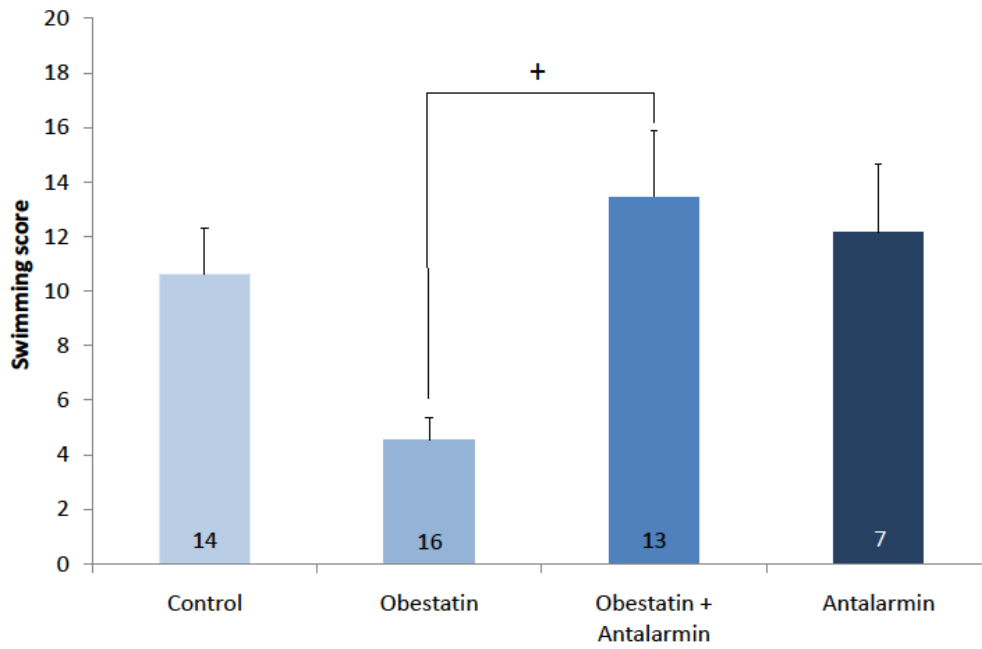
Figure 20. The effect of Antalarmin on Obestatin evoked swimming in the FST test. * p < 0.05 vs. Control, + p < 0.05

Figure 22. The effect of GHRP-6 on Obestatin induced immobility in the FST test. * p < 0.05 vs. Control, + p < 0.05
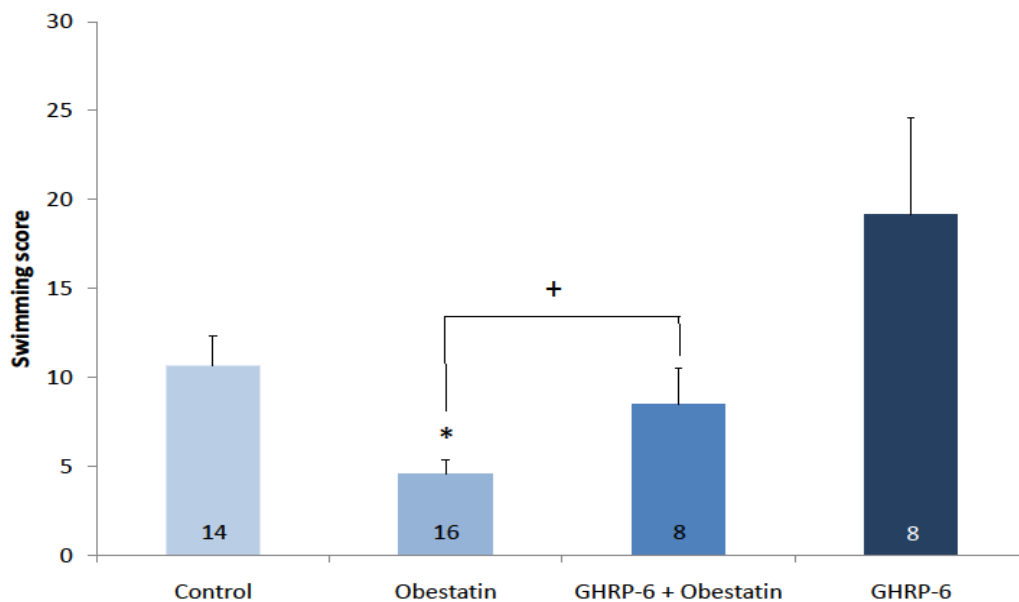
Figure 23. The effect of GHRP-6 on Obestatin induced swimming in the FST test. * p < 0.05 vs. Control, + p < 0.05
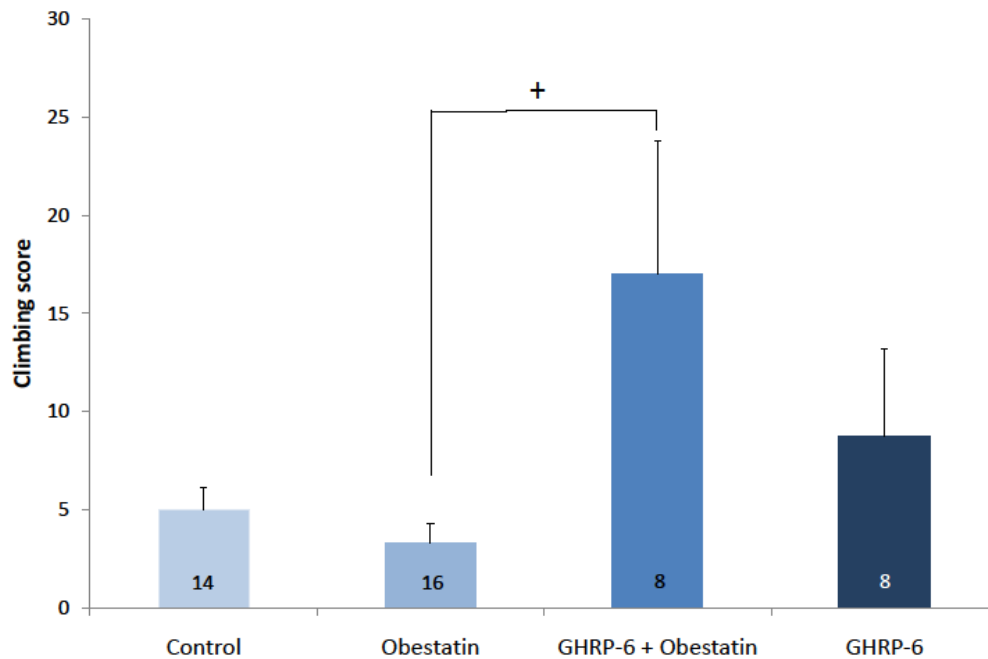
*Figure 24. The effect of GHRP-6 on Obestatin induced climbing in the FST test. * p < 0.05 vs. Control, + p < 0.05*

6. DISCUSSION
Obestatin exerts anxiogenic- like effects in the EPM and OF test

In our studies we demonstrated for the first time that obestatin exerts anxiogenic-like effects in mice in two different paradigms, namely the EPM and OF tests. To note, EPM was shown more sensitive compared to OF testing, since the most effective dose inducing anxiogenic-like behavior were lower in the EPM (1.0 μg vs 1.5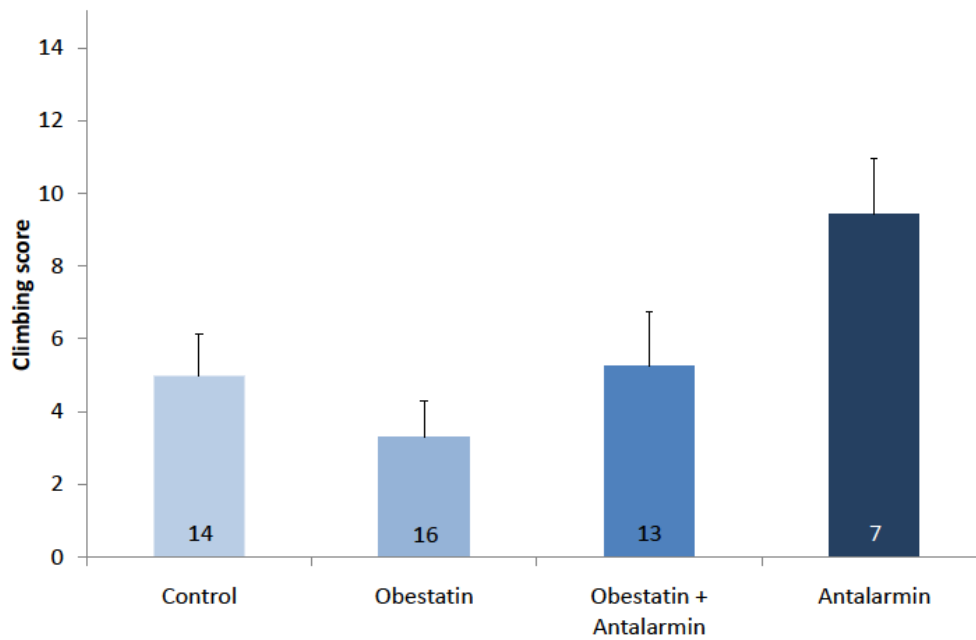 μg). It must be noted, however, that obestatin was originally reported to cause anxiolytic-like effects in rats in the EPM *(77)*. A possible explanation for the contradictory results might be the differences in experimental design (dosage regimen, animal species/strains used) and conditions (basal vs. stressed), as well as feeding state (food available *ad libitum* vs. calorie restriction) which all have high impact on the outcome of behavioral studies related to both ghrelin *(36, 43, 55),* and obestatin *(155, 156).*

Regarding ghrelin's effect, there is still ongoing debate whether ghrelin alleviates or aggravates anxiety-related behavior *(44).* To resolve this conflict, a possible dual role of the peptide was suggested *(9, 44, 55).* Accordingly, in unstressed conditions, or by acute or chronic central or intraperitoneal *(33)* administration, ghrelin would induce an anxiogenic-

like effect in rodents *(17, 32, 33, 35, 36, 157)*. Elevating ghrelin levels by 10 days of caloric restriction and subcutaneous ghrelin injection resulted in anxiolytic-like effects in the EPM *(38)* and acute restraint stress paradigm in mice *(39)*. These findings led to the hypothesis, that ghrelin might have a crucial, survival-promoting role in the adaptive response to ameliorate stress and to help the animal to find food *(44)*.

In our studies obestatin was administered to unstressed mice, therefore, it is reasonable to assume that obestatin, similarly to ghrelin exerts anxiety-like action in basal conditions..

The anxiogenic-lik effects of obestatin are mediated through HPA axis activation

In concert with our behavioral findings, the different doses of obestatin administered also elevated plasma corticosterone levels, which highlights the well-identified correlation between the HPA axis activation and anxiety-related conditions *(125, 129, 136, 158, 159)*. The stimulatory effect of obestatin on ACTH secretion was further demonstrated in pituitary cell cultures from non-human primates (baboons) *in vitro*, and in mice *in vivo,* with a parallel increase in the expression of pituitary CRH receptors *(160)*.

In our studies, administration of the CRHR1 antagonist antalarmin blunted the anxiogenic-like effect in the OF test and the elevation in plasma corticosterone levels induced by obestatin, suggesting the involvement of CRHR1 and HPA axis. In line with our findings, the anxiogenic-like effect of ghrelin was also suggested to be mediated by the stimulation of the HPA axis. Intraperitoneal injection of ghrelin increased hypothalamic CRH expression and produced a significant dose-dependent increase in serum corticosterone levels, while the administration of a CRH receptor antagonist inhibited ghrelin-induced anxiogenic-like effects in the EPM in mice *(33)*. Ghrelin, on the other hand was demonstrated to not only influence anxiety-, but also depression-related behavioral patterns in rodents.

Obestatin exerts depressive-like effects in the FST

As mentioned earlier, elevated ghrelin levels exerted anti-depressive-like responses in the FST, in the chronic social defeat stress model (38), as well as due to acute central ghrelin injection in the tail suspension test or following bilateral olfactory bulbectomy in mice (41). As it was earlier suggested, in conditions of stress, ghrelin, as a part of the adaptive response, induces anxiolytic and anti-depressant responses in order to protect the

subject from excessive anxiety and the development of depression *(8, 57)*. In contrast to these findings, in basal, unstressed conditions chronic central administration of ghrelin, while inducing anxiogenic-like behaviors in the OF and EPM tests, it also exerted depression-like behavior in the FST in rats *(36)*. Similarly, administration of antisense DNA for ghrelin into the lateral ventricles in rats caused anxiolytic-like effects (in the EPM, black and white test or conditioned fear test) and decreased depression-induced immobility in the FST *((161)*.

The depressive-like effects of obestatin are mediated through HPA axis activation

In agreement with these results, in our studies we demonstrated for the first time, that icv injection of obestatin, in a dose of 1 μg (which has also induced anxiety-like behavior in the EPM), significantly increased the immobility score, while decreasing the swimming score in the FST suggesting not only on anxiogenic-like, but also a depressive-like effect for obestatin. These effects were reversed by pretreatment with the CRHR1 antagonist antalarmin, which decreased immobility and increased the swimming score, again highlighting the involvement of HPA axis in the mediation of anxiety- and mood-related behavioral patterns induced by obestatin.

The disruption of normal HPA axis activity is associated with many neuropsychiatric disorders, particularly depression, that represents a major socio-economical and health burden worldwide. According to data from the WHO an estimated 350 million people in all genders (with female predominance), ages, and different social backgrounds are affected with a prognosis of rising tendency in the next decades. Even more severely, depression increases the risk for suicide and is responsible for about 800 000 death cases every year *(139)*.

The conventional antidepressant treatment can be effective in 60-80% of the cases, however, this is only available for fewer than 25% of the affected patients, due to limited economical and human resources, underdiagnosis, or misdiagnosis and unwarranted prescription of antidepressants. These drugs have many known undesirable side effects (sedation, hypotension, weight gain, sexual abnormalities), which often lead to poor compliance, relapse of the disease, and even more, increased risk for suicide. Another major problem is the high percent (up to 40%) of therapy resistant cases *(139)*.

Despite thorough research the etiology of depression has not been elucidated yet, even so both genetic and enviromental factors presumably contribute to the development of the disease. Among the etiological factors, persistent psychological stress and the dysfunction of HPA axis were identified as major neurobiological findings in patients

with depression *(137, 162, 163)*. High cortisol concentrations were found in the plasma and the urine of the patients, and the dexamethasone suppression test has shown an abnormally low ACTH and cortisol suppression, while the ACTH response to CRH was also blunted *(131, 136)*. CRH has an important role in early neurogenesis and neuroprotection, and polymorphism of the gene encoding CRHR1 has been associated with exposure to stressful events in childhood and adulthood depression. Furthermore, early life stress, social deprivation and undernutrition lead to HPA axis dysfunction and hypercortisolaemia, which increase the risk for developing metabolic (hyperlipidemia, tpye II diabetes) and mood disorders such as depression later during life *(163)*.

High amount of animal studies using different stress models have also underscored the importance of HPA axis disruption and consequently hypercortisolaemia in the pathomechanism of depression leading to abnormal anxiety and aggression reactions, alterations of monoaminergic sytem and neurogenesis, reduction in limbic structures and cognitive impairment. HPA axis activity correlated also with the relapse and remission of depressive symptoms. Injection of CRH icv induced anxiety and depression which was blunted by the administration of CRHR1 antagonists *(129, 131)*. These substances produced anxiolytic-like effects in rodent models such as the conditioned fear, neonatal isolation, shock-induced freezing, defensive burying behavior, social interaction test, EPM and OF tests *(164)*.

The oral administration of antalarmin, a CRHR1 antagonist, in male rhesus macaques significantly decreased the social stress-induced plasma ACTH and cortisol elevations and increased the exploratory behavior *(130)*. Several ongoing clinical trials have assessed the therapeutical potential of these substances in anxiety and depression however, the results are still inconsistent *(164)*.

In our experiments we have demonstrated for the first time that obestatin's anxiogenic- and depressive like behavioral effects are mediated by the HPA axis and CRH receptor activation, since these effects were blunted by the CRHR1 antagonist antalarmin *(142)*, which merits further preclinical and clinical studies.

The mechanism of action of obestatin on anxiety and depression involves GHSR signaling

Regarding the mechanism of action of obestatin, no specific signaling pathway or receptor has been identified yet. To note, the involvement of the GPR39 receptor cannot be completely refuted yet, since icv administration of antisense DNA for the GSHR family member, GPR39-1b, which is widely distributed in the central nervous system (e.g. amygdala, hippocampus) caused anxiolytic-like effect in rats in the EPM and black-white box test *(165)*. To evaluate further the mechanism of action of obestatin, we tested the possible role of GHSR signaling in the OF and FST tests. According to our results administration of the ghrelin receptor antagonist [D-Lys3]-GHRP6 blunted the anxiogenic-like responses induced by obestatin in the OF test, by increasing the precentage of central ambulation *(142)*.

Furthermore, pretreatment with the ghrelin receptor antagonist antagonized the depressive-like effects of obestatin in the FST, by increasing the swimming and the climbing scores and decreasing the immobility score *(Szakács J. et al., in preparation)*. As mentioned earlier, the possible involvement of GHS/ghrelin receptor signaling in the mediation of obestatin's action was also suggested in correlation to its anorexigenic role and interaction with ghrelin on feeding behavior *(26, 78)*. This idea was further supported by an in vitro study, in which the administration of the ghrelin receptor antagonist [D-Lys3]-GHRP6 reduced the survival of β cells and human islet cells induced by obestatin *(95)*. Furthermore, the administration of obestatin stimulated GHSR expression *in vitro* in pituitary cell cultures from baboons and mice, while it downregulated in mice, *in vivo (160)*.

Taken together, the present results – that the anxiogenic- and depressive-like effects of obestatin where reversed by the administration of a ghrelin receptor antagonist – indicate that obestatin may act through GHSR signaling. The GHSR1a is widely expressed in brain areas related to stress and anxiety such as the hypothalamus (in PVN, were it can act directly on CRH-containing neurons *(9)*, the anterior pituitary, amygdala and the hippocampus).

The expression of obestatin, so far, was only demonstrated in the anterior pituitary *(160),* however its modulatory effect on feeding, anxiety and depression related-behavior *((142), Szakács J., in preparation),* memory retention *(76),* neuroprotection *((49)* indicates

a complex interaction with the brain regions involved in the integration of these neurobiological processes.

Obestatin influences the behavioral effects of naloxone–precipitated morphine withdrawal

To our knowledge, our results are the first in line to prove that obestatin can influence the behavioral effects induced by naloxone-precipitated morphine withdrawal in the EPM and OF tests. The role of ghrelin in the reward- and addiction-related behaviors is an extensively studied subject *((166, 167)*. However, no data have been pulished on the effects of obestatin previously.

Drug addiction constitutes a severe, continously growing health and socio-economical problem worldwide. Repeated exposure to morphine causes alterations in different neural circuits and neurotransmitters of the brain. The exact mechanism for drug dependence, tolerance, and withdrawal have not been entirely elucidated yet, however it presumably involves alterations in different neuroendocrine structures and pathways such as the mesolimbic-dopaminergic reward system (VTA, amygdala and nucleus accumbens), the noradrenergic neurons of locus coeruleus (LC) and the HPA axis *(168, 169)*. Consequently, the administration of CRH antagonists antagonized drug self-administration and attenuated the anxiogenic-like effects of withdrawal from different drugs such as cocaine, cannabinoids, nicotine and alcohol *(164)*.

The long-term health effects of drug addiction are deleterious, including physical dependence, deficits in learning, memory, concentration and cognitive ability, as well as mood disorders such as anxiety and depression *(168, 169)*. A well-known bidirectional correlation exists between substance abuse and alterations of mood. The negative emotional state highly increases the risk for compulsive and persisting drug use, as well as relapse following abstinence. Exposure to stressful situations on the other hand also exerts a reinforcing effect on maintainance of drug addiction. Therefore, the prevention and treatment of anxiety and depression may be beneficial on the management of addiction-related conditions, as well *(170)*.

The effect of morphine on general locomotion has been widely investigated in rodents. For instance, morphine (4-32 mg/kg) at lower doses decreased, while at higher doses increased locomotion in 15 different mouse strains *(171)* in line with another study using female mice and 10-15-20 mg/kg of intraperitoneal morphine injection *(172)*.

However, research of the other behavioral patterns such as anxiety and depression has been almost completely ignored ((172). In the current decade the majority of studies have indicated that acute or chronic morphine treatment differentially affects the anxiety behavior of rodents. Accordingly, acute intraperitoneal administration of morphine in moderate doses (7.5 and 10 mg/kg) generated anxiolytic-like response to morphine in the EPM (173). Similarly, acute subcutaneous administration of morphine (5.6 mg/kg or 10 mg/kg) in rats resulted in time-dependent increase (as measured at 2, 4 or 8 hours) in anxiolytic-like behavior in the EPM (174). Furthermore, systemic or central treatment with morphine has also induced anxiolytic-like behavior in the EPM in rodents, which was blunted by the opioid receptor antagonist naloxone (170). The evaluation of the pathomechanism of the acute morphine treatment (5 and 6 mg/kg, intraperitoneally) on anxiety has revealed the involvement of dopaminergic pathways via the D1/D2 receptors. Accordingly, the morphine-induced anxiolytic-like behavior in the EPM was blunted by intra-amygdalar administration of dopamin antagonists. To note, the amygdala is a distinct brain area to modulate fear and anxiety responses, furthermore, it also has an abundant number of μ- and δ-opioid, $GABA_A$ and benzodiazepine receptors (175).

In contrast to the previously mentioned findings, a recent comprehensive study in rats has demonstrated that chronic administration of morphine (15-45 mg/kg for 21 days) induces multiple behavioral alterations such as depression-like symptoms in the FST and tail suspension test, anxiety-like behavior as tested in the EPM and OF tests, as well as spatial memory and learning deficits in the Morris water maze. Similarly, rats, which underwent subcutaneous morphine treatment twice a day for 10 days, followed by withdrawal also showed anxiety-like behaviors in the EPM and light/dark box (170).

These results are in line with the findings of our study, since the chronic administration of ascending doses of morphine has also induced a significant decrease in central ambulation distance and a decreasing tendency in central ambulation time in the OF test (141). Treatment protocol of morphine with increasing and irregular intervals, and descending doses significantly attenuated the depression- and anxiety like behavior, and improved cognitive ability, indicating a therapeutical potential in the management of morphine-related mood changes (169). In addition, rodent studies show different alterations in grooming and rearing, a marker of anxiety and exploratory behavior respectively (172). These results highlight the fact that different experimental protocols, for instance the animal strains used and the dosage regimen (drug dose, route of

administration, dosing interval and duration of treatment), can highly influence the morphine-induced behavioral patterns of animals.

Withdrawal from opioids and other drugs of abuse induces many aversive emotional responses including irritation, restlessness, anxiety, dysphoria and anhedonia, which are thought to play a crucial role in the maintenance of drug abuse and relapse after abstinence *(174, 176)*. While physical dependence is characteristic for chronic morphine use, the aversive behavioral signs of withdrawal can be evoked by naloxone following a single morphine injection *(177)*. Notably in rodents, exposed to chronic morphine treatment followed by withdrawal, low doses of opioid antagonists (e.g. 0.2 mg/kg of naloxone) produce affective-aversive signs as detected by increased corticosterone levels *(154)*. Somatic withdrawal signs [e.g. escape jumps, body weight loss, wet dog shakes, diarrhea, profuse salivation *(174)*], on the other hand, are induced by higher doses of naloxone [e.g. 0.4 mg/kg in mice *(154)*]. Accordingly, studies in rats using continous morphine delivery system (subcutaneous pellets of 75 mg morphine) have demonstrated that both spontaneous (removal of pellets) and naloxone-precipitated opioid withdrawal (naloxone doses of 0.01 and 0.03 mg/kg) exerts anxiogenic-like behavior in the EPM *(178)*. Furthermore, the anxiolytic effects of the acute morphine dependence induced by single or repeated morphine injections (5.6 mg/kg or 10 mg/kg) were reversed by pretreatment with the opioid antagonist naloxone *(174)*.

In contrast to the results obtained in humans and rats, unexpected behavioral responses were found in mice, namely that both spontaneous and naloxone-precipitated morphine withdrawal induced an anxiolytic like-response in the EPM model. In point of fact, mice injected subcutaneosuly twice a day with increasing doses of morphine (10, 20 and 40 mg/kg) for 3 days followed by a challenge dose of 20 mg/kg elicited morphine dependence and a decrease in open arm time and the travelled distance in the open arms, indicating an anxiogenic-like behavior. These effects were reversed by the administration of different doses of naloxone (0.1-0.2-0.4 mg/kg) to morphine dependent mice, where all three doses evoked increased open arm time and distance during withdrawal *(154)*.

Notably, by using a similar experimental design, we also demonstrated an anxiolytic-like effect of naloxone-precipitated morphine withdrawal in the EPM and OF tests by increased open arm entries and time in the EPM, and increased central ambulation distance and time in the OF test, respectively *(141)*. Moreover, anxiolytic-like behavior by a significant increase in open arm time and entries was also observed after spontaneous morphine withdrawal in mice receiving chronic treatment with increasing doses of

subcutaneous morphine (10, 20 and 40 mg/kg twice daily for 6 days and 20 mg/kg on the 7. day) *((176)*. As mentioned earlier, these results on opiod withdrawal contradict those found in humans and rats, where an increased anxiety-like effect was recorded.

To underscore further, withdrawal from opiods causes many negative symptoms including conditioned place aversion *(176)* and increased plasma corticosterone levels *(154)*. Therefore, it was suggested that EPM detects not only the anxiety behavior, but also several other aspects of emotionality and motivation, like neophobia (i.e. novelty-induced behavioral inhibition), approach/avoidance- and exploration/fear drive conflict *(176)*, as well as defensive patterns to avoid and escape from withdrawal state *(154)*. Another possible explanation might be that delta and kappa opioid receptors, responsible for inducing and inhibiting anxiety, differentially adapt to the challenges of repeated morphine exposure and opioid withdrawal leading to diverse EPM behaviors in species *(154)*.

The molecular mechanisms of morphine dependence and withdrawal have not been completely elucidated yet. Possible candidates are the mitogen-activated protein kinases (MAPK), which are involved in multiple processes such as learning, memory, synaptic plasticity, and drug addiction *(179)*. The MAPK/ERK1/2 pathway particularly, is known to be activated by drugs of abuse (cocaine, amphetamin, and morphine) in the dopaminergic neurons of the brain reward sytem (VTA, bed nucleus of stria terminalis, central amygdala, the nucleus accumbens). The activation of ERK1/2 pathyways and the subsequent behavioral alterations of drug addiction can be ameliorated by the administration of MAPK inhibitors, dopamin and glutamate receptor antagonists, suggesting the involvement of multiple neurocircuits *(180, 181)*. ERK1/2 activity in the brain is modulated by exposure to acute and chronic morphine treatment, and also by withdrawal from morphine, as demonstrated by a study in which naloxone-precipitated withdrawal increased ERK1/2 phosphorylation in the frontal association cortex, nucleus accumbens and caudate putamen of the mouse forebrain *(179)*.

In our studies obestatin treated mice undergoing withdrawal showed a decreasing tendency in open arm entries and open arm time in the EPM, and a significant decrease in central ambulation and time in the OF test. All the above results indicate that obestatin might have a distinct role not only in anxiety and depression, but also in behavioral responses induced by opioids. The mechanism of action of obestatin on opiod withdrawal might also involve the ERK1/2 signaling, which was recently identified to also mediate obestatin's beneficial effect on glucose metabolism and adipose tissue function *(96, 97)*

7. CONCLUSIONS

In the past two decades an abundant number of extensive studies have focused to reveal the multiple functions of the brain-gut peptide ghrelin. Identified originally as an orexigenic peptide, and an antagonist for ghrelin, in the past few years obestatin has also received growing attention, primarily due to its beneficial effects on glucose and lipid metabolism.   However, the data related to obestatin's central effects are also continously extending, so far it has been proven to have a role in memory, learning, neuroprotection, thirst, sleep and thermoregulation.

Our group has demonstrated for the first time that obestatin affects mood, anxiety and naloxone-precipitated morphine withdrawal in mice.

There is also strong scientific evidence demonstrating the correlation between the disruption of the HPA axis and the development of mood-, addictive- and eating disorders, obesity and metabolic syndrome.

Based upon the data available on its widespread effects, obestatin is a brain-gut neuropeptide which influences multiple physiological and pathological processes. The exact underlying neurocircuits and the specific receptor for obestatin have not been discovered yet. However, the continously developing and more accurate experimental methods will hopefully identify obestatin as a diagnostic and therapeutical potential in different neuropsychiatric and metabolic disorders.

8. SUMMARY

Introduction: Ghrelin and obestatin are both neuropeptides and the products of the same preproghrelin precursor gene, which have been isolated first from the GI tract. Ghrelin is the endogenous ligand of the growth hormone secretagogue (GHS-R1a) receptor, which has multiple functions in appetite regulation, energy homeostasis, anxiety, depression and addictive behaviors. Obestatin is a 23 amino-acid peptide, originally described to antagonise the orexigenic effects of ghrelin. Later, it has also been shown to influence the function of endocrine pancreas and that of the adipose tissue, as well as thirst, sleep, thermoregulation and neurogenesis. However, there are only a few available data on behavioral effects of obestatin. We therefore investigated the effects of this peptide on anxiety, depression and naloxone-precipitated morphine withdrawal.

Materials and methods: Male CFLP mice have been used for the experiments. To test the anxiety-related effects, obestatin was administered intracerebroventricularly in increasing

doses, followed by testing the animals in the elevated plus maze (EPM) and computerized open field (OF) tests. We similarly investigated the effects of the central, acute obestatin administration on depression-related behavior, by using the forced swimming test (FST). In order to elucidate the mechanism of action on anxiety and depression-related behavioral responses, prior to obestatin treatments we administered corticotropin-releasing hormone (CRH) receptor blockage with antalarmin in two different sets of paradigm, the OF test and FST, respectively. We also measured the plasma corticosterone levels by fluorescence assay in the animal groups treated with obestatin and antalarmin, to underscore the involvement of HPA axis. Furthermore, to find out whether obestatin's effect on anxiety and depression are mediated through growth hormone secretagogue receptor (GHSR) signaling, we administered ghrelin receptor antagonist pretreatment with [D-Lys3]-GHRP6 followed by OF test and FST. Next, we investigated the possible effects of the chronic, central obestatin treatment on naloxone-precipitated morphine withdrawal by using graded doses of morphine and obestatin, on day four followed by testing the animals in the OF and EPM tests.

Results: Acute intracerebroventricular administration of obestatin reduced the percent of time spent in the open arms in the EPM test. The basal locomotor activity (ambulation distance and time, rearing, jumping) was not influenced significantly in the obestatin-treated groups in the OF test. The percentage of central ambulation distance was decreased by obestatin. The administration of antalarmin or [D-Lys3]-GHRP6 have both reversed the effect of obestatin on central ambulation. Plasma corticosterone levels were elevated by different doses of obestatin, which effect was antagonized by the injection of antalarmin. In the FST, obestatin increased the immobility score with a parallel decrease in the swimming score. Pretreatment with antalarmin antagonized the effects of obestatin on both immobility and swimming score. Combined treatment with obestatin and the ghrelin receptor antagonist [D-Lys3]-GHRP6 decreased the immobility score, while increasing the swimming and the climbing scores. Naloxone-precipitated withdrawal induced a significant increase in the percentage of open arm time and open arm entries in the EPM, while obestatin treated mice undergoing withdrawal showed a decreased tendency in these parameters. In the OF test, treatment with graded doses of morphine significantly decreased the percentage of central ambulation distance. Naloxone precipitated morphine withdrawal caused a significant increase in the percentage of central ambulation distance

and time. Obestatin pretreatment significantly decreased the percentage of central ambulation and time in mice undergoing naloxone-precipitated morphine withdrawal.

Conclusions: In our studies, we for the first, demonstrated that obestatin exerted anxiogenic- and depressive-like effects, which have been reversed by CRH and ghrelin receptor blockage. These data indicate that obestatin's behavioral effects might be mediated through HPA axis and GHSR signaling. Furthermore, obestatin blunted the anxiolytic-like effect of naloxone-precipitated morphine withdrawal, which suggests that it might be involved in the behavioral responses induced by opioids. The specific receptor for obestatin has not been identified yet, the available results however, indicate that it is a multifunctional brain-gut peptide, therefore merits further investigations.

9. ÖSSZEFOGLALÁS

Bevezetés: A ghrelin és az obestatin neuropeptidek, amelyek a preproghrelin precursor gén származékai és a GI rendszerből izolálták őket először. A ghrelin endogén ligandja a growth hormone secretagogue receptor (GHS-R1a), és számos hatását leírták a táplálékfelvételben, az energiaháztartásban, valamint szorongásban, depresszióban és addiktív zavarokban. Az obestatin egy 23 aminosavból álló peptid, amelyet eredetileg ghrelin antagonista és anorexigén hatása révén azonosítottak. Később számos egyéb hatását is leírták, így képes befolyásolni az endocrin pancreas és a zsírszövet funkcióját, szerepe van a szomjúságérzet, az alvás, a hőháztartás és a neurogenesis szabályozásában. Az obestatin magatartási hatásai azonban lényegesen kevésbé ismertek. Ezért kísérleteinkben vizsgáltuk az obestatin szorongásra, depresszióra valamint a naloxon-kiváltott morfin megvonásra gyakorolt hatásait.

Anyag és módszer: Kísérleteinket hím CFLP egereken végeztük. Az obestatint különböző dózisban, akutan, intracerebroventricularisan adagoltuk, majd vizsgáltuk a szorongásra kifejtett hatását kompjúterizált nyílt tér és emelt keresztpalló tesztben. A depresszió-szerű viselkedésre gyakorolt akut, centralis obestatin hatást erőltetett úszás teszt segítségével vizsgáltuk. A szorongásra és a depresszióra gyakorolt hatásmechanizmusának feltárására corticotropin-releasing hormone (CRH) receptor antagonista antalarmin előkezelést alkalmaztunk, majd nyílt tér és erőltetett úszás teszt segítségével vizsgáltuk az állatokat. A hypothalamus-hipofízis-mellékvesekéreg (HPA) tengely szerepének bizonyítására meghatároztuk a plazma kortikoszteron szintet az obestatinnal és antalarminnal kezelt

állatokban. Továbbá, az obestatin hatásmechanizmusában feltételezve a growth hormone secretagogue receptor (GHSR) jelátviteli út szerepét, [D-Lys3]-GHRP6 ghrelin receptor antagonista előkezelést követően nyílt tér és erőltetett úszás tesztet végeztünk. Ezenkívül megvizsgáltuk a krónikus, centralis obestatin hatását a naloxon-kiváltott opioid megvonásra, amely során emelkedő dózisú obestatin- és morfin kezelést alkalmaztunk, majd a negyedik napon emelt keresztpalló és nyílt tér tesztben vizsgáltuk a kezelések magatartási hatásait.

Eredmények: Az akut, intracerebroventricularis obestatin kezelés emelt keresztpalló tesztben csökkentette a nyílt karban eltöltött időt. Az obestatin kezelés az általános lokomóciót (megtett út, mozgással töltött idő, ugrások és ágaskodások száma, immobilitás) nem befolyásolta nyílt tér tesztben. A centrális zónákban megtett út szignifikánsan csökkent az obestatin kezelés hatására. Az antalarmin valamint a [D-Lys3]-GHRP6 előkezelés felfügesztette az obestatin centralis zónákban megtett útra kifejtett hatását. Az obestatin különböző dózisban növelte a plazma kortikoszteron szintet, amely hatást az antalarmin előkezelés antagonizálta. Erőltetett úszás tesztben az obestatin fokozta az immobilitást, és csökkentette az úszással töltött időt. Az antalarmin előkezelés ezen hatásokat felfügesztette, vagyis növelte az úszás és csökkentette az immobilitás idejét. A [D-Lys3]-GHRP6 ghrelin receptor antagonista szintén csökentette az obestatin hatását az immobilitásra, valamint növelte a mászás és úszás idejét a kombinált kezelésben részesült állatcsoportokban. A naloxon-kiváltott morfin megvonás növelte a nyitott karban eltöltött időt és a nyitott karba történő belépések számát emelt keresztpalló tesztben, amely paraméterek az obestatin előkezelt megvonásos állatcsoportoknál csökkenő tendenciát mutattak. Nyílt tér tesztben a különböző dózisú morfin csökkentette a centrális zónákban megtett utat. A naloxon-kiváltott morfin megvonás ugyanakkor anxiolitikus hatást fejtett ki, mivel növelte a centralis zónában megtett utat és időt, amely hatásokat az obestatin előkezelés felfügesztette.

Konklúzió: Kísérleteinkben először bizonyítottuk, hogy az obestatin anxiogén- és depresszív-szerű hatást fejt ki, amelyet a CRH és ghrelin receptor antagonista előkezelés képes volt felfüggeszteni. Ezek az eredmények arra utalhatnak, hogy az obestatin viselkedési hatásait a HPA tengely és a ghrelin jelátviteli út aktiválása útján fejti ki. Továbbá, az obestatin felfügesztette a naloxon-kiváltott morfin megvonás anxiolitikus hatását, így szerepe lehet az opioidok által kiváltott viselkedési mintázatokban is. Az obestatin specifikus receptorát még nem sikerült azonosítani, az eddigi eredmények

azonban arra utalnak, hogy az obestatin a tápcsatorna-agy tengely egyik multifunkcionális peptidje, amely további vizsgálatokra érdemes.

10. ACKNOWLEDGEMENTS

First of all, I would like to thank to my supervisor **Professor Gyula Szabó,** who provided me the opportunity to join the neuroendocrine study group and to realize my research ideas. I am also indebted for his critical support and guidance to work hard and think individually, and to enjoy science and research.

 I am especially grateful to my colleague and coauthor **Krisztina Csabafi** for her continuous support, friendship, great scientific ideas, and optimism which all have enormously helped me to override the upcoming several difficulties during the performance of our studies and to complete this work.

Special thanks to Nándor Lipták for his approval to include our collaborative studies in the thesis.

I appreciate and thank to our co-workers **Ágnes Pál, Gusztáv Kiss, Ildikó Sipos** and **Éva Dobó** for their technical assistance in performing the laboratory experiments during all these years.

 I especially thank to my colleague **Imre Pataki** for his encouraging attitude, patience and for the correction of the manuscript.

I'm also grateful to professor Árpád Gecse for his warmly supporting attitude during all these years we have been working together.

I thank to professor **Gyula Telegdy** who gave me trust and empolyed me at the Department of Pathophysiology and has followed my carrier with interest all the time.

I would also like to dedicate this thesis in memory of **professor György Lázár,** who generated my interest and enthusiasm for theaching and researching medical science.

I am warmheartedly grateful to my dear parents and family for their endless love, support and encouragement.

It is a pleasure to thank to all my friends, especially Joseph Jung for his friendship, supportive optimism and encouragement both in my private and professional life.

11. REFERENCES

1.Kojima M. The discovery of ghrelin--a personal memory. Regul Pept. 2008;145(1-3):2-6.

2.Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature. 1999;402(6762):656-60.

3.Kojima M, Kangawa K. Ghrelin: structure and function. Physiol Rev. 2005;85(2):495-522.

4.Tschöp M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature. 2000;407(6806):908-13.

5.Gahete MD, Rincón-Fernández D, Villa-Osaba A, Hormaechea-Agulla D, Ibáñez-Costa A, Martínez-Fuentes AJ, et al. Ghrelin gene products, receptors, and GOAT enzyme: biological and pathophysiological insight. J Endocrinol. 2014;220(1):R1-24.

6.Muller TD, Nogueiras R, Andermann ML, Andrews ZB, Anker SD, Argente J, et al. Ghrelin. Mol Metab. 2015;4(6):437-60.

7.Andrews ZB, Abizaid A. Neuroendocrine mechanisms that connect feeding behavior and stress. Front Neurosci. 2014;8:312.

8.Labarthe A, Fiquet O, Hassouna R, Zizzari P, Lanfumey L, Ramoz N, et al. Ghrelin-Derived Peptides: A Link between Appetite/Reward, GH Axis, and Psychiatric Disorders? Front Endocrinol (Lausanne). 2014;5:163.

9.Andrews ZB. The extra-hypothalamic actions of ghrelin on neuronal function. Trends in neurosciences. 2011;34(1):31-40.

10.Briggs DI, Andrews ZB. Metabolic status regulates ghrelin function on energy homeostasis. Neuroendocrinology. 2011;93(1):48-57.

11.Castaneda TR, Tong J, Datta R, Culler M, Tschop MH. Ghrelin in the regulation of body weight and metabolism. Front Neuroendocrinol. 2010;31(1):44-60.

12.Howick K, Griffin BT, Cryan JF, Schellekens H. From Belly to Brain: Targeting the Ghrelin Receptor in Appetite and Food Intake Regulation. Int J Mol Sci. 2017;18(2).

13.Jiao Q, Du X, Li Y, Gong B, Shi L, Tang T, et al. The neurological effects of ghrelin in brain diseases: Beyond metabolic functions. Neurosci Biobehav Rev. 2017;73:98-111.

14.Andrews ZB. Central mechanisms involved in the orexigenic actions of ghrelin. Peptides. 2011;32(11):2248-55.

15.van der Lely AJ, Tschöp M, Heiman ML, Ghigo E. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. Endocr Rev. 2004;25(3):426-57.

16.Delporte C. Structure and Physiological Actions of Ghrelin. Scientifica (Cairo). 2013;2013:518909.

17.Carlini VP, Varas MM, Cragnolini AB, Schiöth HB, Scimonelli TN, de Barioglio SR. Differential role of the hippocampus, amygdala, and dorsal raphe nucleus in regulating feeding, memory, and anxiety-like behavioral responses to ghrelin. Biochem Biophys Res Commun. 2004;313(3):635-41.

18.Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, et al. Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab. 2001;86(12):5992.

19.Zakhari JS, Zorrilla EP, Zhou B, Mayorov AV, Janda KD. Oligoclonal antibody targeting ghrelin increases energy expenditure and reduces food intake in fasted mice. Mol Pharm. 2012;9(2):281-9.

20.Sun Y. Ghrelin receptor controls obesity by fat burning. Oncotarget. 2015;6(9):6470-1.

21.Pradhan G, Samson SL, Sun Y. Ghrelin: much more than a hunger hormone. Curr Opin Clin Nutr Metab Care. 2013;16(6):619-24.

22.Horvath TL, Castañeda T, Tang-Christensen M, Pagotto U, Tschöp MH. Ghrelin as a potential anti-obesity target. Curr Pharm Des. 2003;9(17):1383-95.

23.Schüssler P, Kluge M, Yassouridis A, Dresler M, Uhr M, Steiger A. Ghrelin levels increase after pictures showing food. Obesity (Silver Spring). 2012;20(6):1212-7.

24.Mihalache L, Gherasim A, Niţă O, Ungureanu MC, Pădureanu SS, Gavril RS, et al. Effects of ghrelin in energy balance and body weight homeostasis. Hormones (Athens). 2016;15(2):186-96.

25.Sato T, Ida T, Nakamura Y, Shiimura Y, Kangawa K, Kojima M. Physiological roles of ghrelin on obesity. Obes Res Clin Pract. 2014;8(5):e405-13.

26.Hassouna R, Labarthe A, Tolle V. Hypothalamic regulation of body growth and appetite by ghrelin-derived peptides during balanced nutrition or undernutrition. Mol Cell Endocrinol. 2016;438:42-51.

27.Hassouna R, Zizzari P, Tolle V. The ghrelin/obestatin balance in the physiological and pathological control of growth hormone secretion, body composition and food intake. J Neuroendocrinol. 2010;22(7):793-804.

28.Chen L, Xing T, Wang M, Miao Y, Tang M, Chen J, et al. Local infusion of ghrelin enhanced hippocampal synaptic plasticity and spatial memory through activation of phosphoinositide 3-kinase in the dentate gyrus of adult rats. Eur J Neurosci. 2011;33(2):266-75.

29.Walker AK, Rivera PD, Wang Q, Chuang JC, Tran S, Osborne-Lawrence S, et al. The P7C3 class of neuroprotective compounds exerts antidepressant efficacy in mice by increasing hippocampal neurogenesis. Mol Psychiatry. 2015;20(4):500-8.

30.Steiger A, Dresler M, Schüssler P, Kluge M. Ghrelin in mental health, sleep, memory. Mol Cell Endocrinol. 2011;340(1):88-96.

31.Skibicka KP, Dickson SL. Enteroendocrine hormones - central effects on behavior. Curr Opin Pharmacol. 2013;13(6):977-82.

32.Carlini VP, Monzón ME, Varas MM, Cragnolini AB, Schiöth HB, Scimonelli TN, et al. Ghrelin increases anxiety-like behavior and memory retention in rats. Biochem Biophys Res Commun. 2002;299(5):739-43.

33.Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Fujimiya M, et al. A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. Neuroendocrinology. 2001;74(3):143-7.

34.Kristensson E, Sundqvist M, Astin M, Kjerling M, Mattsson H, Dornonville de la Cour C, et al. Acute psychological stress raises plasma ghrelin in the rat. Regul Pept. 2006;134(2-3):114-7.

35.Currie PJ, Khelemsky R, Rigsbee EM, Dono LM, Coiro CD, Chapman CD, et al. Ghrelin is an orexigenic peptide and elicits anxiety-like behaviors following administration into discrete regions of the hypothalamus. Behav Brain Res. 2012;226(1):96-105.

36.Hansson C, Haage D, Taube M, Egecioglu E, Salome N, Dickson SL. Central administration of ghrelin alters emotional responses in rats: behavioral, electrophysiological and molecular evidence. Neuroscience. 2011;180:201-11.

37.Cabral A, Suescun O, Zigman JM, Perello M. Ghrelin indirectly activates hypophysiotropic CRF neurons in rodents. PLoS One. 2012;7(2):e31462.

38.Lutter M, Sakata I, Osborne-Lawrence S, Rovinsky SA, Anderson JG, Jung S, et al. The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. Nat Neurosci. 2008;11(7):752-3.

39.Spencer SJ, Xu L, Clarke MA, Lemus M, Reichenbach A, Geenen B, et al. Ghrelin regulates the hypothalamic-pituitary-adrenal axis and restricts anxiety after acute stress. Biol Psychiatry. 2012;72(6):457-65.

40.Jensen M, Ratner C, Rudenko O, Christiansen SH, Skov LJ, Hundahl C, et al. Anxiolytic-Like Effects of Increased Ghrelin Receptor Signaling in the Amygdala. Int J Neuropsychopharmacol. 2016;19(5).

41.Carlini VP, Machado DG, Buteler F, Ghersi M, Ponzio MF, Martini AC, et al. Acute ghrelin administration reverses depressive-like behavior induced by bilateral olfactory bulbectomy in mice. Peptides. 2012;35(2):160-5.

42.Chuang JC, Perello M, Sakata I, Osborne-Lawrence S, Savitt JM, Lutter M, et al. Ghrelin mediates stress-induced food-reward behavior in mice. J Clin Invest. 2011;121(7):2684-92.

43.Chuang JC, Zigman JM. Ghrelin's Roles in Stress, Mood, and Anxiety Regulation. Int J Pept. 2010;2010.

44.Wittekind DA, Kluge M. Ghrelin in psychiatric disorders - A review. Psychoneuroendocrinology. 2015;52:176-94.

45.Li E, Kim Y, Kim S, Sato T, Kojima M, Park S. Ghrelin stimulates proliferation, migration and differentiation of neural progenitors from the subventricular zone in the adult mice. Exp Neurol. 2014;252:75-84.

46.Chung H, Li E, Kim Y, Kim S, Park S. Multiple signaling pathways mediate ghrelin-induced proliferation of hippocampal neural stem cells. J Endocrinol. 2013;218(1):49-59.

47.Moon M, Choi JG, Nam DW, Hong HS, Choi YJ, Oh MS, et al. Ghrelin ameliorates cognitive dysfunction and neurodegeneration in intrahippocampal amyloid-β1-42 oligomer-injected mice. J Alzheimers Dis. 2011;23(1):147-59.

48.Martins I, Gomes S, Costa RO, Otvos L, Oliveira CR, Resende R, et al. Leptin and ghrelin prevent hippocampal dysfunction induced by Aβ oligomers. Neuroscience. 2013;241:41-51.

49.Gargantini E, Lazzari L, Settanni F, Taliano M, Trovato L, Gesmundo I, et al. Obestatin promotes proliferation and survival of adult hippocampal progenitors and reduces amyloid-β-induced toxicity. Mol Cell Endocrinol. 2016;422:18-30.

50.Ozsoy S, Besirli A, Abdulrezzak U, Basturk M. Serum ghrelin and leptin levels in patients with depression and the effects of treatment. Psychiatry Investig. 2014;11(2):167-72.

51.Kluge M, Schüssler P, Dresler M, Schmidt D, Yassouridis A, Uhr M, et al. Effects of ghrelin on psychopathology, sleep and secretion of cortisol and growth hormone in patients with major depression. J Psychiatr Res. 2011;45(3):421-6.

52.Kluge M, Schüssler P, Bleninger P, Kleyer S, Uhr M, Weikel JC, et al. Ghrelin alone or co-administered with GHRH or CRH increases non-REM sleep and decreases REM sleep in young males. Psychoneuroendocrinology. 2008;33(4):497-506.

53.Szentirmai E, Kapás L, Krueger JM. Ghrelin microinjection into forebrain sites induces wakefulness and feeding in rats. Am J Physiol Regul Integr Comp Physiol. 2007;292(1):R575-85.

54.Szentirmai E, Hajdu I, Obal F, Krueger JM. Ghrelin-induced sleep responses in ad libitum fed and food-restricted rats. Brain Res. 2006;1088(1):131-40.

55.Spencer SJ, Emmerzaal TL, Kozicz T, Andrews ZB. Ghrelin's Role in the Hypothalamic-Pituitary-Adrenal Axis Stress Response: Implications for Mood Disorders. Biol Psychiatry. 2014.

56.Alvarez-Crespo M, Skibicka KP, Farkas I, Molnár CS, Egecioglu E, Hrabovszky E, et al. The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence. PLoS One. 2012;7(10):e46321.

57.Schellekens H, Finger BC, Dinan TG, Cryan JF. Ghrelin signalling and obesity: at the interface of stress, mood and food reward. Pharmacol Ther. 2012;135(3):316-26.

58. WHO. Depression: A Global Crisis. World Mental Health Day. 2012. http://www.who.int/mental_health/management/depression/wfmh_paper_depression_wmh d_2012.pdf

59.Skibicka KP, Dickson SL. Ghrelin and food reward: the story of potential underlying substrates. Peptides. 2011;32(11):2265-73.

60.Lang UE, Beglinger C, Schweinfurth N, Walter M, Borgwardt S. Nutritional aspects of depression. Cell Physiol Biochem. 2015;37(3):1029-43.

61.Lutter M, Nestler EJ. Homeostatic and hedonic signals interact in the regulation of food intake. J Nutr. 2009;139(3):629-32.

62.Nestler EJ, Hyman SE. Animal models of neuropsychiatric disorders. Nat Neurosci. 2010;13(10):1161-9.

63.Hryhorczuk C, Sharma S, Fulton SE. Metabolic disturbances connecting obesity and depression. Front Neurosci. 2013;7:177.

64.Dallman MF. Stress-induced obesity and the emotional nervous system. Trends Endocrinol Metab. 2010;21(3):159-65.

65.Bazhan N, Zelena D. Food-intake regulation during stress by the hypothalamo-pituitary-adrenal axis. Brain Res Bull. 2013;95:46-53.

66.Kyrou I, Tsigos C. Stress hormones: physiological stress and regulation of metabolism. Curr Opin Pharmacol. 2009;9(6):787-93.

67.Wellman PJ, Clifford PS, Rodriguez JA. Ghrelin and ghrelin receptor modulation of psychostimulant action. Front Neurosci. 2013;7:171.

68.Panagopoulos VN, Ralevski E. The role of ghrelin in addiction: a review. Psychopharmacology (Berl). 2014;231(14):2725-40.

69.Vengeliene V. The role of ghrelin in drug and natural reward. Addict Biol. 2013;18(6):897-900.

70.Rocha-Sousa A, Tavares-Silva M, Fonseca S, Manuel Falcao M, Falcao-Reis, Leite-Moreira AF. Diabetes, Ghrelin And Related Peptides: From Pathophysiology To Vasculopathy. *The Open Circ and Vasc J,*. 2010;3:17-29.

71.Hosoda H, Kangawa K. The autonomic nervous system regulates gastric ghrelin secretion in rats. Regul Pept. 2008;146(1-3):12-8.

72.Tokudome T, Kishimoto I, Miyazato M, Kangawa K. Ghrelin and the cardiovascular system. Front Horm Res. 2014;43:125-33.

73.Tesauro M, Schinzari F, Iantorno M, Rizza S, Melina D, Lauro D, et al. Ghrelin improves endothelial function in patients with metabolic syndrome. Circulation. 2005;112(19):2986-92.

74.Pöykkö SM, Kellokoski E, Hörkkö S, Kauma H, Kesäniemi YA, Ukkola O. Low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes. Diabetes. 2003;52(10):2546-53.

75.Zhang JV RP, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, et al. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. Science. 2005;310:996-9.

76.Cowan E, Kumar P, Burch KJ, Grieve DJ, Green BD, Graham SF. Treatment of lean and diet-induced obesity (DIO) mice with a novel stable obestatin analogue alters plasma metabolite levels as detected by untargeted LC-MS metabolomics. Metabolomics. 2016;12:124.

77.Carlini VP, Schioth HB, Debarioglio SR. Obestatin improves memory performance and causes anxiolytic effects in rats. Biochemical and biophysical research communications. 2007;352(4):907-12.

78.Brunetti L, Di Nisio C, Recinella L, Orlando G, Ferrante C, Chiavaroli A, et al. Obestatin inhibits dopamine release in rat hypothalamus. European journal of pharmacology. 2010;641(2-3):142-7.

79.Holst B, Egerod KL, Schild E, Vickers SP, Cheetham S, Gerlach LO, et al. GPR39 signaling is stimulated by zinc ions but not by obestatin. Endocrinology. 2007;148(1):13-20.

80.Lauwers E, Landuyt B, Arckens L, Schoofs L, Luyten W. Obestatin does not activate orphan G protein-coupled receptor GPR39. Biochem Biophys Res Commun. 2006;351(1):21-5.

81.Santos-Zas I, Gurriarán-Rodríguez U, Cid-Díaz T, Figueroa G, González-Sánchez J, Bouzo-Lorenzo M, et al. β-Arrestin scaffolds and signaling elements essential for the obestatin/GPR39 system that determine the myogenic program in human myoblast cells. Cell Mol Life Sci. 2016;73(3):617-35.

82.Zhang JV, Jahr H, Luo CW, Klein C, Van Kolen K, Ver Donck L, et al. Obestatin induction of early-response gene expression in gastrointestinal and adipose tissues and the mediatory role of G protein-coupled receptor, GPR39. Mol Endocrinol. 2008;22(6):1464-75.

83.Cowan E, Burch KJ, Green BD, Grieve DJ. Obestatin as a key regulator of metabolism and cardiovascular function with emerging therapeutic potential for diabetes. Br J Pharmacol. 2016;173(14):2165-81.

84.Yamamoto D, Ikeshita N, Daito R, Herningtyas EH, Toda K, Takahashi K, et al. Neither intravenous nor intracerebroventricular administration of obestatin affects the secretion of GH, PRL, TSH and ACTH in rats. Regul Pept. 2007;138(2-3):141-4.

85.Nogueiras R, Pfluger P, Tovar S, Arnold M, Mitchell S, Morris A, et al. Effects of obestatin on energy balance and growth hormone secretion in rodents. Endocrinology. 2007;148(1):21-6.

86.Zhang JV, Klein, C., Ren, P.G., Kass, S., Donck, L.V., Moechars, D., Hsueh, A.J.W. Response to Comment on "Obestatin, a Peptide Encoded by the Ghrelin Gene, Opposes Ghrelin's Effects on Food Intake". Science. 2007;315.

87.Van Dijck A, Annemie VD, Van Dam D, Debby VD, Vergote V, Valentijn V, et al. Central administration of obestatin fails to show inhibitory effects on food and water intake in mice. Regul Pept. 2009;156(1-3):77-82.

88.Vergote V, Van Dorpe S, Peremans K, Burvenich C, De Spiegeleer B. In vitro metabolic stability of obestatin: kinetics and identification of cleavage products. Peptides. 2008;29(10):1740-8.

89.Pan W, Tu H, Kastin AJ. Differential BBB interactions of three ingestive peptides: obestatin, ghrelin, and adiponectin. Peptides. 2006;27(4):911-6.

90.Zizzari P, Longchamps R, Epelbaum J, Bluet-Pajot MT. Obestatin partially affects ghrelin stimulation of food intake and growth hormone secretion in rodents. Endocrinology. 2007;148(4):1648-53.

91.Yuan X, Cai W, Liang XF, Su H, Yuan Y, Li A, et al. Obestatin partially suppresses ghrelin stimulation of appetite in "high-responders" grass carp, Ctenopharyngodon idellus. Comp Biochem Physiol A Mol Integr Physiol. 2015;184:144-9.

92.Hassouna R, Zizzari P, Viltart O, Yang SK, Gardette R, Videau C, et al. A natural variant of obestatin, Q90L, inhibits ghrelin's action on food intake and GH secretion and targets NPY and GHRH neurons in mice. PLoS One. 2012;7(12):e51135.

93.Brunetti L, Recinella L, Orlando G, Michelotto B, Di Nisio C, Vacca M. Effects of ghrelin and amylin on dopamine, norepinephrine and serotonin release in the hypothalamus. Eur J Pharmacol. 2002;454(2-3):189-92.

94.Brunetti L, Orlando G, Ferrante C, Chiavaroli A, Vacca M. Peptide YY (3 -36) inhibits dopamine and norepinephrine release in the hypothalamus. Eur J Pharmacol. 2005;519(1-2):48-51.

95.Nagaraj S, Manjappara UV. Studies on the influence of CCK-8 on the ability of obestatin to reduce food intake, gain in body weight and related lipid parameters. Biochimie. 2016;125:126-30.

96.Granata R, Settanni F, Gallo D, Trovato L, Biancone L, Cantaluppi V, et al. Obestatin promotes survival of pancreatic beta-cells and human islets and induces expression of genes involved in the regulation of beta-cell mass and function. Diabetes. 2008;57(4):967-79.

97.Gargantini E, Grande C, Trovato L, Ghigo E, Granata R. The role of obestatin in glucose and lipid metabolism. Horm Metab Res. 2013;45(13):1002-8.

98.Gesmundo I, Gallo D, Favaro E, Ghigo E, Granata R. Obestatin: a new metabolic player in the pancreas and white adipose tissue. IUBMB Life. 2013;65(12):976-82.

99.Granata R, Gallo D, Luque RM, Baragli A, Scarlatti F, Grande C, et al. Obestatin regulates adipocyte function and protects against diet-induced insulin resistance and inflammation. FASEB J. 2012;26(8):3393-411.

100.Anderwald-Stadler M, Krebs M, Promintzer M, Mandl M, Bischof MG, Nowotny P, et al. Plasma obestatin is lower at fasting and not suppressed by insulin in insulin-resistant humans. Am J Physiol Endocrinol Metab. 2007;293(5):E1393-8.

101.Guo ZF, Ren AJ, Zheng X, Qin YW, Cheng F, Zhang J, et al. Different responses of circulating ghrelin, obestatin levels to fasting, re-feeding and different food compositions, and their local expressions in rats. Peptides. 2008;29(7):1247-54.

102.Guo ZF, Zheng X, Qin YW, Hu JQ, Chen SP, Zhang Z. Circulating preprandial ghrelin to obestatin ratio is increased in human obesity. J Clin Endocrinol Metab. 2007;92(5):1875-80.

103.Nakahara T, Harada T, Yasuhara D, Shimada N, Amitani H, Sakoguchi T, et al. Plasma obestatin concentrations are negatively correlated with body mass index, insulin resistance index, and plasma leptin concentrations in obesity and anorexia nervosa. Biol Psychiatry. 2008;64(3):252-5.

104.Granata R, Volante M, Settanni F, Gauna C, Ghé C, Annunziata M, et al. Unacylated ghrelin and obestatin increase islet cell mass and prevent diabetes in streptozotocin-treated newborn rats. J Mol Endocrinol. 2010;45(1):9-17.

105.Ataka K, Inui A, Asakawa A, Kato I, Fujimiya M. Obestatin inhibits motor activity in the antrum and duodenum in the fed state of conscious rats. Am J Physiol Gastrointest Liver Physiol. 2008;294(5):G1210-8.

106.Bresciani E, Rapetti D, Donà F, Bulgarelli I, Tamiazzo L, Locatelli V, et al. Obestatin inhibits feeding but does not modulate GH and corticosterone secretion in the rat. J Endocrinol Invest. 2006;29(8):RC16-8.

107.Bassil AK, Häglund Y, Brown J, Rudholm T, Hellström PM, Näslund E, et al. Little or no ability of obestatin to interact with ghrelin or modify motility in the rat gastrointestinal tract. Br J Pharmacol. 2007;150(1):58-64.

108.Gourcerol G, Taché Y. Obestatin--a ghrelin-associated peptide that does not hold its promise to suppress food intake and motility. Neurogastroenterol Motil. 2007;19(3):161-5.

109.Xing YX, Yang L, Kuang HY, Gao XY, Liu HL. Function of obestatin in the digestive system. Nutrition. 2017;34:21-8.

110.Wang WM, Li SM, Du FM, Zhu ZC, Zhang JC, Li YX. Ghrelin and obestatin levels in hypertensive obese patients. J Int Med Res. 2014;42(6):1202-8.

111.Li ZF, Guo ZF, Cao J, Hu JQ, Zhao XX, Xu RL, et al. Plasma ghrelin and obestatin levels are increased in spontaneously hypertensive rats. Peptides. 2010;31(2):297-300.

112.Ku JM, Andrews ZB, Barsby T, Reichenbach A, Lemus MB, Drummond GR, et al. Ghrelin-related peptides exert protective effects in the cerebral circulation of male mice through a nonclassical ghrelin receptor(s). Endocrinology. 2015;156(1):280-90.

113.Alloatti G, Arnoletti E, Bassino E, Penna C, Perrelli MG, Ghé C, et al. Obestatin affords cardioprotection to the ischemic-reperfused isolated rat heart and inhibits apoptosis in cultures of similarly stressed cardiomyocytes. Am J Physiol Heart Circ Physiol. 2010;299(2):H470-81.

114.Xin X, Ren AJ, Zheng X, Qin YW, Zhao XX, Yuan WJ, et al. Disturbance of circulating ghrelin and obestatin in chronic heart failure patients especially in those with cachexia. Peptides. 2009;30(12):2281-5.

115.Shi JB, Guo ZF, Zheng X, Wang ZB, Ma YJ. Circulating obestatin is increased in patients with cardiorenal syndrome and positively correlated with vasopressin. Peptides. 2012;38(2):377-80.

116.Samson WK, White MM, Price C, Ferguson AV. Obestatin acts in brain to inhibit thirst. Am J Physiol Regul Integr Comp Physiol. 2007;292(1):R637-43.

117.Samson WK, Yosten GL, Chang JK, Ferguson AV, White MM. Obestatin inhibits vasopressin secretion: evidence for a physiological action in the control of fluid homeostasis. J Endocrinol. 2008;196(3):559-64.

118.Szentirmai E, Krueger JM. Obestatin alters sleep in rats. Neurosci Lett. 2006;404(1-2):222-6.

119.Szentirmai E, Kapás L, Sun Y, Smith RG, Krueger JM. The preproghrelin gene is required for the normal integration of thermoregulation and sleep in mice. Proc Natl Acad Sci U S A. 2009;106(33):14069-74.

120.Aimone JB, Li Y, Lee SW, Clemenson GD, Deng W, Gage FH. Regulation and function of adult neurogenesis: from genes to cognition. Physiol Rev. 2014;94(4):991-1026.

121.Erşahin M, Ozsavcı D, Sener A, Ozakpınar OB, Toklu HZ, Akakin D, et al. Obestatin alleviates subarachnoid haemorrhage-induced oxidative injury in rats via its anti-apoptotic and antioxidant effects. Brain Inj. 2013;27(10):1181-9.

122.Dun SL, Brailoiu GC, Brailoiu E, Yang J, Chang JK, Dun NJ. Distribution and biological activity of obestatin in the rat. J Endocrinol. 2006;191(2):481-9.

123.Berridge MJ. Calcium signalling remodelling and disease. Biochem Soc Trans. 2012;40(2):297-309.

124.Tsigos C, Chrousos GP. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res. 2002;53(4):865-71.

125.Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci. 2009;10(6):397-409.

126.Herman JP, McKlveen JM, Ghosal S, Kopp B, Wulsin A, Makinson R, et al. Regulation of the Hypothalamic-Pituitary-Adrenocortical Stress Response. Compr Physiol. 2016;6(2):603-21.

127.Myers B, McKlveen JM, Herman JP. Neural Regulation of the Stress Response: The Many Faces of Feedback. Cell Mol Neurobiol. 2012.

128.Chrousos GP. Stress and disorders of the stress system. Nat Rev Endocrinol. 2009;5(7):374-81.

129.Zoumakis E, Rice KC, Gold PW, Chrousos GP. Potential uses of corticotropin-releasing hormone antagonists. Ann N Y Acad Sci. 2006;1083:239-51.

130.de Kloet ER, Joëls M, Holsboer F. Stress and the brain: from adaptation to disease. Nat Rev Neurosci. 2005;6(6):463-75.

131.Patterson ZR, Abizaid A. Stress induced obesity: lessons from rodent models of stress. Front Neurosci. 2013;7:130.

132.Spencer SJ, Emmerzaal TL, Kozicz T, Andrews ZB. Ghrelin's Role in the Hypothalamic-Pituitary-Adrenal Axis Stress Response: Implications for Mood Disorders. Biol Psychiatry. 2015;78(1):19-27.

133.Gastón MS, Cid MP, Salvatierra NA. Bicuculline, a GABAA-receptor antagonist, blocked HPA axis activation induced by ghrelin under an acute stress. Behav Brain Res. 2017;320:464-72.

134.Smith SM, Vale WW. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogues Clin Neurosci. 2006;8(4):383-95.

135.Nicolaides NC, Kyratzi E, Lamprokostopoulou A, Chrousos GP, Charmandari E. Stress, the stress system and the role of glucocorticoids. Neuroimmunomodulation. 2015;22(1-2):6-19.

136.Yang L, Zhao Y, Wang Y, Liu L, Zhang X, Li B, et al. The Effects of Psychological Stress on Depression. Curr Neuropharmacol. 2015;13(4):494-504.

137.Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM. Neurobiology of depression. Neuron. 2002;34(1):13-25.

138.Lang UE, Borgwardt S. Molecular mechanisms of depression: perspectives on new treatment strategies. Cell Physiol Biochem. 2013;31(6):761-77.

139.Rossetti C, Halfon O, Boutrel B. Controversies about a common etiology for eating and mood disorders. Front Psychol. 2014;5:1205.

140.Liptak N, Dochnal R, Csabafi K, Szakacs J, Szabo G. Obestatin prevents analgesic tolerance to morphine and reverses the effects of mild morphine withdrawal in mice. Regul Pept. 2013;186:77-82.

141.Szakács J, Csabafi K, Lipták N, Szabó G. The effect of obestatin on anxiety-like behavior in mice. Behav Brain Res. 2015;293:41-5.

142.Paxinos GF, KBJ. The Mouse Brain in Stereotaxic Coordinates. 2nd edition ed. San Diego CA: Academic Press; 2001.

143.Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology. 1987;92(2):180-5.

144.Petit-Demouliere B, Chenu F, Bourin M. Forced swimming test in mice: a review of antidepressant activity. Psychopharmacology (Berl). 2005;177(3):245-55.

145.Porsolt RD, Bertin A, Jalfre M. "Behavioral despair" in rats and mice: strain differences and the effects of imipramine. Eur J Pharmacol. 1978;51(3):291-4.

146.Porsolt RD. Animal model of depression. Biomedicine. 1979;30(3):139-40.

147.Powell TR, Fernandes C, Schalkwyk LC. Depression-Related Behavioral Tests. Curr Protoc Mouse Biol. 2012;2(2):119-27.

148.Tanaka M, Schally AV, Telegdy G. Neurotransmission of the antidepressant-like effects of the growth hormone-releasing hormone antagonist MZ-4-71. Behav Brain Res. 2012;228(2):388-91.

149.Tanaka M, Csabafi K, Telegdy G. Neurotransmissions of antidepressant-like effects of kisspeptin-13. Regul Pept. 2013;180:1-4.

150.Can A, Dao DT, Arad M, Terrillion CE, Piantadosi SC, Gould TD. The mouse forced swim test. J Vis Exp. 2012(59):e3638.

151.Jaszberenyi M, Bagosi Z, Thurzo B, Foldesi I, Szabo G, Telegdy G. Endocrine, behavioral and autonomic effects of neuropeptide AF. Hormones and behavior. 2009;56(1):24-34.

152.Zenker N, Bernstein DE. The estimation of small amounts of corticosterone in rat plasma. The Journal of biological chemistry. 1958;231(2):695-701.

153.Hodgson SR, Hofford RS, Norris CJ, Eitan S. Increased elevated plus maze open-arm time in mice during naloxone-precipitated morphine withdrawal. Behav Pharmacol. 2008;19(8):805-11.

154.Li JB, Asakawa A, Cheng K, Li Y, Chaolu H, Tsai M, et al. Biological effects of obestatin. Endocrine. 2011;39(3):205-11.

155.Tang SQ, Jiang QY, Zhang YL, Zhu XT, Shu G, Gao P, et al. Obestatin: its physicochemical characteristics and physiological functions. Peptides. 2008;29(4):639-45.

156.Currie PJ, Schuette LM, Wauson SE, Voss WN, Angeles MJ. Activation of urocortin 1 and ghrelin signaling in the basolateral amygdala induces anxiogenesis. Neuroreport. 2014;25(1):60-4.

157. Koob GF, Thatcher-Britton K. Stimulant and anxiogenic effects of corticotropin releasing factor. Progress in clinical and biological research. 1985;192:499-506.

158. Graeff FG ZHJ. The hypothalamic-pituitary-adrenal axis in anxiety and panic. Psychology & Neuroscience. 2010;3(1):3-8.

159. Luque Rú M, Córdoba-Chacón J, Ibáñez-Costa A, Gesmundo I, Grande C, Gracia-Navarro F, et al. Obestatin Plays an Opposite Role in the Regulation of Pituitary Somatotrope and Corticotrope Function in Female Primates and Male/Female Mice. Endocrinology. 2014;155(4):1407-17.

160. Kanehisa M, Akiyoshi J, Kitaichi T, Matsushita H, Tanaka E, Kodama K, et al. Administration of antisense DNA for ghrelin causes an antidepressant and anxiolytic response in rats. Prog Neuropsychopharmacol Biol Psychiatry. 2006;30(8):1403-7.

161. Pariante CM, Lightman SL. The HPA axis in major depression: classical theories and new developments. Trends Neurosci. 2008;31(9):464-8.

162. Lima-Ojeda JM, Rupprecht R, Baghai TC. Neurobiology of depression: a neurodevelopmental approach. World J Biol Psychiatry. 2017:1-33.

163. Koob GF, Zorrilla EP. Update on corticotropin-releasing factor pharmacotherapy for psychiatric disorders: a revisionist view. Neuropsychopharmacology. 2012;37(1):308-9.

164. Ishitobi Y, Akiyoshi J, Honda S, Ninomiya T, Kanehisa M, Tanaka Y, et al. Administration of antisense DNA for GPR39-1b causes anxiolytic-like responses and appetite loss in rats. Neurosci Res. 2012;72(3):257-62.

165. Dickson SL, Egecioglu E, Landgren S, Skibicka KP, Engel JA, Jerlhag E. The role of the central ghrelin system in reward from food and chemical drugs. Mol Cell Endocrinol. 2011;340(1):80-7.

166. Engel JA, Jerlhag E. Role of appetite-regulating peptides in the pathophysiology of addiction: implications for pharmacotherapy. CNS Drugs. 2014;28(10):875-86

167. Koob GF, Volkow ND. Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatry. 2016;3(8):760-73.

168. Motaghinejad M, Fatima S, Banifazl S, Bangash MY, Karimian M. Study of the effects of controlled morphine administration for treatment of anxiety, depression and cognition impairment in morphine-addicted rats. Adv Biomed Res. 2016;5:178.

169. Miladi-Gorji H, Rashidy-Pour A, Fathollahi Y. Anxiety profile in morphine-dependent and withdrawn rats: effect of voluntary exercise. Physiol Behav. 2012;105(2):195-202.

170.Belknap JK, Riggan J, Cross S, Young ER, Gallaher EJ, Crabbe JC. Genetic determinants of morphine activity and thermal responses in 15 inbred mouse strains. Pharmacol Biochem Behav. 1998;59(2):353-60.

171.Patti CL, Frussa-Filho R, Silva RH, Carvalho RC, Kameda SR, Takatsu-Coleman AL, et al. Behavioral characterization of morphine effects on motor activity in mice. Pharmacol Biochem Behav. 2005;81(4):923-7.

172.Rezayof A, Assadpour S, Alijanpour S. Morphine-induced anxiolytic-like effect in morphine-sensitized mice: involvement of ventral hippocampal nicotinic acetylcholine receptors. Pharmacol Biochem Behav. 2013;103(3):460-6.

173.Zhang Z, Schulteis G. Withdrawal from acute morphine dependence is accompanied by increased anxiety-like behavior in the elevated plus maze. Pharmacol Biochem Behav. 2008;89(3):392-403.

174.Rezayof A, Hosseini SS, Zarrindast MR. Effects of morphine on rat behavior in the elevated plus maze: the role of central amygdala dopamine receptors. Behav Brain Res. 2009;202(2):171-8.

175.Buckman SG, Hodgson SR, Hofford RS, Eitan S. Increased elevated plus maze open-arm time in mice during spontaneous morphine withdrawal. Behav Brain Res. 2009;197(2):454-6.

176.Kest B, Hopkins E. Morphine tolerance after chronic intracerebroventricular injection in male and female mice. Brain Res. 2001;892(1):208-10.

177.Schulteis G, Yackey M, Risbrough V, Koob GF. Anxiogenic-like effects of spontaneous and naloxone-precipitated opiate withdrawal in the elevated plus-maze. Pharmacol Biochem Behav. 1998;60(3):727-31.

178.Li T, Hou Y, Cao W, Yan CX, Chen T, Li SB. Naloxone-precipitated withdrawal enhances ERK phosphorylation in prefrontal association cortex and accumbens nucleus of morphine-dependent mice. Neurosci Lett. 2010;468(3):348-52.

179.Chen Y, Sommer C. The role of mitogen-activated protein kinase (MAPK) in morphine tolerance and dependence. Mol Neurobiol. 2009;40(2):101-7.

180.Girault JA, Valjent E, Caboche J, Hervé D. ERK2: a logical AND gate critical for drug-induced plasticity? Curr Opin Pharmacol. 2007;7(1):77-85.

APPENDIX

I.

II.