Ph.D. thesis

Examination of the ghrelin-induced oxytocin release in an *in vivo* animal model

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List of abbreviations

ARC: nucleus arcuatus

CVO: circumventricular organ

GHRP-6: growth hormone-releasing peptide-6

GHS-R: growth hormone secretagogue receptor/ghrelin receptor

i.c.v.: intracerebroventricular treatment

i.v.: intravenous treatment

NaCl: 0,9% physiological saline

PVN: nucleus paraventricularis

SON: nucleus supraopticus

VMN: ventromedial nucleus

VTA: ventral tegmental area

Introduction

The hypothalamic-pituitary axis is a complex pathway with a central role in the maintaining of body homeostasis. Neurohormones are known to be released not only via physiological stimulation but also via coexisting various peptides. Previous studies verified that the secretion of neurohypophyseal oxytocin is regulated by different aminergic and peptidergic neurotransmitters, including adrenaline, noradrenaline, dopamine, serotonin, histamine, and galanin.

Describing the role of the gut-brain axis has opened up new possibilities for gastroenterology and neurobiological researches over the past few decades. Ghrelin is a 28-amino-acid hormone, mainly secreted by the stomach. Beside the stomach, other tissues possess ghrelin production, such as the pancreas, kidneys, small intestine, and hypothalamus. Ghrelin is the natural ligand for G protein-coupled growth hormone secretagogue receptor (GHS-R) that is expressed in various regions of the brain and also in peripheral tissues. In the central nervous system, the density of the GHS-R is especially high in the nucleus arcuatus (ARC), and dorsal vagal complex, which mediate the effect of ghrelin on food intake, water consumption, as well as metabolic processes.

Oxytocin is a peptide of nine amino acids (nonapeptide), which is primarily produced in the magnocellular hypothalamic neurons (i.e. supraoptic nucleus, SON and paraventricular nucleus, PVN). Beside magnocellular neurons, parvocellular oxytocin neurons can be also divided that play a role in the modulation of cardiovascular function, respiration, nutrition as well as nociception via axonal connections. In addition to the axonal connection of oxytocin to posterior pituitary gland, there are projections to other regions, including the arcuate nucleus, ventromedial nucleus (VMN), ventral tegmental area (VTA), and the spinal cord. As a result of the central and peripheral regulatory mechanisms, endogen oxytocin level can be associated with metabolic processes.

The ghrelin-oxytocin interaction could contribute to the understanding and analysis of physiological and neurobiological properties.

Aims

While previous data clearly support the hypophyseal expression of GHS-R, only few studies are available on their relationship related to the hypothalamic-pituitary axis. The aim of our work was to determine the effect of the ghrelin-oxytocin interaction in animal model *in vivo*.

There were four aspects of our investigation:

- How can centrally injected ghrelin change the plasma oxytocin level in a rat model?
- What kind of alterations could be observed in the plasma oxytocin concentration as a result of systemic ghrelin administration?
- Whether the using of [D-Lys^3]-GHRP-6 ghrelin receptor antagonist is capable of detecting the ghrelin-mediated effects?
- How does the ghrelin receptor antagonist applied at different time points modify the ghrelin-oxytocin interaction in the rat model?

Materials and methods

Experimental protocol

The experiments were performed on 3- to 4-month-old male Wistar rats, ranging in weight from 180 to 250 g. Animals were kept in cages with free access to water and standard rat chow at controlled conditions (temperature, light, and humidity).

In order to examine the ghrelin-oxytocin interaction, rats were injected with ghrelin either centrally or systemically.

For central administration of the ghrelin, rats were anesthetized, and a cannula was inserted into the right lateral cerebral ventricle (intracerebroventricularly, *i.c.v.*). After a 1-week resting period, the doses of 1, 10, or 100 pmol ghrelin in 10 μl final volume were injected to the brain in 1 min, via a Hamilton syringe. Control animals were treated *i.c.v.* with vehicle that was 10 μl physiological saline (0.9% NaCl).

To verify the efficiency of ghrelin receptor antagonist, 10 pmol [D-Lys^3]-GHRP-6 antagonist was adjusted to the cerebral ventricle in 10 μl final concentration via 1 min. The ghrelin receptor antagonist was administrated in a dose of 10 pmol in itself, and 15 min before or after the ghrelin. The position of the *i.c.v.* cannula was checked by the injection of 1% methylene blue.

For systemic measurements, the rats were treated with ghrelin (1 or 10 nmol) in 200 μL of 0.9% NaCl intravenously (*i.v.*), whereas the control rats were injected with 0.9 % NaCl. [D-Lys^3]-GHRP-6 receptor antagonist treatment was similar to the *i.c.v.* treatments. The administration of the ghrelin antagonist was 10 nmol solved in 200 μL of 0.9% NaCl in 1 min, 15 min before/after the ghrelin injection ore in itself.

Thirty min following the administrations of *i.c.v.* or *i.v.* ghrelin or ghrelin antagonist, plasma samples were collected for the detection of oxytocin level.

Oxytocin radioimmunassay

The oxytocin levels were measured by radioimmunassay (RIA) technique. Synthetic oxytocin served as reference preparation for radiolabelling and specific oxytocin antibody was used (Bachem, CA, USA). Reverse-phase chromatography was used to purify the labelled hormone. The standard curves covered the range from 1 to 128 pg per assay tube. Oxytocin was extracted

from supernatants with an Amprep C8 minicolumn, with a recovery of more than 95 %. The dry residue was redissolved in 125 µL of assay buffer and 50-µL aliquots were used in duplicate for the RIA. The sensitivity of the assay for oxytocin was 1 pg/tube. A modified Lowry method was used for the determination of total protein content. The intra- and interassay coefficients of variation proved to be 13.3% and 16.3%, respectively. Oxytocin values are reported in pg/mg protein.

Statistics

The data are expressed as means ± S.E.M. Statistical analysis was performed by using the Tukey–Kramer multiple comparison test. P-values less than 0.05 were considered significantly different.

Results

Effects of centrally administrated (i.c.v.) ghrelin on the plasma oxytocin secretion

Following the injection of 1 pmol ghrelin to the right cerebral ventricle, the plasma oxytocin level significantly increased as compared with the control/intact group. Higher ghrelin doses (10 pmol and 100 pmol) further enhanced the oxytocin secretion; however, there was no significant difference between the effects of 10 or 100 pmol doses. Administration of the vehicle (0.9% NaCl) did not cause changes in oxytocin concentration, which verify that the vehicle did not influence the hormone level.

Effects of centrally administrated [D-Lys^3]-GHRP-6 antagonist on the plasma oxytocin secretion

In order to verify the ghrelin-oxytocin mechanism, the animals were treated with a dose of 10 pmol [D-Lys^3]-GHRP-6 antagonist. The antagonist in itself and prior to the ghrelin injection resulted in a significant decrease in the plasma oxytocin concentration as compared with the 10 pmol ghrelin-treated group. Administration of the antagonist prior to treatment with ghrelin clearly demonstrates that the reduction in oxytocin levels occurs through the modulation of ghrelin.

Contrary to this result, the ghrelin receptor antagonist, injected after ghrelin treatment, did not alter the oxytocin concentration.

Effects of systemically administrated (i.v.) ghrelin on the plasma oxytocin secretion

During systemic measurements, different doses of ghrelin were injected to the lateral tail vein of the rats. Both 1 and 10 nmol doses resulted in a same elevation in the plasma oxytocin level as compared with the control/intact group. Similar to the *i.c.v.* treatments, the vehicle (0.9% NaCl) did not influence the oxytocin concentration relative to the control group.

Effects of systemically administrated [D-Lys^3]-GHRP-6 antagonist on the plasma oxytocin secretion

Our results clearly show that the animals treated with 10 nmol ghrelin exhibited a significantly higher oxytocin release as compared with the control/intact group. While the post-treatment with 10 nmol [D-Lys^3]-GHRP-6 was inefficient in the oxytocin release, the receptor antagonist caused a significant decrease used in itself and prior to the ghrelin treatment as compared with the ghrelin-treated group.

Summary

Our *in vivo* measurements prove that ghrelin plays a role in the modulation of the hypothalamic-pituitary axis-mediated effects. Central administration of the ghrelin regulates plasma oxytocin release via binding to its locally expressed receptors or projecting to the adjacent brain areas. If ghrelin is systemically injected, it need to pass the blood-brain barrier to enhance the oxytocin level. Both circumventricular organs (CVO) as well as the blood-cerebrospinal barrier are well positioned areas in the brain, which facilitate the ghrelin penetration. Passing the blood-brain barrier, ghrelin is able to enhance the nucleus paraventricularis-derived oxytocin secretion as well as its release from the neurohypophysis.

The receptor-bound ghrelin is able to stimulate oxytocin release *in vitro* cell cultures as well as *in vivo* animal models. Interactions of peptide hormones hold the promise of being a potential regulator of metabolic processes as a potential target for treatment of disorders related to food intake and consumption.

Publications

Rudolf Ménesi MTMT code:

https://m2.mtmt.hu/gui2/?type=authors&mode=browse&sel=10066795

Publications related to the doctoral process

1. Szabó R.*, Ménesi R.*, Molnár H A., Szalai Z., Daruka L., Tóth G., Gardi J., Gálfi M., Börzsei D., Kupai K., Juhász A., László F A., Varga C., Pósa A.

New Metabolic Influencer on Oxytocin Release: The Ghrelin

Molecules 2019 Feb 18;24(4)

*= co-first author

2. Pósa A., Kupai K., Ménesi R., Szalai Z., Szabó R., Pintér Z., Pálfi G., Gyöngyösi M., Berkó A., Pávó I., Varga C.

Sexual dimorphism of cardiovascular ischemia susceptibility is mediated by heme oxygenase

Oxid Med Cell Longev. 2013;2013:521563

Publications in peer-reviewed journals

2.1. Publications related to the dissertation

Szabó R.*, Ménesi R.*, Molnár H A., Szalai Z., Daruka L., Tóth G., Gardi J., Gálfi M., Börzsei D., Kupai K., Juhász A., László F A., Varga C., Pósa A.

New Metabolic Influencer on Oxytocin Release: The Ghrelin

Molecules 2019 Feb 18;24(4)

IF: 3.060

*= co-first author

2.2. Other publications

Pósa A., Kupai K., Ménesi R., Szalai Z., Szabó R., Pintér Z., Pálfi G., Gyöngyösi M., Berkó A., Pávó I., Varga C.

Sexual dimorphism of cardiovascular ischemia susceptibility is mediated by heme oxygenase

Oxid Med Cell Longev. 2013;2013:521563

IF: 4.58

Pósa A, Szabó R, Kupai K, Baráth Z, Szalai Z, Csonka A, Veszelka M, Gyöngyösi M, Radák Z, Ménesi R, Pávó I, Magyariné Berkó A, Varga C:

Cardioprotective effects of voluntary exercise in a rat model: role of matrix metalloproteinase-2,

Oxid Med Cell Longev, 2015;2015:876805., doi: 10.1155/2015/876805.

IF: 4.44

Pósa A, Szabó R, Csonka A, Veszelka M, Magyariné Berkó A, Baráth Z, Ménesi R, Pávó I, Gyöngyösi M, László F, Kupai K, Varga C:

Endogenous estrogen-mediated heme oxygenase regulation in experimental menopause, *Oxid Med Cell Longev*, 2015;2015:787063., doi: 10.1155/2015/429713.

IF: 4.44

Cumulative IF: 16,52