Investigation of the role of nesfatin-1/NUCB2 in the central nervous system

Ph.D. thesis

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1. INTRODUCTION

It is essential for living beings to form the right energy balance. The energy source needed to maintain vital functions (basal metabolic rate) is food. Part of the metabolic process consumes calories through physical activity and heat. If the energy intake is greater than the energy expenditure this leads to body weight gain. In the reverse situation weight loss occurs. Obesity as well as pathological leanness is detrimental to health and increases the risk factors for many diseases.

The energy intake occurs by ingestion of food, which is regulated by many factors. The information about the energetic status of the organism and the fullness of the digestive tract reaches the central nervous system by neural and humoral pathways. This information is processed in the hypothalamus, which is the primary food intake regulator center. Various orexigenic (appetite stimulant) and anorexigenic (appetite inhibiting) neuropeptides mediate the information within the hypothalamus. This brain region has efferent connections with the autonomic nervous system, the corticolimbical reward centers and humoral output.

The main part of the energy intake is used for the basal metabolic rate, which is regulated by the hypothalamus-pituitary-thyroid axis. The starting point of this axis is the thyrotropin-releasing hormone (TRH) producing neurons of the paraventricular nucleus (PVN).

Beside the basal metabolic rate, energy expenditure can be realized by physical activity and heat production. The thermoregulation center is

also in the hypothalamus. In a warm environment the heat loss rate rises, there is vasodilatation of skin vessels together with sweating and an increase in saliva production. In a cold environment to minimize the heat loss, vasoconstriction of the skin vessels takes place and hair becomes erect. Moreover animals produce heat by shivering and by activating the brown adipose tissue (BAT). In thermoregulatory circuits the serotonin expressing medullary raphe pallidus (RPa) and raphe obscurus (Ro) cells are relevant. They act as sympathetic premotor neurons regulating skin blood flow, muscles and BAT.

The regulation of the energy balance is very complex; its set point could be affected by various factors, for example by stress. The best part of the stress information reaches the PVN through the A1 (in the area of the ventrolateral medulla - VLM) and A2 (on the territory of the nucleus of the solitary tract – NTS – and the dorsal motor nucleus of vagus complex) catecholamine cell groups in the brainstem. After the integration of the data in the PVN, two kinds of reaction start in the organism. The first is the activation of the hypothalamus-pituitary-adrenal axis. Its center is in the corticotrophin-releasing hormone (CRH) producing PVN neurons. The second is the activation of the sympathetic nervous system.

The anorexigenic nesfatin-1 is in the center of our investigations, it is a new element of the complex system of the energy balance regulation. Nesfatin-1 is a secreted, N-terminal fragment of the nucleobindin-2 precursor molecule. It is widely distributed in the central nervous system and in the periphery. Furthermore it is co-expressed with various

neurotransmitters, like CRH, TRH in the PVN; with serotonin in the RPa and with tyrosine-hydroxilase (catecholamine cell marker) in the NTS. On the basis of the localization of nesfatin-1 in the central nervous system it is supposed that nesfatin-1 is not only an anorexigenic peptide, but it plays a role in other aspects of the regulation of energy balance.

2. OBJECTIVES

To provide answers to the following questions:

I.)

1. What is the time course of the nesfatin-1 effect?
2. Does nesfatin-1 affect the thermoregulatory system of rats?
3. Does a cold environment activate the nesfatin-1/NUCB2 expressing cells?

II.)

4. Does the restraint stress affect the nesfatin-1/NUCB2 positive neurons?
5. Could the exogenous nesfatin-1 directly or indirectly influence the activity of the HPA-axis?
6. Does the negative glucocoricoid feedback have effect on the expression of the nesfatin-1/NUCB2 in the PVN?

3. METHODS

Male Wistar rats weighing 250-300 g were used for the studies. Experiments were performed according to the regulations set up by the Institutional Animal Care and Use Committee of the Semmelweis University and met the guidelines of the Animal Hygiene and Food Control Site (animal protocol number: 1894/003/2004).

The dynamics of the nesfatin-1 effects were determined by intracerebroventricular (icv) administration followed by telemetric recordings. This was used to monitor the rats' locomotion, core body temperature, heart rate and food intake frequency. The rats were observed over a 48h period during which time their body weight, food and water intake was measured in grams daily. All the effects of nesfatin-1 were measured by comparing a control group of rats with a treated group.

Next we subjected rats to cold conditions (4°C) for two hours, and analyzed the activation of the nesfatin-1/NUCB2 expressing cells by Fos immunostaining. In the region of the PVN we performed a nesfatin-1/NUCB2, prepro-TRH and Fos triple immunostaining. Control brain sections were used to co-localize the nesfatin-1/NUCB2 and prepro-TRH in the medullar RPa and Ro.

To determine the effect of exogenous nesfatin-1 on the HPA axis we monitored the ACTH and corticosterone levels in time after icv administration. After that the pituitary cell cultures were treated with nesfatin-1, and the ACTH release was measured. Hormone measurements were by radio-immunoassay.

A long-duration (4h) restraint was performed to establish the role of the endogenous nesfatin-1/NUCB2 in stress reactions. Nesfatin-1/NUCB2 mRNA level changes in the PVN and in the A1 and A2 cell groups were determined by quantitative *in situ* hybridization. In the mentioned brain areas the neuronal activation were detected by performing nesfatin-1/NUCB2, Fos double immunohistochemistry.

Last we tested the sensitivity of the nesfatin-1/NUCB2 mRNA expression in the PVN to the negative glucocorticoid feedback by bilateral adrenalectomy followed (1 week later) by a quantitative *in situ* hybridization.

4. RESULTS

Data collected by means of telemetry emitters revealed that the anorexigenic action of the nesfatin-1 is long lasting. The nesfatin-1 (25pmol) applied either at the beginning of the light or the dark phase decreased the nocturnal food intake duration (rats are nocturnal animals, they are active in the dark phase). This effect occurred during both dark periods in the observation time (48h). Data from daily food intake measurements revealed that the dark phase application of the nesfatin-1 decreased the amount of consumed food followed by a compensatory food intake on the second day. After the light phase treatment there were no significant changes in this parameter. The water intake on the first day was

strongly reduced, and the rats compensated on the second day both in the case of the dark or the light phase application. The body weight changes reflected the tendencies observed in food and water intake, but they were not significant in any cases.

Data regarding body temperature revealed the nesfatin-1 core temperature elevating influence became significant 1,5h after application in case of the light phase injection. In case of the dark phase application the temperature started to rise immediately. In both experimental schedules the difference between treated and control animals was the most outstanding in the light period, when the body temperature of the treated rats failed to reduce according the normal circadian rhythm and remained high. This resulted in a flattened circadian curve in treated rats.

We investigated whether the elevation of the body temperature could be the consequence of the higher locomotion activity, but we did not find any correlation between treatment and locomotion. Remarkably, nesfatin-1 application did not affect the heart rate.

Experiments were repeated with a higher dose of nesfatin-1 (100pmol) applied at the beginning of the light phase. The effects of the nesfatin-1 were the same as in the case of the lower dose (25pmol), but often they were enhanced or appeared sooner.

Since nesfatin-1 elevated the body temperature, we wanted to examine how nesfatin-1/NUCB2 expressing neurons could influence the thermoregulation. After a cold stress (2 hours, 4°C) we established neuronal activation (Fos positivity) in the hypothalamus in the supraoptic,

paraventricular and arcuate nucleus and in the brainstem in the RPa, Ro and NTS.

In the PVN, because of its integrating function, we used a triple immunostaining that exposed that many activated nesfatin-1/NUCB2 producing cells express prepro-TRh too.

The double immunostaining showed that nesfatin-1/NUCB2 positive cells in the RPa and Ro also expessed prepro-TRH.

It is known that stress affects food intake regulation and in consequence the energy balance. There are many neuropeptides that regulate food intake and stress reactions too. So we wanted to investigate the possible role of nesfatin-1 in stress.

Exogenously administrated nesfatin-1 (25pmol) time dependently elevated the plasma ACTH and corticosterone levels. On the other hand nesfatin-1 had no effect on ACTH release of the pituitary cell cultures.

After the exogenously added nesfatin-1, we investigated the endogenous nesfatin-1 role in a restraint (4 hours) stress model. In response to restraint stress we found intensive Fos expression in nesfatin-1/NUCB2 expressing cells of the parvocellular PVN. Beside this, restraint also induced nesfatin-1/NUCB2 mRNA expression in this area. As regards the brainstem, we found elevation in the nesfatin-1/NUCB2 mRNA levels only in the VLM, but not in the NTS. In the investigated medullary regions we did not observe neuronal activation as a result of restraint stress. This could be explained by the different reaction time to restraint stress of these areas compared to PVN.

Due to bilateral adrenalectomy the nesfatin-1/NUCB2 mRNA expression was elevated in the hypophysiotropic areas of the parvocellular PVN. The degree of the increase was especially pronounced in the dorsal medial parvocellular subdivision, where the CRH producing cells are located. So we confirmed the regulatory role of the negative glucocorticoid feedback on the nesfatin-1/NUCB2 expressing cells in the PVN.

5. CONCLUSIONS

Based on our results we established the following:

1. Nesfatin-1 has a long term action on food intake, and this effect is in interaction with the circadian rhythm. The daily measurements of the food and water consumption and body weight after a single administration indicate that the body weight reduction evolves only after a chronic treatment.
2. Nesfatin-1 increases the body temperature of the animals; this effect also interacts with the circadian rhythm of rats. Its action on the body temperature is also long lasting. The elevation of the core body temperature is not the result of intensive locomotion.
3. Nesfatin-1/NUCB2 expressing neurons are present in key brain regions of the thermoregulatory pathway, and they are activated by a cold environment.

4. Nesfatin-1/NUCB2 neurons in the medial parvocellular subdivision of the PNV are activated by restraint stress, furthermore the nesfatin-1/NUCB2 mRNA level is elevated too.

5. Nesfatin-1/NUCB2 mRNA level is increased in the VLM after restraint stress. This brain area plays a pivotal role in relay of the stress information.

6. The exogenous nesfatin-1 activates the HPA axis, but do not affect directly the ACTH release of the pituitary cells.

7. The nesfatin-1/NUCB2 mRNA expression in the hypophysiotropic subdivision of the PVN is under the control of the negative glucocorticoid feedback.

6. LIST OF PUBLICATIONS

Publications related to the thesis:

Könczöl K, Bodnar I, Zelena D, Pinter O, Papp RS, Palkovits M, Nagy GM, Toth ZE. (2010) Nesfatin-1/nucb2 may participate in the activation of the hypothalamic-pituitary-adrenal axis in rats. Neurochem Int, 57: 189-197. IF: 3,601

Könczöl K, Pinter O, Ferenczi S, Varga J, Kovacs K, Palkovits M, Zelena D, Toth ZE. (2012) Nesfatin-1 exerts long-term effect on food intake and body temperature. Int J Obes (Lond), 36: 1514-1521. IF: 5,221

Publications not related to the thesis:

Vas S, Ádori C, **Könczöl K**, Kátai Z, Pap D, Papp RS, Bagdy G, Palkovits M, Toth ZE. (2013) Nesfatin-1/NUCB2 as a potential new element of sleep regulation in rats. PLoS One 8(4): e59809. IF: 3,73