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Neuropeptides influencing food intake in the nuclei of lateral septum of rat

Thesis of PhD Dissertation

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1. Introduction

The most difficult effort of mankind was the struggle for the daily food during the human evolution. We may suppose that this battle was finished with victory all over the world by the end of 21st century. However the question is still contradictory, since starvation and plenty are existing next to each other in our world. A considerable part of mankind – around 800 million people – are undernourished, and a daily 23 thousand dies from starvation, whereas at the rich part of the world day by day hundreds of thousands die of overfeeding, the epidemy of overweight and the related civilisation diseases. How does the organism know that it needs nutrients? Special nuclei sensing the signals coming from the periphery via their receptors are present in the hypothalamus of the brain and their neurons send commands for initiating food intake or upon need stop feeding depending on the momentary energy demand of the organism. Scientific research of the neuronal regulation of food intake is focussed on the structure and function of hypothalamus. In addition several other brain areas including the nucleus of solitary tract, or nucleus Edinger-Westphal were proven to take part in this complicated procedure. The lateral septum (LS) also seems to belong to this system since in addition to its vegetative functions (maternal behaviour, anxiety) it maintains reciprocal connections with the arcuate nucleus (ARC), paraventricular nucleus (PVN), and lateral hypothalamus (LHA).

The LS and its connections

The septal complex has been in the spotlight of neural research during the last decades. As a part of the limbic system it maintains manifold connections with several other brain areas. Among these, the reciprocal connections with hypothalamus, the center of neuroendocrine regulation and with the hippocampal formation, the center of memory and learning are probably the two most important. Via these connections with the neuroendocrine system the LS may influence the food and water intake, blood pressure, various behavioural aspects such as anxiety, maternal behaviour, nursing, suckling, etc. The LS fulfills important relay function between cortical limbic areas and hypothalamic and brainstem centers. It is not a simple relay station, it also integrates endocrine, autonomic functions and cortical cognitive processes. Autoradiographic and anterograde tract tracing studies proved that the LS sends topographically organized efferents to the medial septum/diagonal band of Broca, to substantia innominata, hypothalamus, the midline nuclei of thalamus and several brainstem structures and at the same time receives input from the same areas.

Leucine-enkephalin (Leu-enk), neuropeptid Y (NPY), galanin (Gal) and food intake

The energy homeostasis of the organism is under complex neuroendocrine regulation, including – among others – the peripheral signals ghrelin and leptin, and the neuropeptides of regulatory centers in the brain. Reseach of the regulation of food intake mostly has been limited to the hypothalamic nuclei (ARC, PVN, LHA, ventromedial and dorsomedial nuclei). NPY and Gal were shown to be important anabolic effector molecules of hypothalamic neuronal regulatory circuits. NPY is the most frequently expressed neuropeptide, playing role in the regulation of many physiological processes (cognitive functions, food intake, circadian rythm, neuroendocrine processes, reproduction, and cardiovascular functions). It has strong orexigenic effect. ARC, an area where the effect of blood-brain barrier is not prominent, is very rich in NPY-expressing neurons sensing the peripheral signal molecules (leptin, insulin, ghrelin) of energy homeostasis, and then these neurons may send inputs to other brain areas of food intake regulation. Neurons with mRNA of NPY were shown to be present in LS, septohippocampal nucleus and close to septofimbrial nucleus.

Galanin was identified first in the small intestine, later it has also been found in the central nervous system, in the hypothalamic nuclei (ARC and PVN contains neurons expressing Gal). Though Gal posesses similar effects as NPY – its injection to the central nervous system enhanced food intake –its repeated use did influence neither obesity nor hyperphagie. Multiple synaptic contacts were identified between NPY-ergic (ARC) and galaninergic (PVN) neurons. Gal stimulates the release of NPY in PVN.

Since their discovery in the 70-es, many data were collected about the role of opioids in the regulation of food intake. In general, opioids stimulate food intake. According to the so-called „opioid-taste hypothesis” consuming tasty food causes release of endogenous opioids in the brain, and this leads to enhanced food intake. Application of opioid agonists caused increased amount of consumed food in mammals. Another study proved that for the orexigenic effect of NPY the stimulating central opioid signal is essential. An important representative of endogenous opioids is leucine-enkephalin synthesized from proenkephalin. LS receives very dense enkephalinergic innervation.

Several publications report changes in the amount of immediate early gene-products such as c-fos after reduced food intake. Among others certain cell-populations of the LS exhibited increased c-fos activity, referring to the role of LS in the regulation of food intake. Electrophysiological studies also measured increased neuronal activity upon food intake in LS. However none of the above methods give information about the neurochemical nature of the activated cells. The LS was also shown to contain considerable amount of immunocytochemically detectable orexigenic and anorexigenic neuropeptides. Based on these facts we have chosen the LS as experimental area to study immunocytochemically detectable density changes of 3 neuropeptides as a result of food deprivation.

2. Aims

Since only little information is available about the role of LS in the regulation of food intake, our aim was to study the rostrocaudal distribution of NPY, Gal and Leu-enk along the rostrocaudal axis of rat LS and the changes in their immunocytochemically detectable amount as a result of food deprivation. These studies were carried out on male, female, and ovariectomized (OVX) female rats in order to reveal possible sexual differences.

We aimed at answering the following questions:

- Where and in what sort of neuronal elements do occur Leu-enk, NPY and Gal within the nuclei of LS?
- Is there any difference in the immunocytochemically detectable amount of the three neuropeptides along the rostrocaudal axis of LS?
- Does change the density of Leu-enk, NPY and Gal upon one week complete food deprivation in male rats?
- What sorts of changes take place in the density of the three neuropeptides after 1-4 weeks partial (40 %) food deprivation?
- Do the female gonadal hormones influence the expression of the three neuropeptides in the LS?
- Are the density changes in females after 1-4 weeks 40 % food deprivation similar to those in males?
- How do the gonadal hormones influence the effect of 40 % food deprivation on the density of the three neuropeptides? (Comparison of OVX and intact female results).

3. Materials and methods

Experimental animals

Our experiments were carried out on young adult Wistar male (n=20) and female (n=32) rats weighing 250-280 g. The experiments were permitted by the Local Experimental Animal Welfare Committee of the Veterinary Faculty of Szent István University; moreover we followed the guidelines of EU Law (86/609EEC) and the government's animal welfare order of 243/98.

Operations

We carried out ovariectomy in deep anaesthesia on a part of the young adult female rats. We allowed two weeks recovery time before the food deprivation experiments.

Food deprivation experiments

The control groups in each experiment received regular rat chow and drinking water *ad libitum*. We carried out the following experiments:

- One week complete food deprivation of male rats: the food deprived group receieved no food only drinking water *ad libitum*.
- One week 40 % food deprivation of male, intact female and OVX rats: the food deprived groups received only 60 % of the average daily amount consumed by controls and drinking water *ad libitum.* At the end of the week we measured the body weight of each animal, then, preceeding the perfusion with fixative, we took blood test from the left ventricle of anaesthesized rats in order to measure the glucose, cholesterol, triglycerole and free fatty acid levels in the blood.
- 2-3-4 weeks 40 % food deprivation of male, intact female and OVX rats: the experimental design was the same as in the former experiments, only the duration of food deprivation was extended to 2-3-4 weeks respectively.

Perfusion and light and electron microscopic immunocytochemistry

The animals were perfused with physiological salt followed by a buffered fixative through the left ventricle of the heart in deep anaesthesia. The brains were removed and postfixed overnight in buffered 4% paraformaldehyde. 60 μm serial vibratome sections were cut at the coronal plane from the part of brain containing the septal complex. The conventional pre-embedding immunocytochemical procedure for Leu-enk, NPY and Gal on free-floating sections was applied. Following the visualization of the immunoreactions sections were mounted on gelatine-coated glass slides, dehydrated and covered with coverslip in DePeX. Sections for electron microscopy were postfixed in OsO_4, dehydrated and flat-embedded in Durcupan resin. Areas of LS rich in immunoreactive elements were re-embedded into blocks and 60 nm ultrathin sections were cut with a Reichert ultramicrotome**.** Electron micrographs were taken in a JEOL 100C electron microscope.

Densitometric evaluation of the sections

Serial sections from control and food deprived animals were photographed with an Olympus light microscope equipped with a digital camera at a magnification of ×40. The

Scion Image for Windows v4.02 program (Scion Corporation, Frederick MD, USA) was used for the densitometric evaluation of the digital photos.

Statistical analysis of the results

From the densitometric data of every single control and experimental groups an average±standard deviation was calculated with the help of Excel program (Microsoft). The differences between the control and food deprived groups were confirmed by Student t-test. We considered the differences as significant, if $p<0.05$. This statistical analysis was done with the help of Kaleidagraph (Synergy Software) program, summarizing graphs were prepared with Microsoft Excel program.

4. Results and discussion

In our study we followed density changes of three neuropeptides, Leu-enk, NPY and Gal, caused by food deprivation in the lateral septum of male, intact female and ovariectomized rats.

Distribution and rostrocaudal changes of Leu-enk in the control rat LS

A dense Leu-enk IR fiber network was detected in the medial part of the dorsal subnucleus and in the lateral part of the intermedial and ventral subnuclei of LS. Varicose fibers formed pericellular baskets around immunonegative cell bodies. These targets are suggested to be calbindin IR neurons and 10 % of them are GABAergic somatospiny neurons according to the literature. We were not able to detect Leu-enk IR cell bodies since we did not apply colchicine treatment. We studied the rostrocaudal distribution of the immunopositive fibers and we experienced the densest Leu-enk fiber network in the middle of the section series, whereas the most rostral and caudal sections contained much less (roughly 1/8) Leu-enk IR fibers. This pattern suggests that the neuron population situated in the middle of LS receives considerable Leu-enk input.

Changes in the density of Leu-enk as a result of OVX

The loss of female gonadal hormones caused well detectable increase in the density of Leu-enk of LS. Earlier *in situ* hybridization studies showed cycle-dependent changes in the opioid peptide mRNA level in intact females and in OVX animals following estrogen application. At the ventromedial hypothalamus the reduction of proenkephalin mRNA expression was detected after gonadectomy, at the same time there was no change in the striatum and olfactory bulb. On the basis of these we can assume an area-dependent connection between the gonadal hormones and central opioid signals. The increased amount of Leu-enk in the LS may be due to the transport from the hypothalamic somata to the axons terminating and branching within the LS and this would also explain the decrease in the hypothalamus.

Effect of fasting and partial food deprivation to the density of Leu-enk in the LS of male, female and OVX rats

Independent of the type of food deprivation the amount of Leu-enk was decreased in three of our experiments. The decrease was smallest in the case of male 40 % food deprived group. In addition, as a result of the prolonged food deprivation in this group, the changes were slower and also prolonged. Changes were not experienced in the OVX 40 % food deprived group. This can be explained by the opposite effects of ovariectomy and food reduction. The decrease could be experienced only after the longer (2-4 weeks) food deprivation period. Earlier studies describe the opioids as short-term appetizers. In another study the authors write about the appetite-reducing effect of opioid antagonists in rodents and human. In our experiments this effect could not be manifested. Our experimental desing can be considered as long-term food deprivation and the animals tried to adapt to the circumstances with the reduction of the amount of appetite-enhancing opioid peptides. On the other hand starving is a stressful effect and habituation to this may be behind the decreased expression of opioids as well.

Neuropeptide Y and its rostrocaudal distribution in the rat LS

Similarly to the Leu-enk the NPY is also present along the whole rostrocaudal axis of LS. A part of cell bodies and varicose fibers are present at the lateral part, close to the ependyma lining the lateral ventricles, and another group of cells and fibers are detectable in the dorsal LS. We observed that the somata in the lateral part send their processes into the ependymal layer, an arrangement which may refer to liquor-monitoring function. NPY-cells in hypothalamus were shown to express leptin receptors and this may be true for the lateral NPY-IR cell groups of LS as well being in a position directly sensing the leptin-level of liquor. The rostrocaudal distribution of NPY showed two smaller peaks in accordance with earlier results reporting NPY mRNA in septohippocampal nucleus and its vicinity near septofimbrial nucleus. Our studies in addition report another density increase as we approach to the caudal end of the brain area.

Density changes in NPY as a result of ovariectomy

After OVX the density of NPY decreased to 28 % of the control value. Removal of ovaries and the loss of gonadal hormones leads to obesity and hyperphagie. In ARC and PVN an increased NPY gene expression was detected after OVX which was reduced to normal after the application of gonadal hormones. Earlier it also was shown that NPY neurons express estrogen receptors and this supports a direct effect of these hormones to the NPY system. In our non-published double labelling study we colocalized NPY and GABA within the LS. We observed pericellular multiple contacts of NPY-IR boutons around GABA-IR cells. Taken this together with the reciprocal connection between the above areas and LS we can assume both direct and indirect effects of estrogens or more ecxactly the loss of estrogens as a result of OVX. Further studies are needed to elucidate the exact mechanism of this effect.

Effect of fasting and food deprivation on the density of NPY in male, intact female and OVX rats

NPY is known to be the most orexigenic neuropeptide. Its orexigenic effect can be evoked by its intracerebral application as well as by reduced level of leptin as a consequence of lack of food. Our results are in accord with these facts. As a result of 40 % food deprivation an increased NPY density was detected in both males and females after one week. The complete food deprivation and OVX-combined 40 % food deprivation seems to result in contradictory reductions of NPY. It is worth to take into consideration however, that upon complete food deprivation down-regulation probably may take place at an earlier phase of fasting. We experienced such reduction of NPY level in our long-term 2-4 weeks food deprivation from the second week. Moreover the down-regulation of NPY happens together with the upregulation of Gal, which is considered as another orexigenic neuropeptide, able to take over the role of NPY. The 4 weeks partial food deprivation caused reduction in NPY density in the long run (from 2-3 weeks). The tendency of reduction was the same in male and female animals but it was quicker and larger in females and OVX females. This also may be the result of gonadal hormone fluctuation.

Galanin and its rostrocaudal distribution in the rat LS

The occurrence of galanin partially overlapped with that of NPY in the LS. In the intermedial nucleus and in the lateral part of ventral nucleus there was a dense plexus of varicose Gal-IR fibers, whereas they were missing from the dorsal nucleus. We did not identify Gal-IR cell bodies. In earlier studies such cells were present in colchicine-pretreated mice but also missing from rats. The density of Gal-IR fibers was the lowest from among the 3 studied neuropeptides. The rostrocaudal distribution followed the pattern of NPY with two lower peaks referring to the complementary role of the two orexigenic neuropeptides. However further studies are needed to elucidate the exact structural and functional relationships between the two peptidergic systems.

Effect of ovariectomy on the density of Gal

We experienced nearly 50 % reduction of Gal density upon OVX. In mediobasal hypothalamus Gal-IR neurons were expressing estrogen receptors. Elevated estrogen level during proestrus was shown to increase the amount of Gal together with other publications supporting that estrogens increase Gal-expression. Our observation of reduced Gal-density is in accordance with these results, thus, the loss of gonadal hormones may well be behind the depletion of Gal-IR.

Effect of fasting and food deprivation on the density of Gal in male, intact female and OVX rats

After one week complete food derpivation of males the density of Gal increased in LS. NPY neurons of ARC enhance the expression of Gal in PVN neurons. We can assume similar connections between NPY and Gal in the LS too for the explanation of this phenomenon. It may well be that following the reduction of NPY level, Gal took over the orexigenic role of NPY. In our long-term experiments during the first two weeks an increased Gal density was detected, which changed into gradual reduction by the third and fourth weeks. This pattern also follows the changes int he density of NPY and comfirms the functional connection between the two peptidergic systems. The increased density values by the end of the first week refer to the shifting of metabolic processes to katabolic ditection (e.g. enhanced fatty acid oxidation) in order to maintain energy balance, while the reduced values by the third week may be due to the adaptation of energy metabolism to the reduced energy sources.

In the case of intact and ovariectomized females we observed that the changes of Gal density follow the changes of NPY density with a certain delay, thus, the downregulation of NPY is followed by the downregulation of Gal with a detectale delay.

Our results allow the conclusion that the LS being in strong reciprocal connections with the primary hypthalamic regulatory centers, at least indirectly inferes with the complicated regulatory processes and the LS can be considered as a part of the neuroendocrine system regulating food intake.

5. New scientific results

We firstly studied the effect of food deprivation and gonadal hormones on the density of some orexigenic and anorexigenic neuropeptides in the lateral septum of rats.

The most important results of our experiments are:

1. In control animals we established the distribution of Leu-enk, NPY and Gal along the rostrocaudal axis of LS and we found that the Leu-enk was the most densely occuring neuropeptide followed by NPY and Gal. Whereas the density of Gal was near to even along the rostrocaudal axis, the immunoreactive Leu-enk plexus showed a peak roughly at the middle region. At the same time NPY showed two lower peaks at one third and two third of the rostrocaudal axis of LS.
2. After one week complete food deprivation of male rats the density of Leu-enk and NPY decreased, and the density of Gal was doubled. The long term partial food deprivation of males showed a gradual decrease in Leu-enk, NPY and Gal changed parallel with each other, namely they increased in the first two weeks, then they went down the control values.
3. As a result of OVX alone the density of Lu-enk increased, that of NPY and Gal decreased.
4. In intact females after long-term partial food deprivation we experienced that the tendency of changes in the amount of Leu-enk and Gal was reduction, whereas NPY showed increase by the end of first week followed by gradual decrease by the end of the fourth week.
5. In OVX females the tendency of changes were similar to those in intact males.

6. Own publications

Related to the PhD thesis

In peer reviewed scientific journals

Kovács, É.G., Szalay, F., Halasy, K. (2005): Fasting induced changes of neuropeptide immunoreactivity in the lateral septum of male rats. Acta Biologica Hungarica. 2005, 56: 185-197. Impact factor: 0,474

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Other publications

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Presentations on international conferences:

Kovács, É.G., Bagyánszki, M., Resch, B., Fekete, É. (2001): Quantitative differences in the distribution of myenteric nitrerg neurons along the longitudinal axis of the developing human fetal intestine. - Poster on the VIIIth Annual Meeting of the Hungarian Neuroscience Society, 24th-27th January, 2001, Szeged, Hungary

Halasy, K., **Kovács, É.G.**, Tri-Lam, T., Leranth, C. (2003): Vesicular glutamate transporters in the hippocampus of the rat: effect of lesions on immunostaining. Clinical Neuroscience. 56, pp 33.

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