Szent István Egyetem

Állatorvos-tudományi Doktori Iskola

Functional hypothalamic asymmetry and introduction to a novel estrogen/estrous phase-dependent regulatory mechanism in mitochondrial energy levels in the female rat hypothalamus

(A hipotalamusz funkcionális aszimmetriája és új ösztrogénfüggő szabályzómechanizmusok a hipotalamikus neuronok mitokondriális metabolizmusában)

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Introduction

The hypothalamus plays a key role in the central regulation of various homeostatic systems and related functions, such as energy metabolism, reproduction and sleep-wake behavior. Our research group has investigated the neuronal mechanisms underlying the hypothalamic regulation of gonadotropin hormone-releasing hormone (GnRH) secretion/release and consequential pituitary luteinizing hormone- (LH) surge. Those studies have made it clear that the cyclic nature of female reproductive physiology is the consequence of fluctuating synaptic reorganization in the neuroendocrine hypothalamus. The latter synaptic events, also known as morphological synaptic plasticity, determine the actual number of stimulatory and inhibitory synapses in the hypothalamus, thus continuously imposing a limit to the functional intensity of the two basic types (excitation-inhibition) of neuronal functions. Today, it is generally accepted that the aforementioned synaptic plasticity is responsible for the final shaping of the patterns detectable in hypothalamic functions, with special regard to the regulation of GnRH-release (but also including a number of other hypothalamus-driven mechanisms, e.g., the food-intake, etc.).

The brain is an organ with symmetric tissue organization; in the mature organism paired brain areas usually have distinct physiological functions. Experimental results raising the possibility of functional asymmetry in the neuroendocrine hypothalamus, particularly in the hypothalamus-gonad axis, have emerged long ago. Based on these findings, our research group reopened this question examining the mitochondrial metabolic activity throughout the estrous cycle differentially in the two halves of the rat hypothalamus. The reason for applying mitochondrial metabolic measurements is supported by the fact that synapse generation and neuronal functions, especially neurotransmission, are highly energy dependent. Therefore, the actual ATP level and the regulation of it must be a suitable indicator for all those neuronal functions and dynamic plastic events, which occur during the phases of the estrous cycle.

Considering the aforementioned data, we proposed two hypotheses: 1) The regulation of hypothalamic cellular energy levels is asymmetric, and 2) NTPDase3, as an ATP-hydrolyzing enzyme, plays a role in the regulation of hypothalamic mitochondrial ATP-levels.

Given that the cyclic activity of the female hypothalamus periodically enters the state of high energy (ATP) need, our first working hypothesis states that if functional hypothalamic asymmetry existed, it should be detectable at some point of the reproductive cycle on the level of a general parameter of neuronal metabolism, the mitochondrial respiration.

Earlier, our research group was the first to identify type 3 ecto nucleoside triphosphate diphosphohydrolase 3 (NTPDase3) in the CNS and map its distribution in the rat brain. Particularly high expression levels of NTPDase3 were found in the mitochondria of stimulatory neurons, but not in other cell types of the hypothalamus. Based on these findings, our second working hypothesis states that if NTPDase3 is present in mitochondria, experimental inhibition of its ATP-hydrolyzing activity should significantly decrease ADP-dependent state3 mitochondrial respiration (St3), and the enzyme's expression and/or activity should be estrogen- (E2) dependent. Experimental support of our second working hypothesis would make NTPDase3 a likely candidate for the regulation of mitochondrial energy levels in hypothalamic stimulatory neurons.

Aims of the study

In terms of hypothalamic asymmetry in mitochondrial metabolism, we attempted to answer the following questions:

I. Is there any difference in the overall oxygen (O_2) content and consumption between the two sides of the hypothalamus in normal cycling female rats?

II.

- **A.** If yes, is there any recognizable pattern of this metabolic sidedness in the course of the estrous cycle?
- **B.** What are the characteristics of the mitochondrial sidedness in different states of mitochondrial metabolism?

III.

- **A.** Is there any difference between the proportion of the left and right sided hypothalami?
- **B.** If yes, how does it change during the estrous cycle phases and in different states of mitochondrial metabolism?

In terms of the role of NTPDase3 in hypothalamic regulatory mechanisms, our goal was to

I.

- **A.** determine the neuron type-specificity of the enzyme's location.
- **B.** determine the subcellular distribution of the enzyme.

II.

- **A.** demonstrate if there is any effect of E2 on the NTPDase3 expression, and if yes,
- **B.** is it reflected in the enzyme's activity?

III.

- **A.** show the effects of E2 on ADP-dependent St3 in the lateral-medial parts of the hypothalamus, and the
- **B.** effects of fasting versus fasting/re-feeding on ADP-dependent St3 in ovariectomized rats.

Materials and Methods

Normal cycling female Sprague-Dawley rats were used to examine the metabolic asymmetry of the hypothalamus throughout the phases of the estrous cycle. In addition, we also investigated E2 effects in ovariectomized rats. Reproductive cycling was determined and verified by periodic examination of vaginal smears.

Mitochondrial respiration measurements were carried out using samples obtained from the left and right sides of the hypothalami; tissue blocks were homogenized and fractionated by means of differential separation followed by Percoll-gradient fractionation. Mitochondrial respiratory rates were registered in synaptosomal fractions using Clark-type oxygen electrode.

Immunohistochemical labeling techniques were followed by electron microscopic analysis to identify the histological distribution and subcellular localization of NTPDase3, and to co-localize NTPDase3 with glutamic acid-decarboxylase (GAD).

Western blot studies were performed to determine NTPDase3 expression levels. To demonstrate the activity of NTPDase3 in mitochondrial metabolism, we blocked the enzyme with suramin before the mitochondrial respiratory measurements.

Results and Discussion

Hypothalamic asymmetry in mitochondrial metabolism

In general, there are two important aspects of our results: 1. The mitochondrial metabolism showed a fluctuation that corresponded with the phases of the estrous cycle; 2. The fluctuation in mitochondrial metabolism occurred in only one side of the hypothalamus (referred to as the „active" side), while mitochondrial respiration rate (MRR) values in the contralateral side remained nearly steady (balanced) throughout the estrous cycle (referred to as the „silent" side). Therefore, it is reasonable to assume that the regulation of GnRH secretion/release is based on asymmetric/sided hypothalamic activity. We are aware that presently there is no direct evidence available to prove whether functional inhibition of the „active side" would prevent the GnRH surge (ongoing experiments in our laboratory aim to clarify this question). However, for the sake of creating a new hypothesis/theory from the present results, we will attempt to interpret the data in a relatively speculative manner, assuming that the excess mitochondrial metabolic activity of the „active" side over the „silent" side is responsible for the generation of GnRH-release.

Determination of the neuron type-specificity distribution of NTPDase3 in the hypothalamus

Light microscopic analysis of immunoreactive (IR) profiles showed NTPDase3-IR cell bodies and neural-like processes in the lateral hypothalamic nucleus (LHN) and arcuate nucleus (AN), whereas in the rest of the hypothalamus only immunostained cell processes were found, many of which were morphologically closely associated with the vasculature. According to the comparison of GAD and NTPDase3 immunostainings, none of the 2540 GAD-IR neurons examined contained NTPDase3, indicating that NTPDase3 may be predominantly expressed in excitatory neurons of the hypothalamus.

Subcellular localization of NTPDase3 in the hypothalamus

Our light- and correlated electron microscopic studies showed that NTPDase3-IR is present at certain well-demarked segments of the plasma membrane, supporting the generally accepted view of NTPDases being transmembrane proteins and hydrolyzing phosphorylated

nucleotides outside of the cell. Electron microscopic analysis, however, clarified that NTPDase3 is present in both dendrites and axons, and most interestingly labeled particles were found bound to ribosomes and in the mitochondrial matrix or closely associated with the inner mitochondrial membrane. Based on these findings and our present knowledge about NTPDase function, it is suggested that this ATP-hydrolyzing protein may play a role in the regulation of the ATP/ADP ratio in hypothalamic excitatory neurons.

Estrogen effects on hypothalamic NTPDase3 expression

Since the neuroendocrine hypothalamus is highly E2-responsive, it was reasonable to assume that E2 may influence the expression level of NTPDase3 within this brain area. In samples containing the LHN, NTPDase3 expression levels increased significantly 4–12 hrs after a single subcutaneous injection of E2 in ovariectomized rats, and gradually returned to nearly control levels by 16–26 hrs after E2 treatment. In contrast, temporal changes in medial hypothalamic samples containing the AN showed an initial increase in NTPDase3 expression between 6–10 hrs after E2 treatment, followed by a sharp decrease to control level, and again followed by a second rise between 22–26 hrs after E2 treatment. Thus, in the lateral hypothalamus a single-, whereas in the medial hypothalamus a double-peaked curve was determined, suggesting that NTPDase3 expression in the hypothalamus is regulated by E2 and this regulation in the two parts of the hypothalamus is likely associated with the distinct functions of the two hypothalamic regions.

Since the mediobasal hypothalamus, including the AN, is a major player in the biphasic (positive- and negative feedback) regulation of the gonadotrophin secretion and release, it is reasonable to speculate that in the medial part of the hypothalamus, NTPDase3 may be involved in the estrogenic control of gonadotrophins. This hypothesis is consonant with our observation that inhibition of NTPDase activity decreases St3 and the total mitochondrial respiratory capacity, ergo an increased amount of mitochondrial NTPDase3 would well serve the energy needs of a transient intensification in excitatory neuronal activity that accompanies the E2-dependent synaptic reorganization on hypothalamic neurons right before the GnRH surge.

Demonstration of NTPDase3 activity in hypothalamic mitochondria

Inhibition of NTPDases in male rats by suramin had a significant inhibitory effect on St3 (45.05 ± 4.9 nmol O_2 mg protein/min with suramin versus 65.1 ± 6.6 of control). The total mitochondrial respiratory capacity decreased as well (87.8 ± 5.5 nmol O_2/mg protein/min in control synaptosomes versus 57.7 ± 6.5 nmol O_2/mg protein/min). These results suggest that mitochondrial NTPDase3 activity likely influences O_2-consuming biochemical processes in the mitochondrial matrix. All these suggest that the yet unidentified endogenous regulation of intramitochondrial NTPDase3 activity is a likely candidate mechanism for the energetic regulation of excitatory neurotransmission.

Estrogen's differential effects on St3 in the lateral-medial parts of the hypothalamus

Ten hours after a single subcutaneous injection of E2, St3 increased by 63 % (lateral hypothalamus) and 43 % (medial hypothalamus), respectively. The differences between NTPDase3-expression and enzyme activities in respective hypothalamic areas suggest additional mechanisms that, besides the regulation of E2-dependent NTPDase3-expression, might be involved in the regulation of NTPDase-activity.

Effects of fasting versus fasting/re-feeding on ADP-dependent St3 in ovariectomized rats

Effects of 24 hrs fasting in E2-deprived animals increased St3 by 44 % in the medial hypothalamus as compared to the 81 % increase in the lateral hypothalamus, latter which was reverted 4 hrs after refeeding. E2 may exert its effects on the regulation of feed-intake

targeting the lateral hypothalamic (LHT) neurons rather than the medial hypothalamic (MHT cells.

Summary

The hypothalamus is one of the most important brain areas that are responsible for the regulation of homeostatic processes, such as reproductive events, feed-intake, body temperature and sleep-wake behavior, and as such, it is a central target for major peripheral hormones, such as estrogens, thyroid hormones, ghrelin, leptin, etc.

Based on early studies that raised the possibility of the functional lateralization of the hypothalamus, we aimed to examine one of the general parameters, the mitochondrial metabolism, that could reliably indicate a possible functional sidedness of the hypothalamus. Our experiments on normal cycling female rats indicated that the intensity of mitochondrial metabolism in hypothalamic samples followed the phases of the estrous cycle, however, this cyclicity was only observed in one of the hypothalamic hemispheres ("active" side). The contralateral side of the hypothalamus did not show such fluctuation in the examined metabolic aspect ("silent" side). Thus, based on these results we concluded that there is a sidedness in hypothalamic functions, and because of its dependence on the estrous phase, it is most likely related to the regulation of GnRH-secretion/release.

The regulation of GnRH (the ovarian cycle) is the consequence of cyclic reorganization of hypothalamic synapses; hence, morphological and functional synaptic plasticity regulates the secretion and release of GnRH. Synapse generation and neurotransmission are highly energy-dependent. Therefore, it was reasonable to assume that energy levels in hypothalamic mitochondria are constantly adjusted to the actual physiological needs of the relevant regulatory cells.

Based on its ATP-hydrolyzing function, one of the likely candidates to regulate mitochondrial energy (ATP) levels in the hypothalamus was the NTPDase3. This assumption was further sustained by our findings on the brain distribution and the subcellular localization of the enzyme. Our immunohistochemical co-localization studies showed that NTPDase3 expression is highly restricted to excitatory neurons of the hypothalamus. Electron microscopic studies identified NTPDase3-IR particles incorporated in the plasma membrane, linked to ribosomes and present in the mitochondrial matrix of excitatory hypothalamic neurons.

Results from morphological studies were confirmed by successive functional data, as inhibition of NTPDase3 in hypothalamic synaptosomes significantly decreased the ADP-dependent St3. Thus, the evidence we have obtained this far strongly suggested that the hypothalamic neuronal metabolism that is associated with the regulation of female reproductive functions (GnRH-release) is asymmetric in nature, and that NTPDase3 most likely plays a role in the regulation of GnRH-secretion by the regulation of the availability of mitochondrial-cellular ATP needed for stimulatory neuronal actions.

Experiments were carried out on ovariectomized rats to demonstrated further functional link between estrogen-regulated hypothalamic mechanisms and NTPDase3. E2 administration differentially increased the expression level of NTPDase3 in the medial and lateral parts of the hypothalamus, suggesting that E2 may regulate neuronal ATP-hydrolysis in these two parts of the hypothalamus in a functionally relevant manner. In consonance with these, an increase in ADP-dependent St3 values was detected after the administration of E2 to OVX animals, further implying that an E2-caused increase in NTPDase3-expression manifests in increased NTPDase3-activity.

Since the hypothalamus is the regulatory center of feed-intake as well, it seemed reasonable to compare the effects of E2 and hunger on St3 in the lateral and medial parts of the hypothalamus. Results showed that St3 is differentially regulated in those two parts of the hypothalamus, and this finding was in line with our results from the described Western-blot experiments on NTPDase3-expression.

As a final conclusion, the neuroendocrine hypothalamus is functionally asymmetric, including the regulation of female reproductive processes and most probably that of feeding as well. This functional asymmetry includes blood/O_2 supply, mitochondrial metabolism and the regulation of neuronal cellular energy levels, in which NTPDase3 plays a functional role through the down-regulation of mitochondrial ATP levels in a manner that depends on both estrogen and satiety levels. Our present results suggest that future consideration of hypothalamic functional laterality in hypothalamus research would lead to radically new observations regarding probably all known hypothalamic functions.

New scientific results

In the presented series of experiments, we

- reopened the question regarding sidedness in hypothalamic functions;
- determined asymmetry in mitochondrial oxygen consumption in the two hypothalamic hemispheres related to GnRH release;
- demonstrated the cyclic changes in mitochondrial activity of the mediobasal hypothalamus;
- identified NTPDase3, an ATP-hydrolyzing enzyme, expressed in excitatory hypothalamic neurons; as a potential effector in the regulation of synaptic plasticity and GnRH-release in the hypothalamus;
- determined the subcellular distribution of NTPDase3;
- proved that estrogen can upregulate NTPDase3 protein expression in the hypothalamus;
- presented evidence that NTPDase3 can regulate ADP-dependent St3 mitochondrial respiration;
- demonstrated estrogen's differential effects on ADP-dependent St3 mitochondrial respiration in the lateral and medial parts of the hypothalamus;
- demonstrated that fasting differentially affects St3 ADP-dependent mitochondrial respiration in the lateral and medial parts of the hypothalamus.

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