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

Ph.D. Thesis

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1. Introduction

The preproghrelin gene encodes several peptides with different structure and function, acyl ghrelin, desacyl ghrelin and obestatin. 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 and pathological processes.

Ghrelin is a 28 amino acid peptide and the endogenous ligand of the growth hormone secretagogue receptor 1a (GHS-R1a), originally identified from the rat stomach (*Kojima et al.,1999),* as having orexigenic activity and regulating food intake, body weight and energy homeostasis. The normal ghrelin signaling is disturbed in eating disorders such as obesity, anorexia and bulimia nervosa. Later, ghrelin was also shown to have a crucial role in the integration of feeding signals with those regulating emotion and mood. Furthermore, it is involved in motivated and rewarding behaviours induced by food and different addictive substances (alcohol, cocaine, amphetamin). Considering the effects on anxiety and mood, multiple studies conducted in rodents have demonstrated that ghrelin can induce not only anxiety and depression-like behaviors but it also has anxiolytic- and anti-depressant-like effects. Accordingly, in unstressed animals the administration of ghrelin would favour anxiety- and depression-like behavior. The anxiolytic- and antidepressant-like effect of ghrelin on the other hand may be a critical counter- regulatory mechanism to cope with different stress conditions, promoting food seeking, maintanance of energy homeostasis and survival advantage during evolution. 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.

Obestatin is a 23 amino-acid peptide, originally isolated from the rat stomach and identified as an anorexigenic hormone and antagonist of ghrelin *(Zhang et al., 2005).* 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, whith both central and peripheral effects. Later, most scientific papers refuted the anorexigenic effect of obestatin under a variety of conditions and only its acute food-intake inhibiting effect was reproducible. Despite these controversies,

obestatin has been shown to antagonise acyl ghrelin's effect on growth hormone secretion and food intake in rodents and fish, while fasting resulted in elevated ghrelin- and reduced obestatin levels. Furthermore, in a variety of obesity syndromes with different etiology (monogenic obesity, metabolic syndrome, Prader-Willi syndrome), in anorexia- and bulimia nervosa, the values and the ratio of the preproghrelin products (total ghrelin, acyl ghrelin and obestatin) were found either reduced, unchanged or increased.

In the past years much of the scientific interest has focused on obestatin's effect on glucose metabolism, accordingly obestatin was identified as an important regulator of pancreatic endocrine function and antiapoptotic/ survival factor for pancreatic islet cells, promoting cell proliferation and insulin secretion. Obestatin levels are altered in patients with obesity, type 2 diabetes mellitus, insulin resistance and metabolic syndrome. Obestatin can also modulate the function of adipose tissue, by promoting adipocyte differentiation and survival, and regulating lipogenesis/lipolysis and circulating lipid levels. 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.

Acting as a neuropeptide, it also exerts central effects, participating in the control and integration of neurocircuits which regulate metabolism, thirst, thermoregulation, and sleep. Additionally obestatin inhibits apoptosis and promotes proliferation and survival of hippocampal progenitor cells *in vitro*, which suggests a possible beneficial role in the treatment of neurodegenerative disorders. 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.

2. The aim of the study

- Until present, a single study has examined the effects of obestatin on anxiety in rats, indicating that it exerts anxiolytic-like effects in the elevated plus maze (EPM) test.
- In contrast to ghrelin, no data is available at present on obestatin's possible role in the behavioral responses related to depression or different drugs of addiction.
- For the above mentioned 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 (open field) tests.

- In order to reveal the depression-related responses, by using a similar treatment regimen, we also investigated the effects of obestatin in the forced swimming test (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 (forced swimming test), 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, followed by testing the animals in the OF and EPM tests.

2. Materials and methods

Male CFLP mice (25-35 g body weight) of an outbred strain *(Domaszék, Hungary)* were used for the experiments in accordance with the rules of the Ethical Committee for the Protection of Animals in Research (Faculty of Medicine, University of Szeged, Hungary). They were kept under a standard light–dark cycle (lights on between 07.00 and 19.00 h) with food and water available *ad libitum.*

2.1. Treatment protocols

Different animal groups were treated intracerebroventricularly (icv) with obestatin [(1-23), *AnaSpec, Inc. USA*] in doses of 0.5 µg, 1 µg, or 1.5 µg, or pretreated with either 1 µg/ [D-Lys3] -GHRP-6, *(Sigma-Aldrich),* or 0.1 µg antalarmin (*Bachem, Switzerland*). Control groups received 2 µl artificial cerebrospinal fluid (aCSF), which also was the vehicle for peptide treatments.

2.2. The effect of acute obestatin administration in the Open Field (OF) and EPM tests

The following behavioral parameters were detected in the OF (Conducta system, EXPERIMETRIA LTD. Hungary): ambulation distance and time, rearing, jumping, immobility time, as well as the percentage of distance and time spent in the center squares (center/total ambulation distance and time%).

In the EPM 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%) were registered.

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

In order to determine plasma corticosterone concentrations, trunk blood was collected in heparinized tubes. The plasma corticosterone concentration was measured by fluorescence assay (described by Zenker and Bernstein as modified by Purves and Sirett).

2.4. Forced swimming test (FST)

In our laboratory the modified mouse FST was performed as described by *Porsolt.* During the test session the durations of swimming, climbing and immobility were registered for 5 min, 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.

2. 5. Obestatin, morphine and the naloxone-precipitated withdrawal

Chronic morphine treatment was administered as described earlier. Mice received subcutaneous (sc), twice-daily injections of ascending doses of morphine (10-20-40 mg/kg), Mice were also treated daily with obestatin (1.5 μg/2 μl, aCSF icv). On the test day (day 4), a single dose of morphine (20 mg/kg,sc.) was given, followed by icv injection of obestatin. Withdrawal from morphine was achieved by the injection of 0.2 mg/kg of naloxone *(naloxone–HCl, Sigma-Aldrich),* followed by the testing of mice in the EPM or OF. Control mice received sc saline or icv aCSF.

3. Statistical analysis

Behavioral data were assessed by one-way ANOVA. Post-hoc individual means comparisons were conducted by Holm-Sidak method. Data with morphine treatment was analyzed by 2-way repeated measure. $P < 0.05$ was accepted as a significant statistical value.

4. Results

4.1. The effect of acute obestatin treatment in the OF and EPM tests

Acute central administration of obestatin had no effect on the overall locomotor activity, as measured by ambulation distance/time and the number of rearings and jumpings, respectively.

Central ambulation distance%: Was decreased by obestatin administration, and this effect was reversed both by the preatreatment with antalarmin and [D-Lys3] GHRP-6.

Central ambulation time %: The percentage of time spent in the central areas showed a decreasing tendency in obestatin-treated groups.

OAT% was significantly reduced by obestatin and a decreasing tendency in *OAE%* results was observed. Treatment with different doses of obestatin did not influence the number of total entries.

4.2. The effect of obestatin treatment on plasma corticosterone levels

Plasma corticosterone levels were elevated by the different doses of obestatin, this effect has been reversed by the administration of antalarmin.

4.3. The effects of obestatin on naloxone -precipitated morfin withdrawal

4.3.1. EPM results

Treatment with the graded doses of morphine and obestatin did not influence significantly the total activity, OAT% and OAE%, respectively. Naloxone-precipitated withdrawal induced a significant increase in both OAT% and OAE%.

4.4.2. OF test results

Treatment with graded doses of morphine significantly decreased the percentage of center ambulation distance, 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. Naloxone-precipitated morphine withdrawal caused a significant increase in the percentage of central ambulation distance and time, the effect on central ambulation time was reversed by obestatin.

4.5. Results in the FST

Immobility score was significantly increased and the swimming score was decreased by obestatin. Pretreatment with antalarmin antagonized the effect of obestatin on both immobility and swimming score. Combined treatment with obestatin and the ghrelin receptor antagonist [D-Lys3]-GHRP6 decreased the immobility score, and increased both the swimming and the climbing scores.

5. Discussion

Obestatin exerts anxiogenic- and depressive-like effects via HPA axis and ghrelin receptor signaling

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. It must be noted, however, that obestatin was originally reported to cause anxiolytic-like effects in rats in the EPM. 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 and obestatin. 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. Furthermore, 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. 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.

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. 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. 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 which merits further preclinical and clinical studies.

Regarding the mechanism of action of obestatin, no specific signaling pathway or receptor has been identified yet. 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. 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. 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, the anterior pituitary, amygdala and the hippocampus. The expression of obestatin, so far, was only demonstrated in the anterior pituitary, however its modulatory effect on feeding, anxiety and depression related-behavior, memory retention, neuroprotection 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. 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. 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.

In the current decade the majority of studies have indicated that acute or chronic morphine treatment differentially affects the anxiety behavior of rodents. Accordingly, the acute morphine treatment was shown to decrease anxiety, while chronic administration of morphine induced 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. 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 in the OF test, suggesting an anxiogenic-like effect.

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.

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. 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.

A possible explanation for the contradictory results related to the behavioral effects of naloxone-precipitated morphine withdrawal might be the differences in experimental design (dosage regimen, animal species/strains used).

It was also 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), exploration behavior, as well as defensive patterns to avoid and escape from withdrawal state.

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.

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.

The mechanism of action of obestatin on opiod withdrawal might involve the ERK1/2 signaling, which was recently identified to also mediate obestatin's beneficial effect on glucose metabolism and adipose tissue function.

6. 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. primarly 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.

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.

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Publications related to the topic of PhD thesis

1. LIPTÁK N, DOCHNÁL R, CSABAFI K, SZAKÁCS J, SZABÓ G.
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   In preparation; 2017