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

PhD Thesis

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Abbreviations

ADP	adenosine diphosphate
AgRP	agouti-related protein
ATP	adenosine triphosphate
AN	arcuate nucleus
BSA	bovine serum albumin
CNS	central nervous system
DE	diestrus
DMSO	dimethyl sulfoxide
E	estrus
E2	17 β -estradiol
EGTA	ethylene glycol tetraacetic acid
EP	early proestrus
ER	estrogen receptors
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
GAD	glutamic acid-decarboxylase
GnRH	gonadotropin-releasing hormone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LH	luteinizing hormone
LHN	lateral hypothalamic nucleus
LHT	lateral hypothalamus
LP	late proestrus
M	malate
MBH	mediobasal hypothalamus
ME	metestrus
MHT	medial hypothalamus
MnSOD	superoxide dismutase
MPOA	medial preoptic area
MRR	mitochondrial respiration rate
NADH/NAD ⁺	nicotinamide adenine dinucleotide
NPY	neuropeptide Y
NTPDase	ecto nucleoside triphosphate diphosphohydrolase
OVX	ovariectomized
P	pyruvate
St1-5	mitochondrial respiratory state (typ 1-5)
UCP	uncoupling protein

I. Introduction and hypothesis

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-releasing hormone (GnRH) secretion/release and consequential pituitary 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.).

Synapse generation and neuronal functions, especially neurotransmission, are highly energy dependent (Laughlin *et al.*, 1998). This statement applies to both inhibitory and excitatory neuronal activities. Therefore, the regulation of neuronal ATP levels is of particular importance for all neuronal-cellular functions, as well as for intercellular signaling. Based on recent reports, the regulation of energy availability in excitatory and inhibitory neurons are distinct, although all those mechanisms seem to take place in neuronal mitochondria. In brief, one of the avenues through which mitochondrial ATP levels are regulated in inhibitory (but not excitatory) neurons of the mediobasal hypothalamus (MBH) is the mitochondrial expression (and regulation) of uncoupling proteins (UCPs, specifically UCP2). UCPs establish a proton leak in the inner mitochondrial membrane, thereby uncoupling mitochondrial respiration, decreasing mitochondrial ATP synthesis and dissipating energy in the form of heat. In contrast, the availability of cellular energy in MBH excitatory neurons is based on a different cellular strategy: mitochondria in these neurons appear to maintain a continuous surplus in mitochondrial ATP levels followed by the adjustment of ATP levels to the actual cellular needs by ATP-hydrolyzation (down-regulation).

The brain is an organ with symmetric tissue organization. Because of its symmetrical nature, there are basically two types of brain structures: paired areas on the two sides of the brain and unpaired structures along the anatomical midline. In the mature organism, paired brain areas usually have distinct physiological functions. The first known reports on functional

cerebral asymmetry were published in 1861 (Broca *et al.*, 1861). Since then, it became clear that cerebral regions are specialized to distinct functions, i.e., each of the cerebral hemispheres dominate in certain specific functions. In line with these discoveries, few authors reported on the asymmetric lateralization of the rat neuroendocrine system, particularly in the hypothalamus-gonad axis, as reviewed by Gerendai and Halasz (1997). For example, Gerendai and Halasz described that unilateral ovariectomy resulted in changes in hypothalamic protein (Gerendai and Halasz, 1976) and GnRH content (Gerendai *et al.*, 1978) in rats. Further, Klein and Burden (1988) found that in a significant majority of rats, the right-sided ovary is more richly innervated by sympathetic fibers than the left. Supporting these findings, lesion studies suggested that an asymmetry exists in the hypothalamic control of the ovarian cycle (Nance *et al.*, 1983; Fukuda *et al.*, 1984; Cruz *et al.*, 1989; Fukuda *et al.*, 1992). Glick *et al.* (1979) provided evidence that there is more metabolic activity on the right side of the rat hypothalamus. Thus, although reported by only a few research groups, evidence exists for the anatomical, hormonal and metabolic laterality in the rat hypothalamus. Unfortunately, those early findings received little attention. The history of hypothalamus-based research testifies that the hypothalamus, a crossroad and center of many homeostatic regulatory pathways, has most frequently been investigated as an unpaired midline structure, despite its seemingly symmetric histological characteristics. However, thorough examination of hypothalamic laterality could radically re-shape our knowledge of the ontogeny, physiology and pathophysiology of hypothalamic functions.

Considering the aforementioned data, we proposed two hypotheses: 1) The regulation of hypothalamic cellular energy levels are 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.

The team I am affiliated with was the first to identify type 3 NTPDase (NTPDase3) in the CNS and map its distribution in the rat brain (Belcher *et al.*, 2006). A 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.

II. Hypothalamic asymmetry in mitochondrial metabolism

Hypothalamic functional asymmetry had been described decades ago. Yet, since then, most studies in hypothalamic research continued to investigate this brain area as a morphologically and functionally compact midline regulatory center. One of the major neural mechanisms involved in the orchestration of integrated, hypothalamus-driven homeostatic functions is the cyclic synaptic reorganization on hypothalamic neurons. Such morpho-functional changes are highly energy dependent and rely on mitochondrial ATP-availability. Therefore, mitochondrial respiration/metabolism plays a permissive role in hypothalamic regulatory events. Here we examined the functional sidedness of the neuroendocrine hypothalamus of rats by measuring a general metabolic parameter, the mitochondrial respiration, in isolated left and right sides of rat hypothalami. We demonstrated that hypothalamic mitochondrial oxygen (O_2) consumption, an indicator of mitochondrial respiration and metabolism, shows an asymmetric lateralization during the estrous cycle. Mitochondrial respiration rates (MRR), during state 1-5 mitochondrial respiration (St1-5), were measured in hypothalamic synaptosomes/mitochondria from normal cycling female rats in each phase of the estrous cycle.

II.1. The Mitochondria: Structure and function

II.1.1. Basic structure of the mitochondrion

Mitochondria are bordered by an outer membrane, however, an inner membrane also exists to separate the inner matrix from the intermembrane compartment. The inner membrane is marked by so-called cristae that arise from the invaginations of the membrane, thus unifying the intermembrane and intercrystal spaces into a continuous compartment. The outer mitochondrial membrane hosts integral membrane proteins called porins that function as transmembrane channels to allow metabolite exchange between mitochondria and the cytoplasm (Ha *et al.*, 1993). The inner membrane is impermeable to H^+ , thereby providing the basis of mitochondrial energy transduction. Integral proteins of the inner membrane are catalysts of the oxidative phosphorylation: the electron transfer and the ATP synthase complexes (Mitchell *and* Moyle, 1965) (Figure 1.).

The function of the mitochondrial respiratory chain involves the action of a series of electron carriers (as redox pairs). Four electron transfer/respiratory complexes (complexes I–IV) are known, each contributing to the catalysis of the electron transfer along the respiratory chain

(Hatefi *et al.*, 1985; Hofhaus *et al.*, 1991; Friedrich and Bottcher, 2004; Hinchliffe and Sazanov, 2005).

II.1.2. Oxidation and electrochemical potentials in the mitochondrial respiratory chain

The electron flow from the electron donors, NADH or succinate, to the acceptor, O₂, occurs following the oxidation potential of the components of the electron transfer chain. Electrons move toward compounds with more positive oxidation potentials as given by the standard redox potential and the ratio of the oxidized and reduced forms, according to the Nernst equation. The differences in redox potential of the electron carriers define the reactions that are exergonic enough to provide the free energy required for the coupled endergonic pumping of H⁺ into the intermembrane space (Muraoka *and* Slater, 1969).

Complexes I, III, and IV function as H⁺ (proton) pumps, where complex IV (cytochrome *c* oxidase, cytochrome oxidase; cytochrome *c*-O₂ oxidoreductase) is the final catalyst of the respiratory chain. The H⁺ pumps are powered by the free energy of the coupled oxidation. The stream of H⁺ is unidirectional, from the matrix to the intermembrane space, resulting in relative negative charge surplus in the matrix and positive charge surplus in the intermembrane space (Figure 1.) (Mitchell *and* Moyle, 1965; Hatefi *et al.*, 1985; Hofhaus *et al.*, 1991; Friedrich and Bottcher, 2004; Hinchliffe and Sazanov, 2005).

II.1.3. Electrochemical potential propels ATP-synthase molecular rotor to phosphorylate ADP

Based on the chemiosmotic coupling hypothesis, introduced by Nobel Prize winner Peter D. Mitchell and Moyle (1965), the electron transport chain and oxidative phosphorylation are coupled by a proton gradient across the inner mitochondrial membrane. According to this, the free energy generated during the fall in redox potential of the electrons carried through the respiratory complexes is used to generate a H⁺ electrochemical potential gradient, expressed in electric potential units as the proton-motive force as Δp . The Δp propels ADP-phosphorylation and stops electron flow in the controlled absence of ADP.

The phosphorylation of ADP into ATP is carried out by the F₀F₁-ATP-synthase (or complex V), which has two distinct components: F₁, a transmembrane protein complex acting as a proton channel, which permits hydrogen ions to get back into the matrix releasing free energy, and F₀, which uses this free energy catalyzing ATP (Pullman *et al.*, 1958; Walker *et*

al., 1995; Boyer, 1998). In addition, hydrogen ions, accumulating this way in the mitochondrial matrix, will be consumed by cytochrome oxidase (Complex IV) with two electrons carried by the respiratory chain to produce H_2O in the presence of O_2 (Figure 1.).

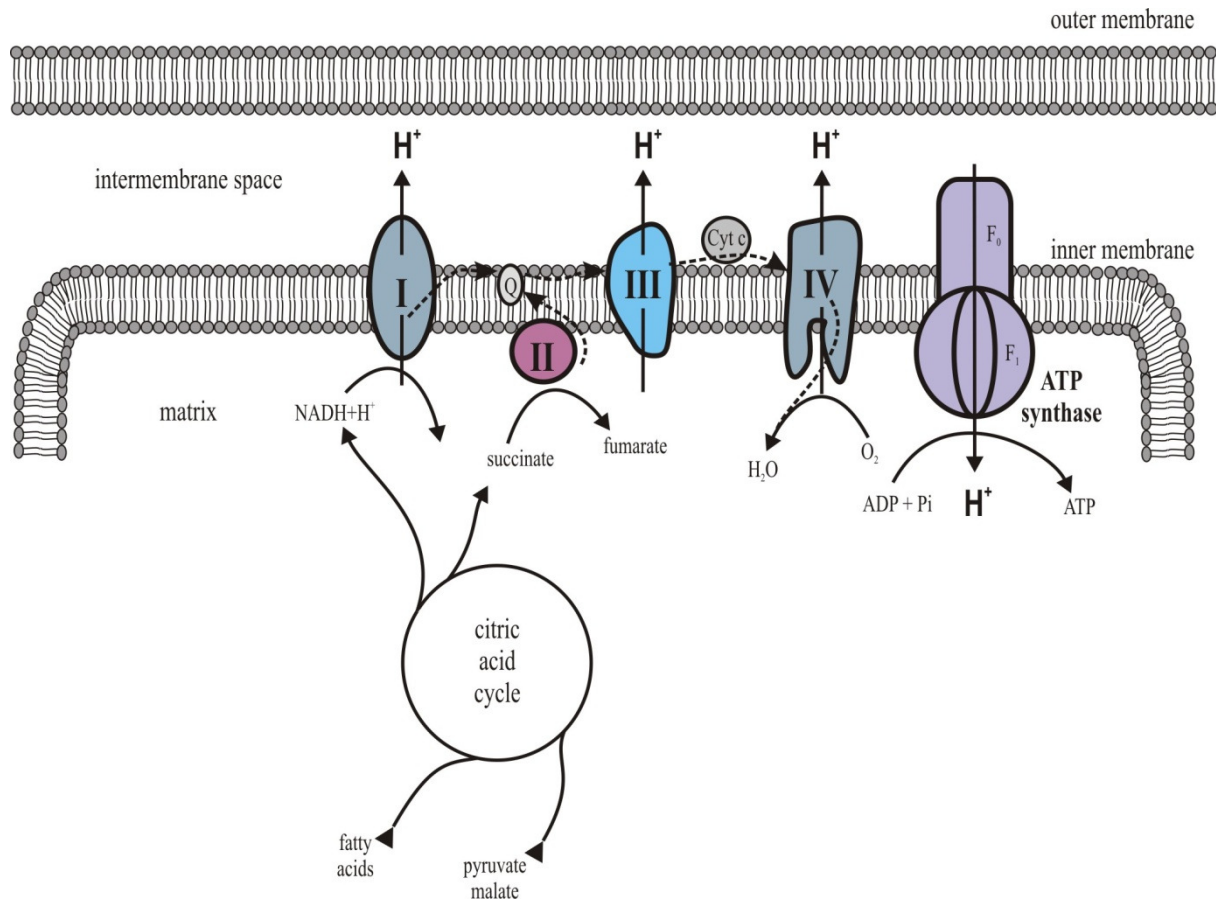


Figure 1. Schematic representation of mitochondrial respiratory chain.

II.1.4. Regulation of mitochondrial respiration

It is not surprising that oxidative phosphorylation depends on the integrity and impermeability of the inner mitochondrial membrane. First, the chemical potential of NADH and succinate oxidation is converted into an H^+ electrochemical gradient, followed by the catalysis by the ATP-synthase that uses the H^+ electrochemical gradient to propel the endergonic ATP synthesis.

It should be noted that the F_0 part of the ATP-synthase is not the only transmembrane complex that provides a way for protons to stream into the matrix (thereby consuming oxygen). The members of uncoupling protein (UCP) family are inner membrane proton carriers that are able to dissipate the H^+ gradient preventing ATP production (reviewed in Rousset *et al.*, 2004). Their regulated function in the inner membrane results in a physiological fine tuning of the equilibrium between ATP and heat production, or mediates

pathological events leading to cell death (Diano *and* Horvath, 2012). Beyond this, several ionophore reagents (hydrazones, fatty acids are worth to mention here) and disrupting conditions (physical and chemical impacts) are known that can break the integrity of the inner membrane abolishing the chemiosmotic gradient resulting in diminished ATP synthesis with a simultaneously high rate of oxygen consumption (Kalckar *et al.*, 1979; Wojtczak *and* Schönfeld, 1993).

Therefore, it can be stated that under physiological conditions, mitochondrial respiration rate exclusively depends on the ADP availability to F_1 -ATP synthase and the function of natural uncouplers (Rousset *et al.*, 2004). The impact of uncouplers can be measured experimentally by switching off the F_0 proton channel. A low respiratory rate observed under application of oligomycin, an antibiotic agent extracted of *Streptomyces* bacteria fully inhibiting the F_0 subunit (i.e. resulting in blockage of ATP synthesis), determines the physiological level of uncoupling (Pullman *and* Monroy, 1963). Basically, oxidative phosphorylation is regulated by three metabolites: ADP, O_2 , and NO. The respiration rate and ATP synthesis are set by cellular energy needs, expressed as cytosolic ADP concentration (more exactly the ATP/ADP ratio). When the cellular demand for energy increases, ATP breakdown to ADP and P_i increases and lowers the phosphorylation potential. With more ADP available, the rate of respiration increases, causing regeneration of ATP (Rousset *et al.*, 2004).

II.2. Hypothalamic functions in reproduction

II.2.1. Hypothalamic areas involved in the regulation of estrous cycle

The hypothalamus is one of the most important brain areas that are responsible for the regulation of homeostatic processes, such as reproductive events, food-intake, body temperature and sleep-wake behavior, linking the endocrine and the nervous systems together. Considering the regulation of reproductive hormone secretion, it is well established that GnRH neurons integrate internal and environmental signals to shape the main output of this neuroendocrine network that regulates gonadal events. It is the episodic, estrogen-induced release of GnRH into the pituitary portal bloodstream at the pituitary stalk that is responsible for the initiation of the LH surge that consequentially leads to the ovulation (Knobil, 1980; Levine *et al.*, 1991). GnRH secreting neurons, themselves, are located dispersed in a longitudinal array of cells in the basal forebrain and neighboring regions. In rats, the great majority of them are located in the medial preoptic area (MPOA), and in the medial septum (Merchenthaler *et al.*, 1984; Malik *et al.*, 1991). Nonetheless, the GnRH

neurons from these areas project to the hypophyseal portal system, at least in studied species, such as rodents, primates or ruminants (Silverman *et al.*, 1987; Goldsmith *et al.*, 1990; Jansen *et al.*, 1997). It is widely agreed that in rodents, it is the MPOA that hosts the GnRH cell population that plays the key role in the generation of GnRH/LH surge in response to sharply rising midcycle estrogen (E2) levels (Merchenthaler *et al.*, 1984; Silverman *et al.*, 1994; topic reviewed by Herbison, 1998). Specifically, the direct E2 responsiveness of GnRH cells is currently unclear, however, it is well established that the E2-responsive neural circuit that directly regulates MPOA GnRH cells is anatomically located in the ventrobasal (and to some extent ventrolateral) regions of the hypothalamus (Naftolin *et al.*, 2007). It is important to consider at this point that due to the complexity of hypothalamus-driven homeostatic functions, there are no sharp anatomical boundaries between hypothalamic neuron populations that would be devoted to the neuroendocrine regulation of single, well-defined biological functions. Instead, anatomically (hypothalamic nuclei) or biochemically characterized (different neuropeptide/neurotransmitter containing), distinct neuron populations are rather involved in the hypothalamic regulation of multiple homeostatic processes.

II.2.2. Estrogen-induced morpho-functional plastic changes in the hypothalamus

It is well documented that hypothalamic nuclei involved in the regulation of reproductive functions are targets of gonadal steroids. Estrogen can cross the blood-brain-barrier (Pardridge and Mietus, 1979) and reaches the mediobasal regions of the hypothalamus including AN neurons, and induces plastic changes in synaptic contacts (combination of particular morphological and subcellular characteristics) followed by altered firing activity (Naftolin *et al.*, 1993; Parducz *et al.*, 2002; Parducz *et al.*, 2002).

During most phases of the estrous cycle E2 exerts an inhibitory effect on GnRH secretion and sensitizes the pituitary gonadotrophs to GnRH. This means that as the circulating E2 level rises due to follicular maturation, the suppression of GnRH secretion increases, followed by the simultaneous sensitization of pituitary gonadotrophs through upregulation of GnRH receptor expression (McArdle *et al.*, 1992). GnRH suppression followed by the pituitary sensitization are parts of the E's negative feedback control on LH and FSH (Ropert *et al.*, 1984; Leranth *et al.*, 1986; Witkin *et al.*, 1994; Lopez *et al.*, 1998; Terasawa *et al.*, 1999; Lawson *et al.*, 2002) that is followed by the turn into the positive feedback phase of the cycle.

At a certain high level of circulating E2 (E2 peak) during late proestrus, the inhibitory effect of E2 on the hypothalamus is reversed. As in this phase pituitary sensitization is highly augmented, the episodically rising GnRH release results in LH (and FSH) burst (Corker *et*

al., 1969; Nillius *and* Wide, 1972). This paradoxical event caused by reversed E2 effect disinhibiting GnRH secretion can be interpreted as an apparent positive feedback between the gonadal E2 and the LH/FSH secretion. There is, however, a complex dynamic reorganization of synapses targeting GnRH and other hypothalamic neurons that underlies these E2 effects.

Estrogen is generally (in non midcycle level) synaptogenic in the brain and modulates neurotransmitter secretion. A similar effect has been shown in rat and primate hypothalamic nuclei involved in the control of GnRH secretion. The number of synapses in the AN changes according to the actual concentration of circulating blood E2 (Ferin *et al.*, 1969; Langub *et al.*, 1994). This is supported by the observation that in these species a drastic or even total loss of synapses on GnRH neurons occurs after ovariectomy that can be reversed by E2 treatment (Witkin *et al.*, 1991). In negative feedback mode, estrogen maintains a high inhibitory/excitatory ratio of synapses targeting the GnRH cells to drive the negative feedback suppression on the GnRH cells. As the estradiol increases ensuing the maturation of the dominant ovarian follicle, the total synaptic number reaches a plateau with the further increase of inhibitory/excitatory ratio in the synaptic status (Zsarnovszky *et al.*, 2001).

However, during the preovulatory E2 surge, the synaptogenic effect of estradiol is overthrown and a synaptolytic effect on both inhibitory and stimulatory connections (ultimately targeting the GnRH cells) results in the disinhibition of GnRH cells and leads to GnRH surge. The rising GnRH released into the pituitary portal capillaries targets the pre-sensitized pituitary gonadotrophs to initiate a robust and sharp increase in the amounts of hypophyseal LH production; this process consequentially induces ovulation (Soendoro *et al.*, 1992). This paradoxical response of the hypothalamus to E2 peak is termed as estrogen-induced gonadotropin surge (EIGS) (reviewed by Naftolin *et al.*, 2007).

II.2.3. Estrogen-induced changes in the number and function of brain mitochondria

All the events (remodeling of synapses, increased turnover of membrane proteins, altered firing pattern, etc.) regarding the rewiring of hypothalamic neuronal circuits and changes in the pattern of neuroendocrine activity are inconceivable without highly energy depending molecular mechanisms, suggesting well balanced regulation of mitochondrial ATP synthesis.

Changes in respiratory capacity in distinct brain regions, can derive from the alteration of the size and number of mitochondria per cell; reorganization and activation/deactivation of intramitochondrial components are modulating factors as well. Estrogen exerts neurotrophic and neuroprotective effects sustaining reproductive mechanisms (Nicholls *and* Budd, 2000;

Nilsen *and* Diaz Brinton, 2003). Estrogen receptors (ER), for instance, as they are present in mitochondria in several cell types, supposedly act as transcription factors for mitochondrial genes encoding protein complexes of the electron transport chain (Chen *et al.*, 2005). Beyond that, these steroids fine-tune the metabolic processes adjusting them to the energetic demands of neurotransmission and neurosecretion. Many aspects of mitochondrial functions depend upon circulating levels of ovarian hormones, as E2 replacement or treatment leads to alteration or impairment in neuronal energy expenditure. Among others, it has been reported that the protein complexes of mitochondrial respiratory chain are E2-controlled and their affinity to redox mechanisms can be induced through ER activation in hippocampal and hypothalamic regions (Bettini and Maggi, 1992). Irwin *et al.* (2008) demonstrated that estrogen and progesterone episodically enhance mitochondrial respiration by increasing the activity of cytochrome c oxidase enzyme, and improve the respiratory efficacy by diminishing proton leakage through the inner membrane. Furthermore, it has been reported that ATP synthase in brain mitochondria can be directly supported or blocked, depending on the tissue type and concentration of gonadal steroids. (Keller *et al.*, 1997; Zheng and Ramirez, 1999; Massart *et al.*, 2002). Transport of different substrates through mitochondrial outer membrane, such as fuels for citrate cycle and inorganic phosphate needed for oxidative phosphorylation, are partially ovary-controlled as well. Beside the regulation (activation or inhibition) of respiratory protein complexes, transcription and posttranslational targeting are under the control of estrogen and progesterone, as it has been reported with regard to genes coding for cytochrome c oxidase, cytochrome oxidase and cytochrome c-O₂ oxidoreductase (Van Itallie and Dannies, 1988; Bettini and Maggi, 1992). In hypothalamic neurons, transcription of nuclear encoded ATP-synthase subunits are also induced by E2 possibly in a cell specific manner (Chen *et al.*, 2008; reviewed in Klinge, 2008). This statement is in concert with the finding that brain mitochondria isolated from E2-treated ovariectomized (OVX) rats showed significantly higher O₂ consumption (Nilsen *et al.*, 2006; Mattingly *et al.*, 2008).

This regulatory mechanism may be of further interest, as these protein assemblies are mostly encoded in part by the mitochondrial DNA itself, and in part by the nuclear genome.

A large body of evidence supports that oxidative stress plays a crucial role in aging of brain mitochondria and neuronal decline. Oxidative stress occurs by means of electron leakage of the respiratory chain. Some electrons do not couple with protons to form water in the presence of oxygen molecules, but form oxygen radicals that will be dismutated to H₂O₂, a reaction catalyzed by superoxide dismutase (MnSOD). A proportional peroxidase activity is needed to reduce oxygen radicals to neutral water. In the absence of sufficient peroxidases essential protein and lipid parts of the surrounding microstructures are impaired. This

impairment results in irreversible membrane destruction drastically altering the fine order of compartmentalization. Irwin *et al.* (2008) showed evidence that ovarian hormone treatment prevents harmful lipid peroxidation in neuronal mitochondria (Subramanian *et al.*, 1993; Behl *et al.*, 1995; Shea and Ortiz, 2003; Kii *et al.*, 2005). In addition, enhanced functional efficiency of mitochondria, induced by E2 and progesterone, correlates with lower electron leakage further attenuating production of oxygen radicals (Gridley *et al.*, 1998; Nakamizo *et al.*, 2000).

II. 3. 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 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?

II.4. Materials and Methods

II.4.1. Animals and measurement of mitochondrial respiration

Reproductive cycling of female Sprague-Dawley rats was determined and verified by periodic examination of vaginal smears. Hypothalamic sampling was started two weeks after determination of cyclicity, thus, possible influential effects of vaginal smearing on cyclicity could be discounted. Brains were removed after quick guillotine decapitation, and the actual estrous phase was determined based on the cytology of vaginal smears. It is noteworthy that our rationale for the post mortem vaginal smearing was to avoid hormonal influence through the possible mechanical irritation of the cervix; however, this also resulted in a difference in the number of animals in different estrous phase groups.

Vaginal smears were evaluated on the basis of the following criteria:

Early proestrus (EP): many epithelial cells + few cornified cells (n=13).

Late proestrus (LP): many epithelial cells + many cornified cells (n=8).

Estrus (E): many cornified cells with or without few epithelial cells (n=9).

Metestrus (ME): many leucocytes + few epithelial cells with or without few cornified cells; or many leucocytes + few cornified cells with or without many epithelial cells (n=24).

Diestrus (DE): few leucocytes + few epithelial cells with or without few cornified cells (n=12).

Hypothalami were dissected from the removed brains as follows: in antero-posterior direction: between the caudal margin of the optic chiasm and the rostral margin of the mamillary body; in dorso-ventral direction: below the upper margin of the fornix. Dissected hypothalami were then cut into left and right halves. Hypothalamic samples were homogenized in isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% fatty acid-free BSA, 1 mM EGTA, 20 mM HEPES, pH adjusted to 7.2 with KOH). The homogenate was spun at 1300 x *g* for 3 min, the supernatant was removed, and the pellet was resuspended with isolation buffer and spun again at 1300 x *g* for 3 min. The two sets of supernatants from each sample were topped off with isolation buffer, and centrifuged at 13,000 x *g* for 10 min. The supernatant was discarded; the pellet was resuspended with isolation buffer and layered on 15% percoll. The next centrifugation step was to separate the synaptosomal and perikaryal mitochondrial fractions from cell debris at 22,000 x *g* for 7 min. After this procedure, the final 1 ml of centrifugate contains 3 layers/fractions: the perikaryal mitochondrial fraction is at the lower tip of the tube, the middle layer is the synaptosomal fraction, and the top layer is the myelin-rich debris. Since

myelin can mask the results of mitochondrial respiration, this fraction has been omitted from further sampling. Thus, the lower 200 μl of the centrifugates, containing the mitochondrial and synaptosomal fractions were resuspended and topped off with isolation buffer without EGTA (215 mM mannitol, 75 mM sucrose, 0.1% fatty acid-free BSA, 20 mM HEPES, pH adjusted to 7.2 with KOH), and centrifuged again at 22,000 $\times g$ for 7 min. The supernatant was discarded; the pellet was resuspended in isolation buffer without EGTA, and spun at 13,000 $\times g$ for 10 min. As the last step of the separation procedure, the supernatant was poured off, and the pellet was stored up on ice till the mitochondrial oxygen-consumption measurement. Before the measurement, equal volumes (50 μl) of the samples were placed in the electrode-chamber (Clark-type oxygen electrode, Hansatech Instruments, Norfolk, UK, at 37 °C) and diluted with 450 μl respiration buffer (215 mM mannitol, 75 mM sucrose, 0.1% fatty acid-free BSA, 20 mM HEPES, 2 mM MgCl, 2.5 mM KH_2PO_4 , pH adjusted to 7.2 with KOH). Measured values represent the mitochondrial respiration rate (MRR, given in consumed nmol O_2 per ml of final/measured volume).

II.4.2. State 1-5 mitochondrial respiration

As the name and numeral marking of different mitochondrial respiration states varies in the publications on the subject, here we explain our nomenclature as used in this thesis. In all of our MRR measurements we registered the O_2 consumption of the respiration states (60 secs for 1-4 respiration states, and until full O_2 depletion for St5) in the sequence as follows (Figure 2.).

First step: the mitochondrial O_2 consumption was measured in respiration buffer only, without the addition of any substrates that may affect mitochondrial respiration. Under such conditions, oxygen consumption per unit time depends on the actual metabolic state of the hypothalamic sample and the sample's original O_2 supply. We termed this experimental setup state 1 mitochondrial respiration (St1).

Second step: to fuel the Krebs' cycle, 5 μl pyruvate (P, of the following mixture: 275 mg pyruvate/5 ml distilled water + 100 μl 1M HEPES) and 2.5 μl malate (M, of the following mixture: 335.25 mg malate/5 ml distilled water + 100 μl 1M HEPES) were added to the sample. Under such conditions, the Krebs' cycle intensifies and O_2 consumption increases due to consequential facilitation of the terminal oxidation and oxydative phosphorylation if the prior (*in vivo*) blood/ O_2 supply of the hypothalamic tissue sampled was sufficient and down-regulating mechanisms are not active. We termed this experimental setup state 2 mitochondrial respiration (St2).

Third step: 2.5 μ l ADP (of the following mixture: 64.1 mg ADP/5 ml distilled water + 100 μ l 1M HEPES) was added to the sample. Since ADP is a major upregulator of mitochondrial respiration, under such conditions MRR increases if prior (*in vivo*) blood/fuel supply of the hypothalamic tissue was sufficient. We termed this experimental setup (ADP-dependent) state 3 mitochondrial respiration (St3).

Fourth step: 1 μ l oligomycin (of the following mixture: 1 mg oligomycin/1 ml ethanol) was added to the sample. Oligomycin is an ATP-synthase blocker, therefore inhibits the oxidative phosphorylation (ATP synthesis), while terminal oxidation continues. Under such conditions, O₂ consumption depends on the actual uncoupled stage and alternative oxidation in mitochondria. Under physiological conditions, uncoupling and alternative oxidation play important roles in transient down-regulation of ATP biosynthesis when cellular energy needs drop. Therefore, increased O₂ consumption in this case refers to the decline of a process (that was previously up-regulated or the attempt by the mitochondrion to down-regulate ATP synthesis). We termed this experimental setup state 4 mitochondrial respiration (St4).

Fifth step: 3 μ l FCCP (carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone; of the following mixture: 1,271 mg FCCP/5 ml DMSO) was added to the sample. FCCP is a cyanide derivative, therefore depletes all remaining O₂ from the sample (also acts as uncoupler). Decrease of O₂ level under such conditions depends on the actual/initial (*in vivo*) metabolic state of the tissue sampled and the amount of O₂ consumed during states 1-4 respiration; i. e., the total amount of O₂ consumed in states 1-4 respiration plus the amount of remaining O₂ depleted by FCCP gives good reference to the blood/O₂ supply of the tissue at the time of the animal's sacrifice. Therefore, this experimental setup is also known as total mitochondrial respiratory capacity, hereby referred to as state 5 mitochondrial respiration (St5).

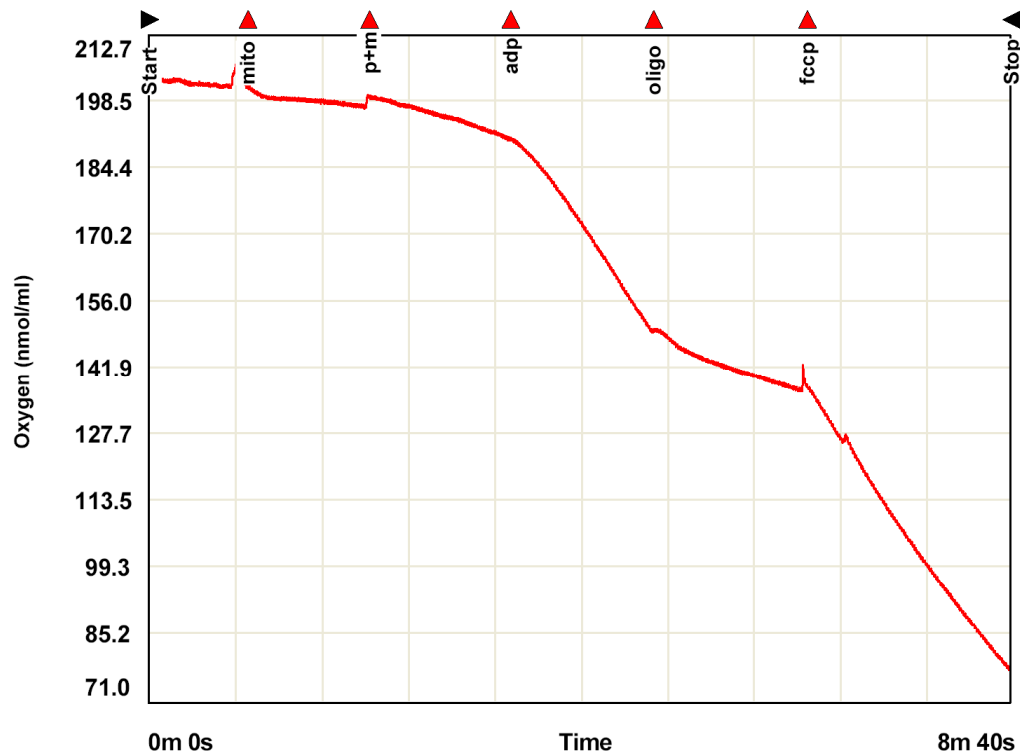


Figure 2. General registration of mitochondrial respiration rates made by a Clarke-type oxygen electrode (Hansatech Instruments). Note the administration of different respiration modulators marked on the top edge of the screen.

II.4.3. Data analysis

As expected, comparison of data from left and right sides of the hypothalami showed that in a given individual, only one of the two sides was metabolically active. This means that one of the hypothalamic sides of each animal displayed only negligible differences in MRR values, regardless of the respiration state (we call it the 'silent' side). Mitochondrial respiration rates measured in the contralateral ('active') side highly varied, depending on the estrous phase and the respiration state. Since mitochondrial respiration rates highly varied between (otherwise) normal cycling individuals (including the steady metabolic activity of the 'silent' hypothalamic side), statistical comparison/analysis of mitochondrial respiration rate values was senseless. Instead, it was more reasonable to use MRR results obtained from the 'silent' sides as reference values to evaluate the magnitude and direction of changes in MRR values in the contralateral side of the same individual, thereby establishing the possibility to interpret MRR changes (in the 'active' side) on the basis of causality.

To support our data interpretation, we also analyzed our results from a second point of view: the share (expressed in percentage) of the left and right sides in hypothalamic sidedness.

Sidedness was considered if, in any given respiration state (St), at least 60 % or more (arbitrary limit) of total, bilateral O₂ was consumed by either the left or the right sides.

II.5. Results and discussion

II.5.1. Hypothalamic asymmetry

As mentioned above, St1-5 measurements were carried out in samples from isolated hypothalamic sides, followed by the comparison of data from the left and right hypothalamic hemispheres. 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 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.

II.5.2. Mitochondrial oxygen content and oxygen consumption

Since sufficient blood/O₂ supply is necessary for normal mitochondrial respiration, we measured the total O₂ content and total O₂ consumption (Figure 3.) of the hypothalamic samples and compared the results obtained from the left and right hypothalamic sides.

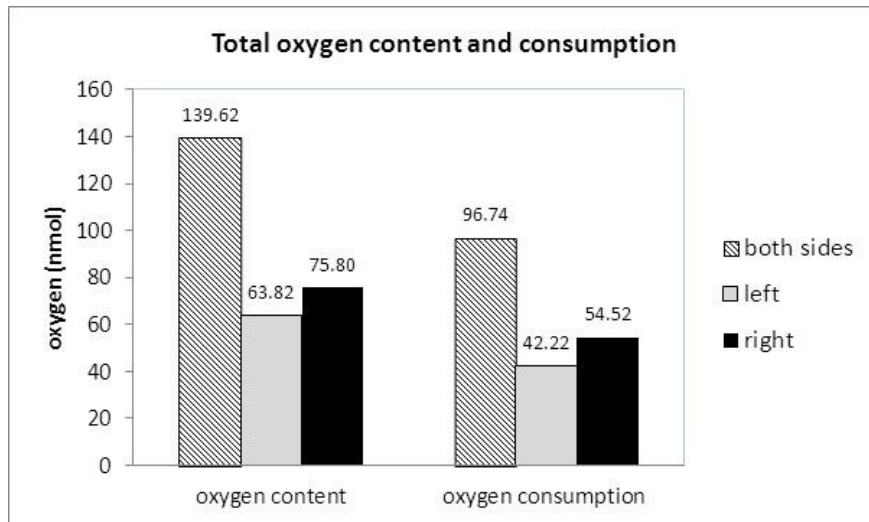


Figure 3. Total oxygen content and oxygen consumption of hypothalami and hypothalamic sides. Columns show means of total O₂ consumption and O₂ content from all phases (early proestrus through diestrus) of the estrous cycle.

Results in Figure 3. indicate that both O₂ consumption and O₂ content were asymmetric in the hypothalamus. It should be noted, however, that values in Figure 3. comprise the means of MRR values from all respiration states (St1-5), therefore the cycling nature of differences between the hemispheres is not demonstrated here in its full magnitude. It is also obvious from Figure 3. that patterns in O₂ consumption and blood/O₂ supply are highly similar. This observation raises the question of whether the regulation of local blood/O₂ supply sets a limit for the intensity of mitochondrial and tissue metabolism, or alternatively, the two parameters are regulated by (a) common mechanism(s) (e.g., autonomic nervous system).

II.5.3. Hypothalamic asymmetry in mitochondrial metabolism

Results of mitochondrial respiration measurements were analyzed from two major aspects:

1. The extent of hypothalamic asymmetry, and
2. the share of the left and right sides in hypothalamic sidedness in all phases of the estrous cycle during St1-5.

II.5.3.1. The extent of hypothalamic asymmetry

Analysis of mitochondrial metabolism in early proestrus (EP) animals (Figure 4., 9.) revealed that about half of the animals showed hypothalamic asymmetry (with either left- or right-sided dominance) in basic (St1) and fuel-dependent (St2) respiration states. However, the ADP-dependent St3 and uncoupled respirations (St4) were more intense in either one of the hemispheres. The highest degree of asymmetry was found in total mitochondrial respiratory capacity (St5). It should be noted that addition of pyruvate and malate ('fuel', P+M) could not increase the MRR in St2 (please compare to St1 vs. St2 in late proestrus [LP]). In contrast, addition of ADP increased St3 MRR in one of the hypothalamic sides, suggesting that a unilateral mechanism exists that facilitates mitochondrial metabolism in EP, and that this mechanism is ADP-dependent. Such a mechanism seems to be absent or blocked in the contralateral ('silent') hemisphere. These results, with special regard to St5 values, suggest that there is a high degree of asymmetry in hypothalamic blood/O₂ supply during EP, which is consonant with earlier findings that the sympathetic nervous system shows asymmetry in the ovaries.

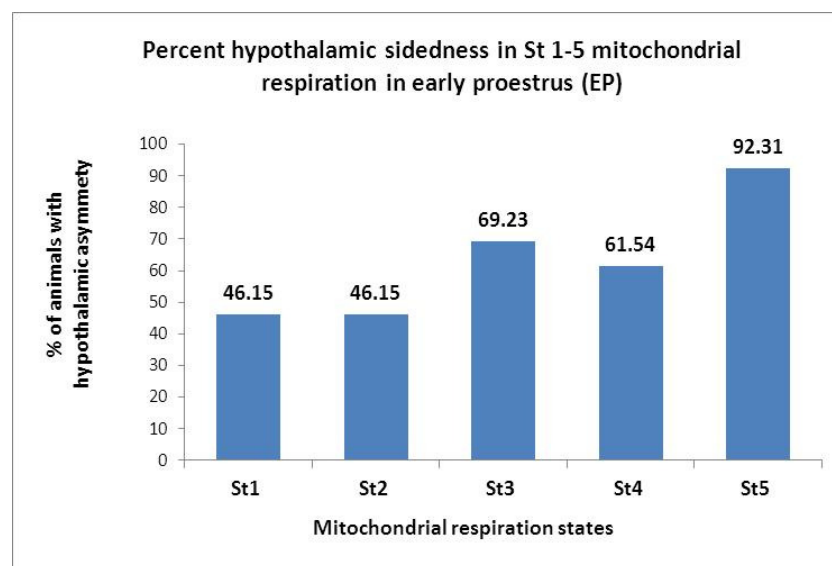


Figure 4. Hypothalamic asymmetry in St1-5 mitochondrial respiration in early proestrus. About half of the animals showed hypothalamic asymmetry (with either left- or right-sided dominance) in basic (St1) and fuel-dependent (St2) respiration states, however, the ADP-dependent St3 and uncoupled respiration (St4) were more intense in one of the hemispheres. The highest degree of asymmetry was found in total mitochondrial respiratory capacity (St5).

The degree and pattern of sidedness over St1-5 was remarkably different in LP than in EP (Figure 5., 9.). The St1 increased, compared to the EP value and St2 was even higher (than in EP). It is important to keep in mind that experimental fueling of the mitochondria (addition of P+M) in St2 has been done in all estrous phases, yet, in EP St2, laterality was not higher than in St1. This suggests that a yet unknown mechanism exists that can activate fuel-dependent mitochondrial respiration. It is also important that, while basic respiration increased from EP to LP, experimental fuel addition induced an even more intense respiration, suggesting that in LP, the mitochondria 'are allowed' to reach more intense levels of metabolism in the potential case of need. In contrast, laterality in ADP dependent St3 substantially decreased (somewhat more than 30 % of animals showed St3 asymmetry) compared to EP. To give a suitable interpretation for this observation, one should consider the high St4 values (please see the explanation of St4 in chapter II.3.2.) and the simultaneous synaptic events that occur during the estrous cycle.

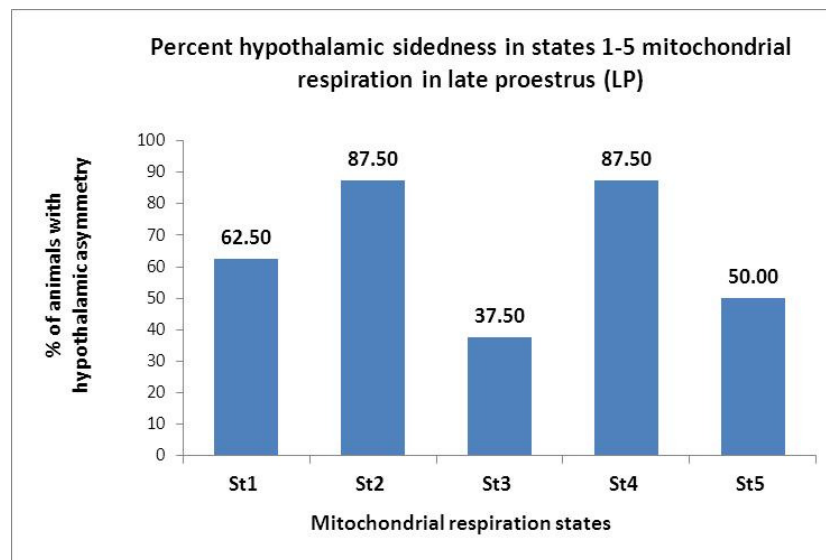


Figure 5. *Hypothalamic asymmetry in St1-5 mitochondrial respiration in late proestrus.*

In estrus (E), base mitochondrial metabolism (St1) was highly asymmetric (Figure 6., 9.), indicating that one side was metabolically more active than the contralateral side in almost 90 % of animals. This asymmetry decreased substantially after administration of P+M (St2) and even more so after ADP-addition (St3), suggesting that a down-regulating mechanism, supposedly reflected in high LP St4 values, could bring E-related neural actions to a halt.

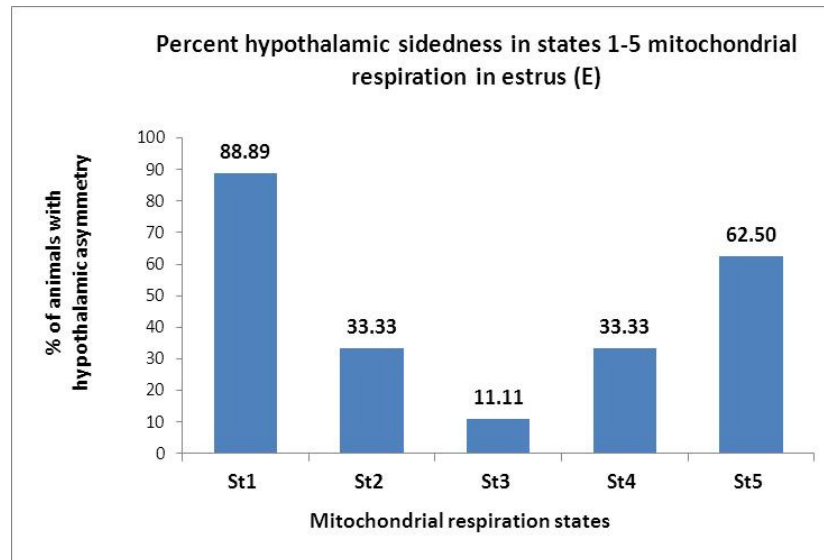


Figure 6. Hypothalamic asymmetry in St1-5 mitochondrial respiration in estrus.

In ME (Figure 7., 9.) sidedness in base metabolism decreased (St1) and a negative feedback-like regulation was still observed in St2-3 as MRR values decreased after addition of P+M (St2) and ADP (St3). Higher than E St4 values may indicate intensifying down-regulation in the 'active' sides. This idea is supported by further decreasing base MRR in DE St1 (Figure 8., 9.). In DE, the down-regulating effects of P+M (St2) and ADP (St3) are still detectable, albeit, at a smaller extent than in ME. St4 values are also substantially decreased compared to E, likely playing a role in turning the negative feedback-like effect of ADP to a positive, facilitatory effect (in sidedness) by EP.

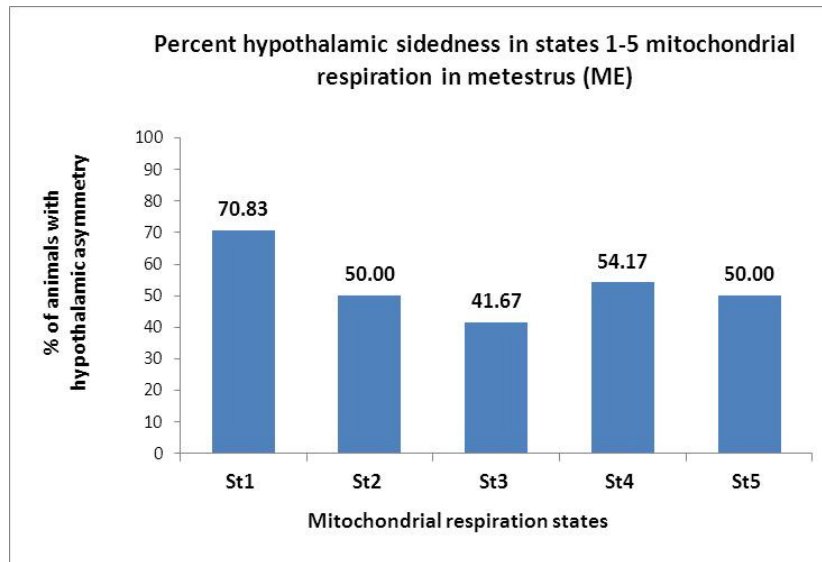


Figure 7. *Hypothalamic asymmetry in St1-5 mitochondrial respiration in metestrus.*

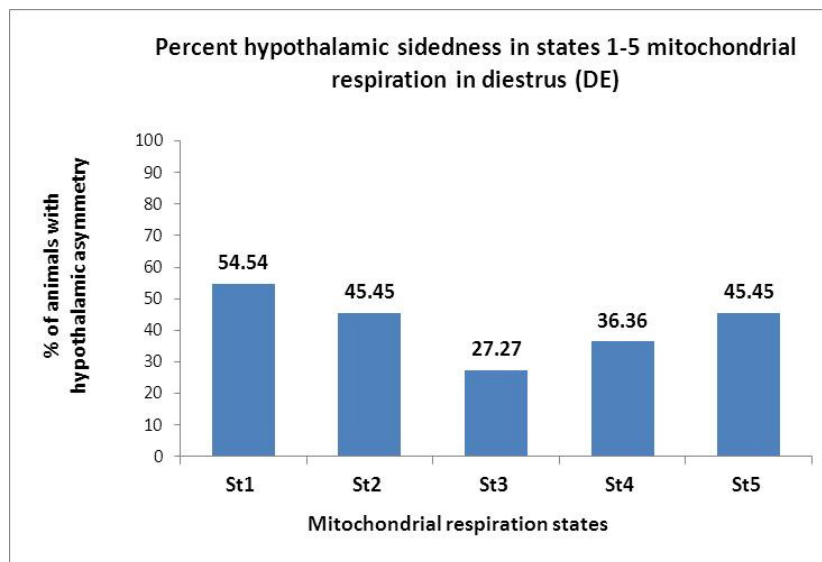


Figure 8. *Hypothalamic asymmetry in St1-5 mitochondrial respiration in diestrus.*

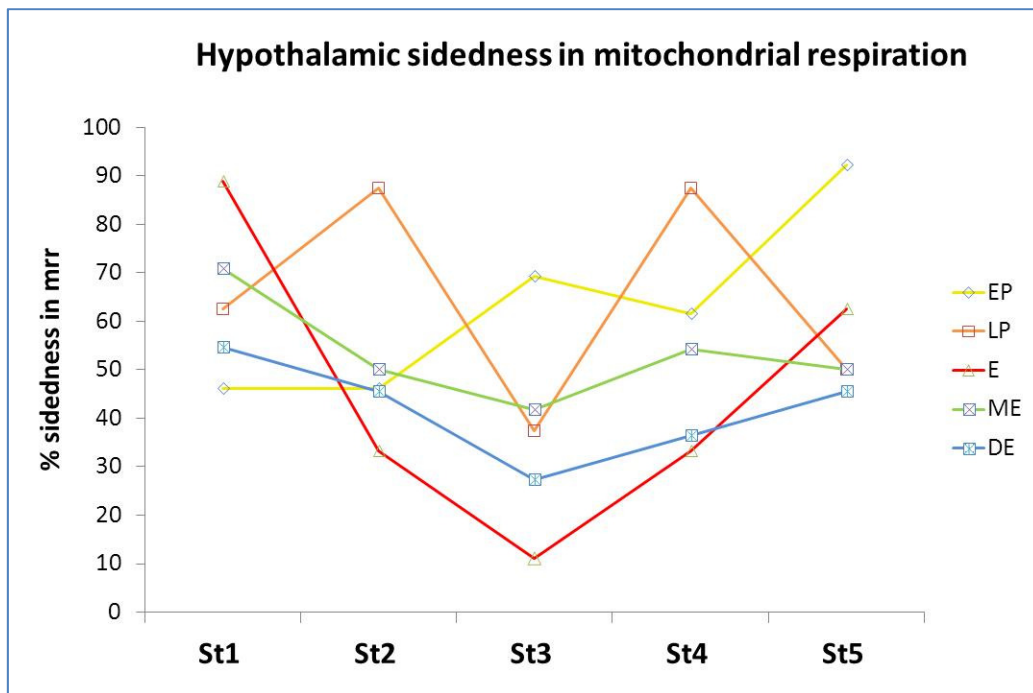


Figure 9. Comparison of hypothalamic sidedness in St1-5 mitochondrial respiration in all phases of estrus cycle.

II.5.3.2. The share of the left and right sides in hypothalamic sidedness

In EP sided animals (Figure 10.) left and right sidedness was balanced in basic mitochondrial metabolism (St1). However, when adding P+M (St2), a right-sided dominance evolved with an increase on the right side and a decrease on the left side. This observation suggests that a mechanism exists that may inhibit metabolism on the left side but facilitate it on the right side. A further increase in the right-sidedness was found after the administration of ADP (St3), which could arise from the facilitatory effect of ADP on mitochondrial metabolism. Such an ADP-effect may have worked in St2 as well, and/or in St3 we observed the additive effects of ADP and a supposed mechanism mentioned earlier regarding our St2 finding. Right-sidedness in St4 (based on the interpretation given in the chapter 'Materials and methods') indicates that a more intense down-regulation is in progress on the right side. In spite of increased right-sided O_2 consumption, St5 values show that in EP, there is still more O_2 left in the right side than in the left.

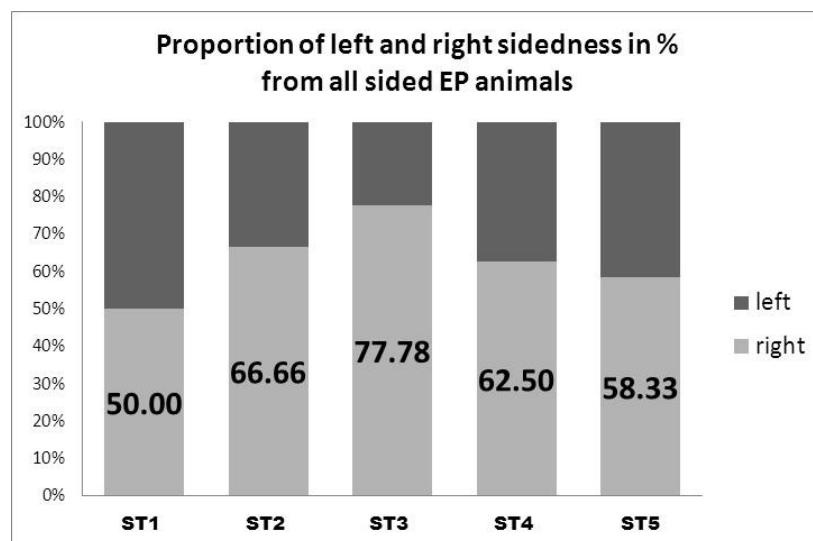


Figure 10. Left-right share in hypothalamic asymmetry in early proestrus.

In LP (Figure 11.) right-sidedness was observed in basic mitochondrial metabolism (St1). This phenomenon is likely the consequence of the right sided facilitatory effect of ADP having its onset in EP. Fuel-dependent asymmetry in St2 supports this idea and indicates the high potential of the right side to further increase the intensity of metabolism. Addition of ADP (St3) resulted in full right-sidedness, further suggesting the facilitatory role of ADP on mitochondrial metabolism. Simultaneously, full right-sidedness was detected in St4, meaning that sided mitochondrial metabolism seen in St1 is accompanied by the activation of down-regulating mechanism. Based on this observation one may anticipate that in successive estrous phases stimulatory metabolic effects will fade. Total sidedness in St4 rises the idea that the 'passivity' of the 'silent' hemisphere is the result of the lack of stimulation rather than that of some sort of inhibition. It is interesting to note that after exhausting the samples through St1-4, St5 values are fairly balanced. This means that the surplus in right-sided blood/O₂ supply is proportional to the excess metabolic potentials of the right side over the left side.

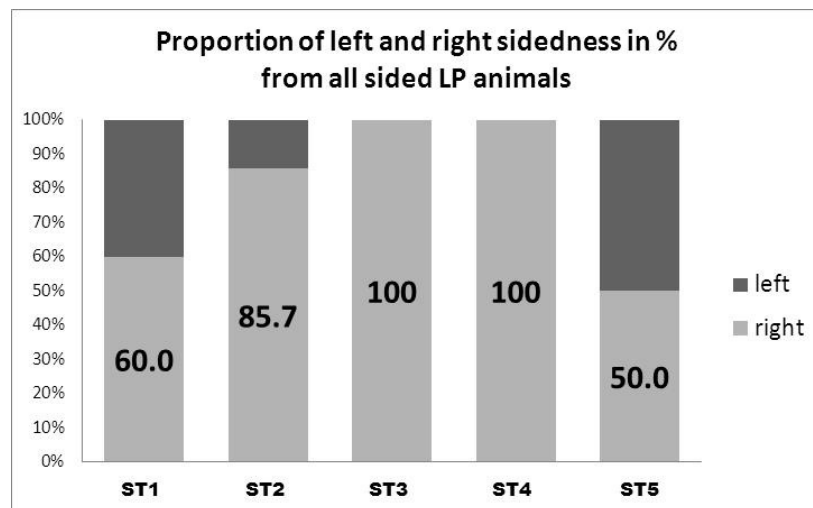


Figure 11. *Left-right share in hypothalamic asymmetry in late proestrus.*

The potentials for full right-sidedness in LP St2-3 appear to be realized in E St1, where full right-sidedness was detected (Figure 12.). Interestingly, addition of P+M in St2 resulted in a robust decrease in right-sidedness accompanied by elevated left-sided metabolism. This phenomenon supports our notion regarding LP St4 and may result from decreased amounts of hypothalamic ADP (and consequential weakened facilitation) on the right side. Addition of ADP in St3 reinstated the full right-sidedness, supporting the idea (mentioned in LP St4 and E St2 discussion) that ADP may be the major regulator of metabolism, especially in the 'active' side. In St4, we found a left-sided dominance. The stronger down-regulation in the left side may be responsible for the maintenance of the more intense mitochondrial metabolism in the right side, and at the same time this raises the possibility that a left-sided (i.e., contralateral) inhibitory mechanism may exist with a yet unidentified nature. In E, less residual O₂ remained in the right side (St5) vs. the left side. One may speculate that a right-sided decrease in blood/O₂ supply might occur in E and that this decrease may set a limit to the fuel/O₂ consumption observed in the right side, thereby bringing the estrus phase to a halt.

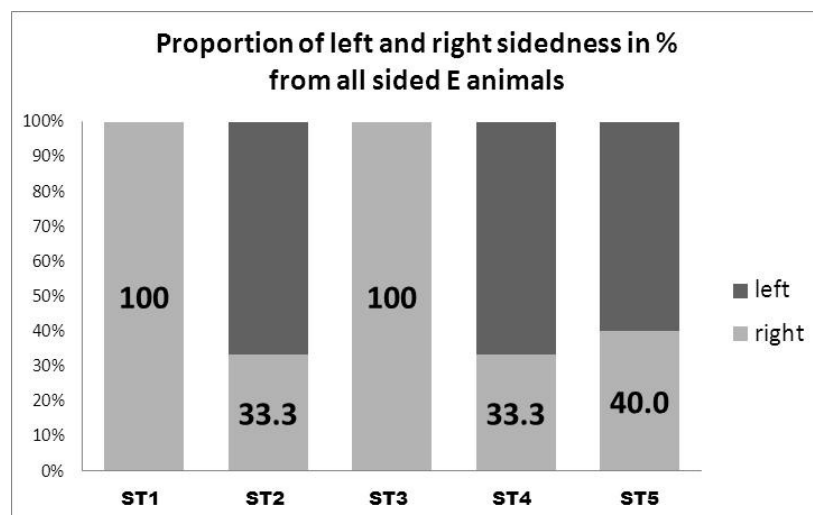


Figure 12. Left-right share in hypothalamic asymmetry in estrus.

Indeed, in metestrus (ME) (Figure 13.), the base mitochondrial metabolism (St1) seems to be balanced between the two hemispheres, nevertheless, addition of 'fuel' or ADP (St2-3) shows that the right side continues to possess the potentials for a more intense metabolism compared to the left. Also, the aforementioned potential left-sided inhibition (in E St2) does not seem to act in ME. St4 results indicate that in ME, balanced base metabolism is accompanied by also balanced down-regulatory processes (St4) in the two hemispheres. St5 values suggest that blood/O₂ supply in the hypothalamic sides has not changed compared to E.

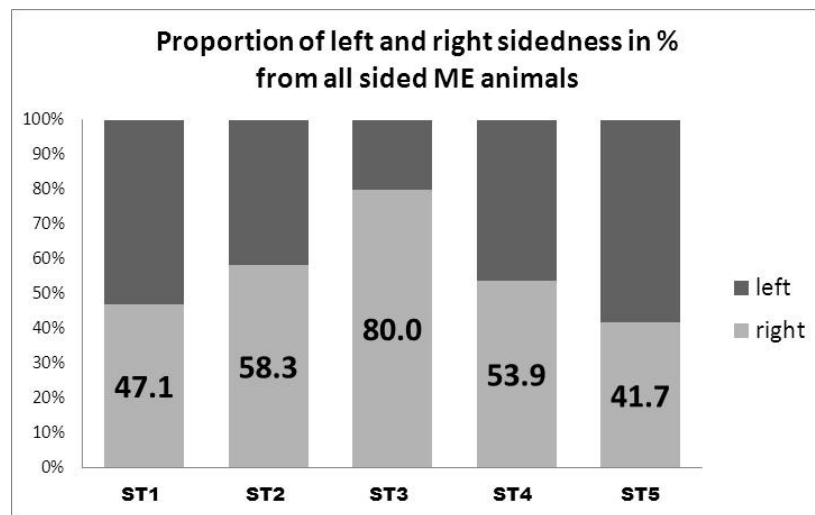


Figure 13. *Left-right share in hypothalamic asymmetry in metestrus.*

In diestrus (DE) (Figure 14.), right-sided dominance was apparent (St1), which could not be further increased by the addition of P+M and/or ADP (St2-3). We currently do not know the explanation of this phenomenon, however, increased down-regulation (St4) in the right side was also observed that, with lower residual O₂ left in the right side (St5) may play a role in equalizing the left-right balance until entering EP (Figure 10.)

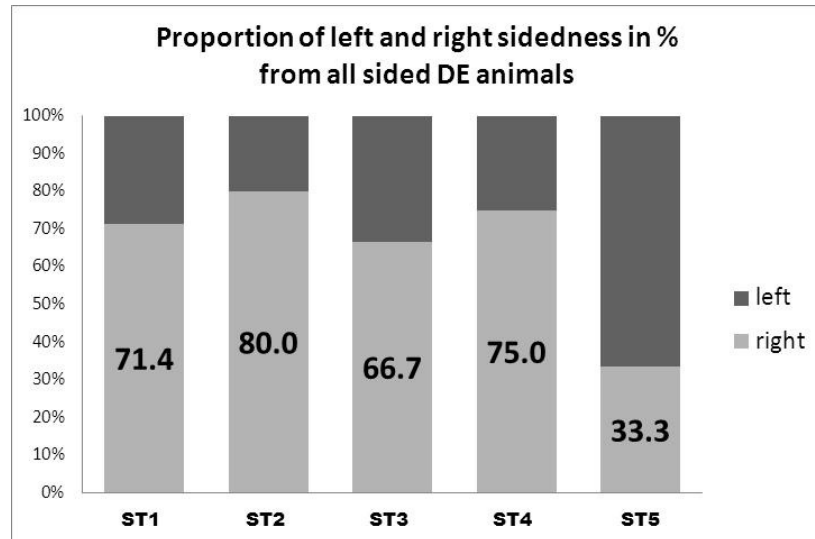


Figure 14. *Left-right share in hypothalamic asymmetry in diestrus.*

III. NTPDase3 expression and activity in the hypothalamus

Recently, our research group identified a novel ATP-hydrolyzing protein (ecto nucleoside triphosphate diphosphohydrolase 3, NTPDase3) in the mediobasal hypothalamus (MBH) that is a member of the ectonucleotidase family of enzymes (Robson *et al.*, 2006). Ectonucleotidases (NTPDase1-8) have been known as transmembrane enzymes that hydrolyze ATP to ADP and AMP outside of the cell, thus providing specific ligands (ADP and AMP) for purinergic intercellular signaling and for 5'ectonucleotidase for the production of adenosine from AMP. As key regulators of purinergic intercellular signaling, this enzyme family has been the subject of intense research, however, an increasing body of knowledge on the biological effects of NTPDase-inhibition could not have been explained by impaired purinergic signaling. Therefore, the possibility has risen that one or more of these enzymes may function intracellularly to regulate integrated cell responses to extracellular cues. Because of the lack of relevant research, little was known about the NTPDases' role in the central nervous system (CNS). Although previous studies (Chadwick *and* Frischauf, 1998; Smith *and* Kirley, 1998) identified transcripts for NTPDase1-3 in brain tissue homogenates, that information was insufficient to formulate a conclusion regarding these enzymes' function/role in the CNS. There are four known cell-surface NTPDases capable of controlling the concentrations of nucleotide agonists near purinergic receptors (NTPDase1–3, 8). The mRNA for the newly discovered mouse NTPDase8 was reported to be most abundant in liver, jejunum, and kidney, and not detectable in brain (Bigonnesse *et al.*, 2004). The expression of two other cell surface NTPDases, NTPDase1 and NTPDase2, has been studied in brain. NTPDase1 (also known as CD39) was reported to have widespread expression in the CNS of the rat (Wang and Guidotti, 1998), being present in neurons (in the cerebral cortex, hippocampus and cerebellum) glial cells, and endothelial cells. Pinsky *et al.* (2002) reported that NTPDase1, present in the vascular endothelial cells in the brain, exerts a protective thromboregulatory function, since NTPDase1 null mice exhibited increased infarct volumes following cerebral arterial occlusion. In contrast to NTPDase1, NTPDase2 expression is less ubiquitous. It was found in the germinal zones of the rat brain (Braun *et al.*, 2003), and was also seen in the subventricular zone and the rostral migratory stream. Double-labeling using probes against NTPDase2 and a glutamate transporter revealed that type-B cells also express NTPDase2 in the rat brain (Braun *et al.*, 2003), and that study suggests the possibility that NTPDase2 may be involved in ATP-mediated pathways that play an important role in neural development and differentiation. A few biochemical (Kukulski and Komoszynski, 2003; Nedeljkovic *et al.*, 2003; Kukulski *et al.*, 2004) and histochemical

(Vlajkovic *et al.*, 2002a,b) studies examined the expression of both NTPDase1 and NTPDase2, reinforcing the notion that they have distinct patterns of expression in the brain. However, no studies to-date have reported the immunolocalization of NTPDase3 in the brain, or in any other tissues. NTPDase3 was first cloned and characterized from a human brain cDNA library (Smith and Kirley, 1998). The enzymology of NTPDase3 is intermediate between NTPDase2 (also known as CD39L1 or ecto-ATPase, since it hydrolyzes nucleoside triphosphates at rates of about 50 times the rate of nucleoside diphosphates) and NTPDase1 (also known as CD39, which hydrolyzes ATP and ADP at similar rates). Chadwick and Frischauf (1998) showed that NTPDase3 (also known as CD39L3) mRNA is most abundant in the brain and pancreas, and has a less ubiquitous tissue distribution than either NTPDase1 or NTPDase2. To obtain further insight into the relationship between the NTPDase3 and the CNS, our research group was the first to immunolocalize NTPDase3 in the CNS and map its distribution in the rat brain (Belcher *et al.*, 2006; Zsarnovszky *et al.*, 2007). It was found that NTPDase3 is only expressed in neurons but absent from other cellular elements of the CNS. NTPDase3 showed an uneven distribution in the brain, being present mainly in midline structures, with particularly high amounts in the hypothalamus. Perikaryal cytoplasmic NTPDase3-immunoreactivity (IR) was only detected in the lateral hypothalamic nucleus (LHN) and arcuate nucleus (AN). Its tissue distribution and enzymatic function strongly suggested that NTPDase3 may play an important role in one or more of the integrative functions regulated by the neuroendocrine hypothalamus. Because of its function and localization, NTPDase3 appeared to be a likely candidate for the mediation/regulation of hypothalamic energy (ATP) levels, however, its unknown subcellular localization did not allow for a clearer view into the enzyme's exact cellular role. Therefore, our first goal was to determine: 1) the neuron type-specificity of the enzyme's location (stimulatory or inhibitory or both); 2) subcellular localization; and 3) other possible tissue characteristics in the hypothalamus, with regard to NTPDase3. Once the cellular localization described, we aimed to determine the possible E2-dependency of NTPDase3 expression and activity.

III. 1. Aims of the study

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.

III.2. Determination of the neuron type-specificity and subcellular localization of NTPDase3 in the hypothalamus

III.2.1. Co-localization of NTPDase3 and GAD in the hypothalamus

Gamma-aminobutyric acid (GABA) is the most ubiquitous inhibitory neurotransmitter in the CNS, including the hypothalamus. Determining whether or not NTPDase3 is localized to GABAergic cells could shed some light on the function of this enzyme. Since the metabolic turnover of GABA as a neurotransmitter at most GABAergic neurons may be considerably fast, direct identification of GABA may not display the full spectrum of such a neuron population. Therefore, to visualize GABAergic neurons, we have chosen to identify glutamic acid-decarboxylase (GAD), which is the key enzyme in GABA biosynthesis. Next, we identified NTPDase3 in hypothalamic tissue slices and investigated its cellular localization relative to that of GAD using the classical 'mirror' co-localization technique.

III.2.1.1. Materials and methods

Animal surgery and tissue fixation

Male and female Sprague-Dawley rats (body weight: 230–250 g; vendor: Charles-River Laboratories, Inc.) were used. Animals were kept under standard laboratory conditions, with tap water and regular rat chow *ad libitum* in a 12-h light, 12-h dark cycle. For histological studies, brains of anesthetized (intramuscular injection of a mixture of 200 mg/kg ketamine and 6.6 mg/kg xylazine) OVX animals (n = 12) were fixed by transcardial perfusion of a mixture of 5% paraformaldehyde and 2% glutaraldehyde in 0.1 molar phosphate buffer and stored in 4% paraformaldehyde until tissue processing.

Immunohistochemistry

Hypothalami were sectioned and 50 μm thick slices were immunostained for NTPDase3 using an affinity purified rabbit anti-NTPDase3 primary antibody. Omission of the primary antibody resulted in no detectable staining. (The rabbit anti-NTPDase3 [KLH14] primary antibody was kindly provided by Dr. Terence Kirley [University of Cincinnati College of Medicine]). Testing the specificity of this polyclonal antibody was described in details by Belcher *et al.* (2006). To study the possible expression of NTPDase3 in GABAergic inhibitory neurons, we assessed whether NTPDase3 and GAD are co-expressed in hypothalamic neurons. Adjacent hypothalamic slices were used for the comparison of GAD (rabbit anti-

GAD primary antibody, dil.: 1:2000; Sigma-Aldrich Chemie GmbH, Switzerland) and NTPDase3 immunolabelings by the previously described 'mirror technique' (Zsarnovszky *et al.*, 2000). In short, adjacent sections were arranged in pairs and one section of each pair was immunostained for NTPDase3 as described above, whereas their counterparts were single immunolabeled for GAD. Immunolabeling for GAD followed the standard immunohistochemistry protocol referred to above with the addition of a negative control experiment when the primary antibody for GAD was omitted. Omission of the primary antibody resulted in no detectable staining. After the visualization of immunoreactive material by nickel-intensified diaminobenzidine reaction, pairs of sections were thoroughly rinsed in 0.1 molar phosphate buffer and mounted with their matching surfaces on the upper side. Sections were then dehydrated through increasing ethanol concentrations and coverslipped. Focusing the microscope on the upper surface of each section, digital images were captured at various magnifications and corresponding areas were determined based on the pattern of vasculature and matching cell-halves through the overlay of images using Adobe Photoshop v. 7.0 software. After the computer-assisted reconstruction of the histological view, GAD-IR neurons were counted and potential NTPDase3-labeling of the matching cell halves was searched.

Electron microscopy

The formerly immunolabeled sections were processed as detailed by Zsarnovszky *et al.* (2001). The sections were immersed into 1% osmic acid diluted in 0.1 M phosphate buffer (PB) for 15 min, and then dehydrated in increasing ethanol concentrations. In order to enhance ultrastructural membrane contrast 1% uranyl-acetate was added to the 70% ethanol in the course of the dehydration. After dehydration, sections were embedded in water-insoluble Araldite resin (Sigma-Aldrich). After resin blocks were solidified, ultrathin sections were cut on an ultramicrotome, collected on Formvar-coated slot grids. Lead-citrate was used for further contrasting. For the synapse characterization, the guidelines provided by Palay and Chan-Palay (1975) and Colonnier (1968) were followed.

III.2.1.2. Results and discussion

The hypothalamic distribution of NTPDase3-immunoreactivity (IR) found in the present study was consistent with that described in an earlier report (Belcher *et al.*, 2006). Light microscopic analysis of 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 (Figure 15.).

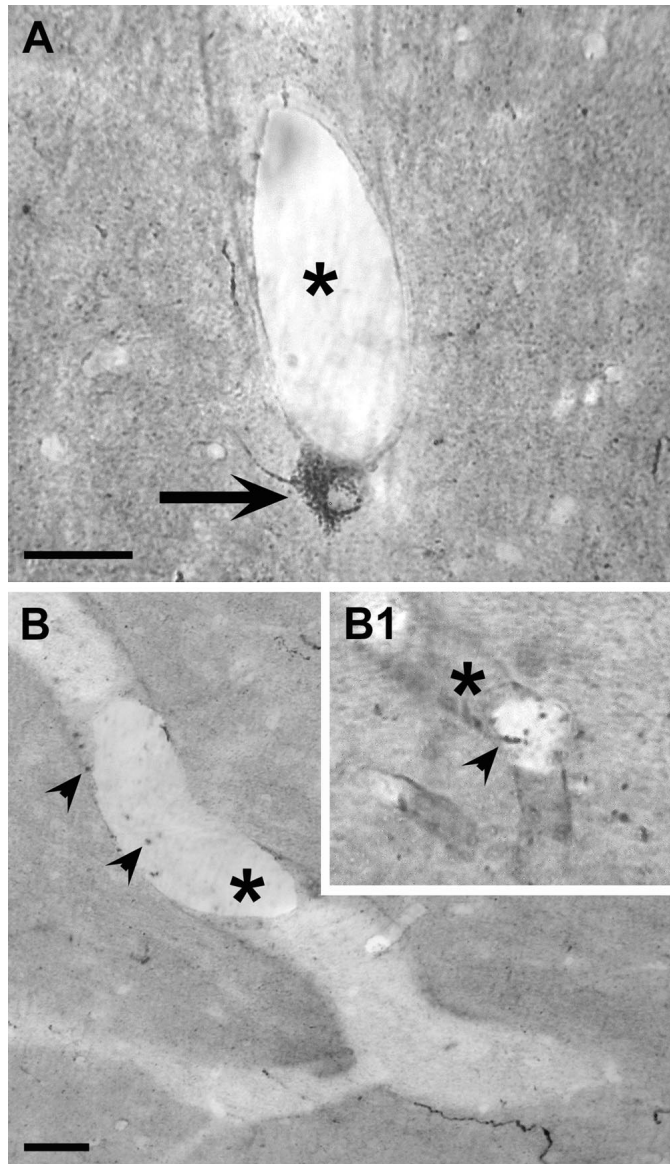


Figure 15. Hypothalamic NTPDase3-immunoreactive (IR) cells in close apposition to hypothalamic vessels. NTPDase3-IR perikarya (**A**, arrow) and putative neuronal processes (**B**, arrowheads) were frequently seen in close apposition to hypothalamic capillaries (asterisks). Scale bars represent 20 μ m.

A more detailed examination revealed that cellular staining occurred either in the form of cytoplasmic staining predominantly aggregated in particle-like dots (Figure 16A.) or as plasma membrane-associated punctate structures (Figure 16B.).

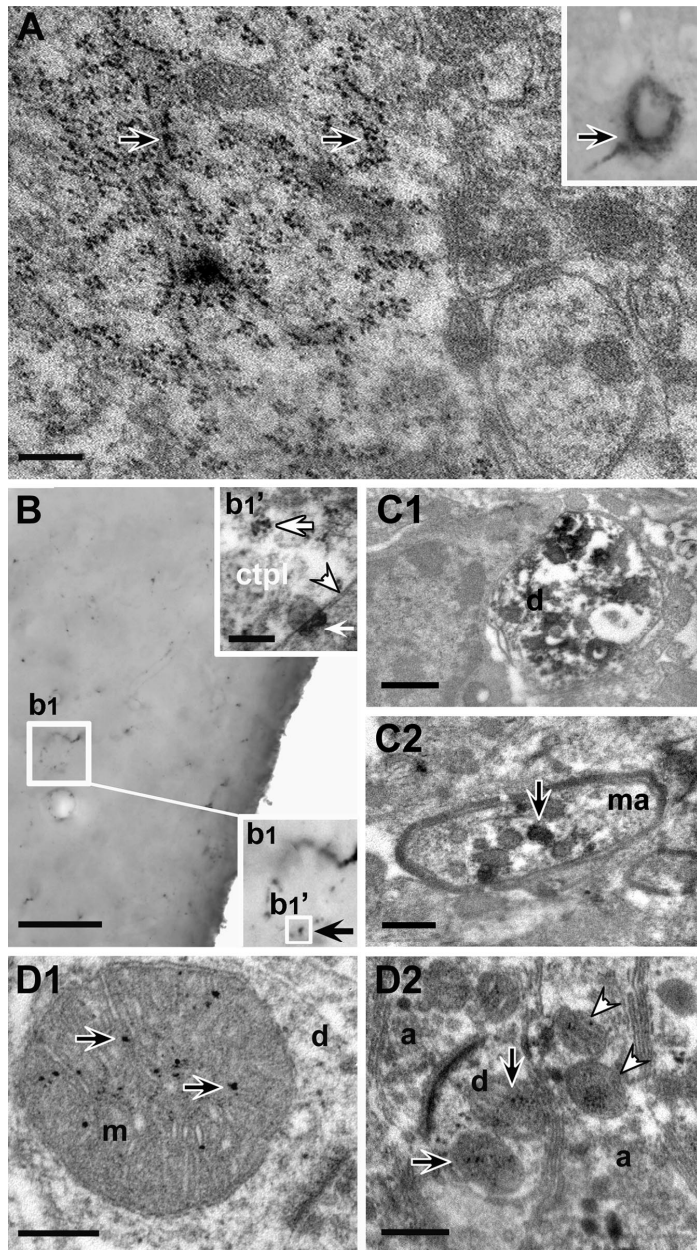


Figure 16. Electron microscopic analysis of ecto-nucleoside triphosphate diphosphohydrolase 3- (NTPDase3) immunoreactive hypothalamic profiles.

A: Grainy cytoplasmic immunoreactivity (inset, black arrow) may result partly from NTPDase3-IR material linked to ribosomes (black arrows). Scale bar: 400 nm.

B: Light microscopic studies showed NTPDase3-immunoreactive dots (b1 inset, black arrow) outlining the boundaries of immunoreactive cells. Scale bar: 50 μ m.

Correlated electron microscopic examination showed that immunoreactive dots shown on b1 represent plasma membrane-linked aggregates with nickel-DAB-labeling on the extracellular side (b1'; Arrowhead points to the plasma membrane. Upper white arrow indicates cytoplasmic immunopositive material; Lower white arrow points to plasma membrane-bound immunoreactive material. Scale bar: 100 nm.) ctpl: cytoplasm.

C1: Representative image of an NTPDase3-immunoreactive dendrite (d). Scale bar: 0.5 μ m.

C2: Representative image of a myelinated axon (ma) containing NTPDase3-immunoreactive mitochondria (arrow). Scale bar represents 1 μ m.

D1: NTPDase3-immunoreactive material in the mitochondrial (m) matrix (arrows) or linked to the inner mitochondrial membrane in a dendrite (d) Scale: 200 nm.

D2: NTPDase3-immunoreactive mitochondria in a dendritic (d) spine (black arrows) near an asymmetric synapse and in an unmyelinated axon (a) segment (white arrowheads). Scale: 400 nm.

Of the 320 NTPDase3-IR cell bodies examined, 29 contained nuclei with invaginations of the nuclear membrane. Since GABAergic neurons of the AN possess infolded cell nuclei (Leranth *et al.*, 1985; Leranth *et al.*, 1991), we assessed whether NTPDase3 is expressed in GABAergic (and thus inhibitory) cells. According to the comparison of GAD and NTPDase3 immunostainings (Figure 17.), none of the 2540 GAD-IR neurons examined contained

NTPDase3, indicating that NTPDase3 may be predominantly expressed in excitatory neurons of the hypothalamus.

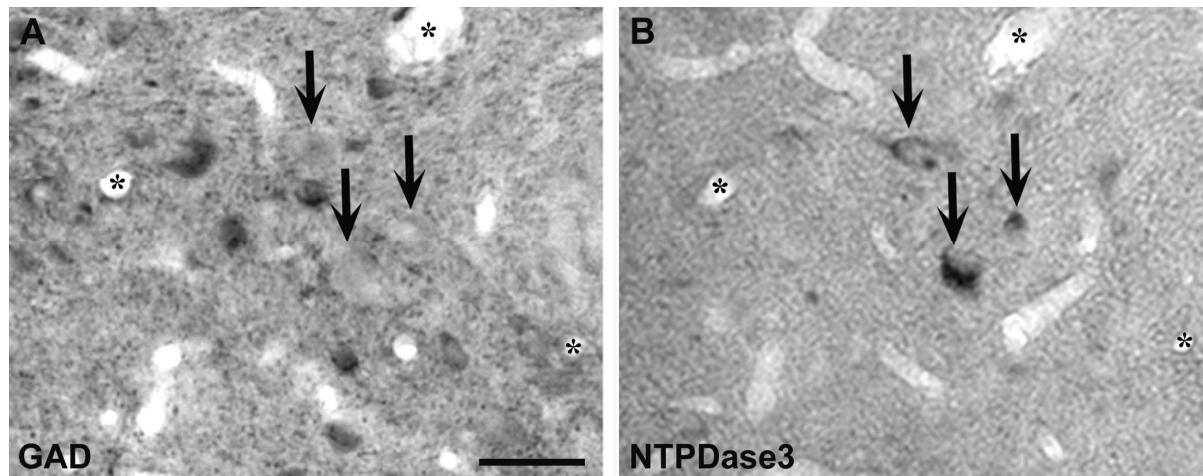


Figure 17. Immunolabeling for glutamic acid decarboxylase (GAD) and ecto-nucleoside triphosphate diphosphohydrolase 3 (NTPDase3) immunoreactivities in the hypothalamus using the mirror technique. Corresponding arrows point to matching cell halves immunostained either for GAD (A) or NTPDase3 (B). None of the examined GAD-immunoreactive neurons contained NTPDase3-immunoreactive material. Asterisks label corresponding vessels. Scale bar: 50 μ m.

Although we have not examined all hypothalamic GAD-IR neurons for the localization of NTPDase3, and some other, non-GABAergic neurons (e.g., dopaminergic) that are inhibitory in function and might also express NTPDase3, our present findings still suggest that the vast majority of hypothalamic NTPDase3 is expressed in excitatory neurons. In order to support the latter suggestion, further electron microscopic studies were performed.

III.2.2. Subcellular localization of NTPDase3 in the hypothalamus

The fine structure of a cell is genetically designed to best serve the actual cell's specific function. Therefore, the localization of subcellular components, be structural or functional, gives a good hint of their role in the parent cell. Following this logic, we investigated the subcellular localization of NTPDase3-IR material in hypothalamic neurons.

III.2.2.1. Materials and Methods

Animals, tissue fixation, cutting of 50 μ m thick hypothalamic slices and immunostaining for NTPDase3 were described in chapter III.2.1.1. Immunostained sections were then processed (osmicated, dehydrated, embedded, sectioned for electron microscopy and contrast-stained) for electron microscopic analysis as described in chapter III.2.1.1. (Zsarnovszky *et al.*, 2001). In order to eliminate potential pitfalls arising from the possible precipitation of lead citrate, in

addition to the general protocols we also examined ultra-thin sections that were only contrast-stained with uranyl-acetate during the dehydration process (1% uranyl-acetate in 70% ethanol for 60 minutes), but omitting subsequent treatment with lead citrate. For morphological characterization of synapses we considered the guidelines provided by Colonnier (1968) and Palay and Chan-Palay (1975).

III.2.2.2. Results and discussion

Our light- and correlated electron microscopic studies showed that NTPDase3-IR is present at certain well-demarcated segments of the plasma membrane (Figure 16Bb1.). This finding is consonant with the generally accepted view of NTPDases being transmembrane proteins and hydrolyzing phosphorylated nucleotides outside of the cell. Electron microscopic analysis clarified that labeled neuronal processes comprise both dendrites (Figure 16C1.) and axons (Figure 16C2.). In dendrites, cytosolic, ribosome-associated, as well as mitochondrial labelings were detected (Figure 16D1-D2.). In contrast, in myelinated axons and axon terminals only mitochondrial immunoreactivity was observed. The ultrastructural appearance of the grainy cytoplasmic and dot-like or line-shaped membrane-associated immunoreactive material was also investigated in neuronal perikarya of the LHN and AN. Ultrastructural examination of the cytoplasmic labeling revealed that part of the cytoplasmic IR particles observed in light microscope are associated with ribosomes, some of which were free cytoplasmic, but the majority of which were linked to the endoplasmic reticulum. This finding is not surprising in light of the fact that the amino acid sequence used for the generation of the antibody is part of the core protein; therefore, it is immunohistochemically detectable readily after translation. Interestingly, we found NTPDase3-IR in the mitochondrial matrix or closely associated with the inner mitochondrial membrane (Figure 16D1-D2.), which is probably the most intriguing finding of this study. Immunolabeled mitochondria were typically linked to asymmetric (putative excitatory) synaptic membrane specializations, present either in presynaptic terminals or at the post-synaptic side, in dendritic spines. Therefore, it is reasonable to assume that mitochondrial NTPDase3 activity is functionally linked to excitatory, rather than inhibitory neuronal functions. Labeled mitochondria were also detected in the perikaryal cytoplasm, frequently concentrated in the vicinity of the plasma membrane.

III.3. Estrogen effects on hypothalamic NTPDase3

The neuroendocrine hypothalamus is a major target of the ovarian hormone estradiol. Estradiol mediates peripheral signals about the reproductive status of the ovaries and evokes neural responses to regulate the secretion/release of hypothalamic GnRH and

(consequentially) pituitary LH. As a result, nearly steady (so-called pulsatile) levels of GnRH/LH are being released for the most part of the ovarian cycle through an E2-controlled negative feedback mechanism, while a midcycle E2 surge induces GnRH- and consequential LH surge in a positive feedback manner to evoke ovulation. Thus, the cyclic/periodic fluctuation of the ovary-derived E2 levels in the blood causes the activation of alternating negative-positive feedback neural actions in the hypothalamus. These neural actions manifest in an alternating (swinging) pattern in the ratio of stimulatory and inhibitory neuronal functions. Therefore, it was reasonable to assume that molecular mechanisms, especially those that regulate the availability of neuronal energy (ATP), such as the mitochondrial NTPDase3, are also influenced by the periodic changes in the circulating E2 levels.

III.3.1. Estrogen effects on hypothalamic NTPDase3 expression

As a first step in testing whether E2 influences hypothalamic NTPDase3 in any way, we investigated the effect of a single subcutaneous injection of 17-beta-estradiol in ovariectomized rats on the hypothalamic expression level of NTPDase3 by Western blot analysis.

III.3.1.1. Materials and Methods

Western blot studies

Rats were ovariectomized and kept under standard laboratory conditions for seven days. One week after ovariectomy, control animals were sacrificed (and processed as described below), while the rest of the animals received a single subcutaneous injection of 17-beta-estradiol (23 µg/100 g body weight; Sigma, water-soluble, cat. no. E4389). Estrogen-primed rats were then sacrificed 2–26 hours after receiving the E2 in two-hour intervals to determine the temporal changes in blood E2 concentrations and hypothalamic NTPDase3 expression (n = 5 for each group). After quick decapitation, a tissue block containing the AN and LHN was excised, following the coordinates of the rat brain atlas (Paxinos and Watson, 1986) as follows: A coronal slice of the entire rat brain was cut with a rostral border at anterioposterior level 2.12 mm behind the bregma (just behind the caudal border of the optic chiasm) and a caudal border at anterioposterior level 4.52 mm behind the bregma (just before the caudal tip of the mamillary body). Slices were divided into two halves along the midsagittal plane. The dorsal border of the tissue block was cut along a horizontal line dorsally tangential to the third ventricle and the remaining cortical tissue and optic tract were removed. The remaining tissue block was further divided into two halves along a sagittal plane passing through the fornix. Thus, we obtained two tissue blocks from the medial part of the hypothalamus

containing the AN, and two from the lateral part containing the LHN from each animal. Tissue blocks were then homogenized in (in mM) 20 Tris-HCl, pH 7.5, 150 NaCl, 1 PMSF, 1 EGTA, 1 EDTA, 2.5 sodium pyrophosphate, 1-beta-glycerol phosphate, and 1 Na₃VO₄ plus 1 mg/ml Pefabloc, 10 µg/ml leupeptin 10 µg/ml pepstatin, 1 µg/ml aprotinin, and 1% Triton X-100, 0.05% sodium deoxycholate. Homogenates were sonicated for 5 sec a total of 5 times and cleared by centrifugation at 14,000 × g for 1 min at 2°C. Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL). Western blotting and densitometric analysis were performed by standard protocols (Jakab *et al.*, 2001; Wong *et al.*, 2003). Membranes were blocked with 5% nonfat dry milk for 1 hr in TBS-T and incubated with appropriate antisera (affinity purified rabbit anti- NTPDase3, KLH14, as described by Belcher *et al.* (2006). Immunoreactive bands were visualized onto preflashed x-ray film by enhanced chemiluminescence. Optical densities were calculated as arbitrary units after local area background subtraction, normalized to the protein concentrations of samples and to the density of controls. Results are reported as fold changes relative to control. All data that have been presented are representative of at least three independent experiments. Serum estradiol concentrations were determined from each animal used for the western blot studies by 3H-RIA, as described by Csernus (1982).

III.3.1.2. Results and discussion

Since NTPDase3-IR perikarya were only detected in two of the hypothalamic regions, i.e., the AN and LHN, isolated medial and lateral hypothalamic tissue samples were collected from ovariectomized and estrogen-primed animals, and examined by Western blot analysis for the expression level of NTPDase3 (Figure 18.).

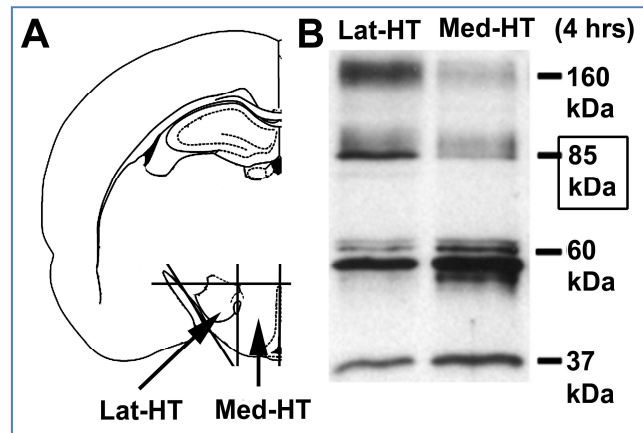
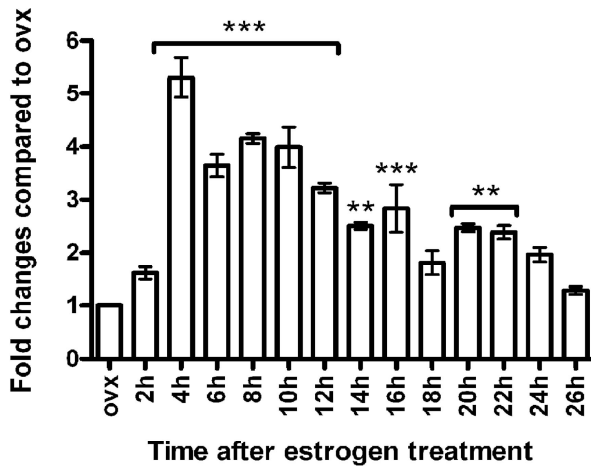


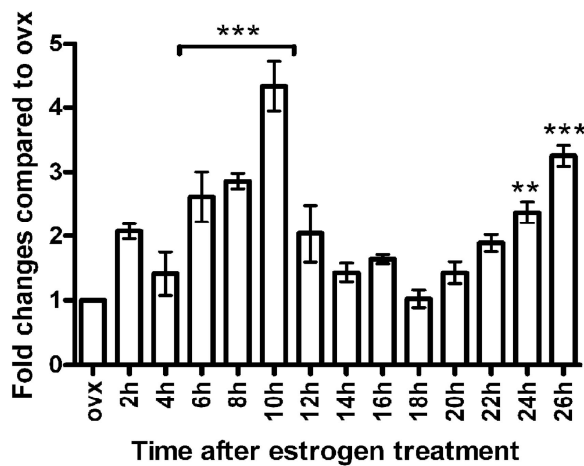
Figure 18. Assessment of NTPDase3 levels in the hypothalamus of female rats. **A:** Sample collection. Tissue blocks of the medial part of the hypothalamus (Med-HT) containing the arcuate nucleus and the lateral part of the hypothalamus (Lat-HT) containing the lateral hypothalamic nucleus were excised as follows: A coronal slice of the entire rat brain was cut with a rostral border at anteroposterior level 2.12 mm behind the bregma (just behind the caudal border of the optic chiasm) and a caudal border at anteroposterior level 4.52 mm behind the bregma (just before the caudal tip of the mamillary body). Slices were cut into two halves along the midsagittal plane. The dorsal border of the tissue block was cut along a horizontal line dorsally tangential to the third ventricle and the remaining cortical tissue and optic tract were removed. The remaining tissue block was further cut into two halves along a sagittal plane passing through the fornix. Thus, we obtained two tissue blocks from both the medial and lateral parts of the hypothalamus from each animal. **B:** Representative image showing NTPDase3-immunoreactive material in the Lat-HT and Med-HT four hours after a single subcutaneous injection of 17β -estradiol. Four immunoreactive bands were detected using homogenates of whole tissue samples. The 82-85 kDa molecular weight form of the enzyme, generally considered as the fully functional form, was further analyzed. Note the difference between the densities of the 82-85 kDa bands of the Lat-HT and Med-HT samples.

Four immunoreactive bands (~160–170 kDa, 82–85 kDa, 60 kDa and 37 kDa) were detected using the rabbit anti-NTPDase3 affinity-purified polyclonal antibody (KLH14, kindly provided by Dr. Terence Kirley, University of Cincinnati, OH, USA). In a previous study, where this antibody was tested and used for Western blot studies, multiple immunoreactive bands were found using COS cell membranes, and one IR band was detected using rat brain thalamus membranes from OVX rats (Belcher *et al.*, 2006). It is, therefore, not surprising that using homogenates of rat hypothalami (a brain area highly sensitive to E2) more IR bands were found, likely representing distinct structural and functional forms of the enzyme. Of the listed bands, the 85 kDa protein is considered as the fully glycosylated form of the protein, while the 160–170 kDa and 60 kDa values correspond to the dimeric form of the enzyme and the core protein. Here we focused on the analysis of the expression level of the 85 kDa protein. Time- and E2-dependent increases in NTPDase3 levels were found in both (medial and lateral) selected hypothalamic areas (Figure 19.); however, the temporal changes in NTPDase3 levels displayed distinct patterns.

A NTPDase3 levels in the lateral hypothalamus



B NTPDase3 levels in the medial hypothalamus



C Estrogen concentrations after a single subcutaneous injection of 17beta estradiol (pg/ml)

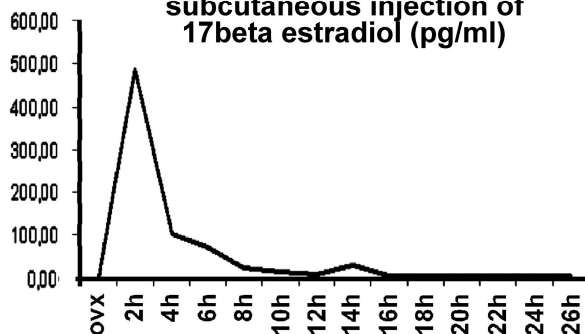


Figure 19. Time-course of changes in hypothalamic NTPDase3 levels after a single subcutaneous injection of 17β -estradiol (E_2). Optical density measurements of the 82-85 kDa immunoreactive bands were analyzed in OVX animals and OVX plus E_2 -treated animals 2-26 hours after a single subcutaneous injection of E_2 . Samples were taken in two-hours intervals after E_2 -priming.

A: Estrogen-treatment induced a sharp rise in NTPDase3-expression in lateral hypothalamic samples, reaching significantly elevated peak levels by four hrs. After the peak, a gradual decrease in NTPDase3-expression was observed reaching nearly control OVX levels by 26 hrs.

B: In medial hypothalamic samples, E_2 -treatment induced a gradual increase in NTPDase3 levels with highest values observed at 10 hrs after E_2 -priming. This was followed by falling of NTPDase3-level that reached a nadir at 18 hrs, followed by a second rise by 24-26 hrs. The difference in the time-course of changes in NTPDase3 levels between the medial and lateral parts of the hypothalamus suggests that NTPDase3 may serve region-specific functions in the neuroendocrine hypothalamus.

C: Mean E_2 blood plasma concentrations measured after a single subcutaneous injection of E_2 (water soluble, $23 \mu\text{g}/100 \text{g}$ body weight).

In samples containing the LHN, NTPDase3 levels increased significantly 4–12 hrs after a single subcutaneous injection of E_2 , and gradually returned to nearly control (OVX) levels by 16–26 hrs after E_2 treatment (Figure 19A.). In contrast, temporal changes in medial hypothalamic samples containing the AN showed an initial increase in NTPDase3 expression between 6–10 hrs after E_2 treatment, followed by a sharp decrease to control level, and

again followed by a second rise between 22–26 hrs after E2 treatment (Figure 19B.). 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, however, its role in the two hypothalamic regions might be different.

As mentioned earlier, multiple NTPDase3-IR bands were detected in Western blot studies using rat hypothalamus homogenates. This phenomenon may indicate that there is 'incomplete' processing of the enzyme, however, it is more likely that the distinct bands observed represent different maturational forms of the enzyme. In this study, we detected NTPDase3-IR linked to multiple subcellular structures (plasma membrane, ribosomes, endoplasmic reticulum) including neuronal mitochondria. Additionally, we provided evidence for NTPDase-activity in synaptosomal preparations (please see below). Therefore, it is also possible that one or more of the protein-forms detected on Western blots represent functional forms of the protein adapted or adjusted to the microenvironment or functional attributes of the cell organelles. 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. Therefore, in a pilot study we investigated whether E2-treatment of OVX animals affects NTPDase3 levels in tissue blocks containing the entire hypothalamus. That study showed that a single subcutaneous injection of E2 results in significantly increased levels of NTPDase3 (unpublished observation). Those results prompted us to examine and analyze the temporal changes in NTPDase3 expression in lateral and medial hypothalamic tissue samples as described above. The present findings indicate that in response to a single subcutaneous injection of E2, NTPDase3 expression increases in just a few hours after E2-treatment in both hypothalamic areas, however, the pattern of temporal changes in the medial hypothalamus differs from that observed in the lateral part of this brain area. Since the mediobasal hypothalamus, including the AN, is a major player in the biphasic (positive- and negative feedback) regulation of the gonadotropin 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 gonadotropins. The idea of a causal coincidence between the two peaks in NTPDase3 levels and the positive/negative gonadotropin feedbacks raises several questions. For example, a number of data suggest that NTPDase3 may be involved in the hypothalamic regulation of gonadotropin release. We have previously described that during the E2-induced gonadotropin surge, an E2-dependent synaptic reorganization on hypothalamic neurons occurs. This phenomenon was termed 'phased synaptic remodeling', that shows specific changes in the ratio of inhibitory/excitatory synapses during the two (positive- and negative-) states of the gonadotropin feedback control (Naftolin *et al.*, 2007). A sharp rise in the number of excitatory synapses was observed at the time of the E2-surge,

and the formation of new synapses may well include ones equipped with NTPDase3-containing mitochondria. This hypothesis is consonant with our observation that inhibition of NTPDase activity decreases state 3 mitochondrial respiration 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. Both medial and lateral hypothalamic functions are known to involve mechanisms mediated by various purinoceptors, such as A1, P2X, and the activity of NTPDase3 containing hypocretin-orexin neurons in the LHN is directly influenced by such receptor actions (Thakkar *et al.*, 2002; Gordon *et al.*, 2005; Wollmann *et al.*, 2005; Florenzano *et al.*, 2006; Kittner *et al.*, 2006; Seidel *et al.*, 2006; Knott *et al.*, 2007; Liu and Gao 2007). Since here we found neuronal membrane-linked NTPDase3-IR in the AN/LHN, and E2 induced a transient increase in NTPDase3 levels in both hypothalamic sites, it is also possible that E2 increases the amount of membrane-incorporated NTPDase3 to transiently intensify purinergic interneuronal signaling. Further studies are underway to clarify this issue. In lateral hypothalamic samples, NTPDase3 levels peaked at 4 hrs after E2-treatment followed by a gradual decrease, and reached OVX levels by 26 hrs. It has been demonstrated by Belcher *et al.* (2006) that LHN NTPDase3-containing cells are nearly all (96–97%) hypocretin-orexin containing neurons. These neurons are known to be direct modulators of the midbrain raphe serotonergic neurons (Liu *et al.*, 2002) to influence sleep-wake states. It is also known that E2 influences arousal mechanisms in many ways (Lee and Pfaff, 2008). Thus, it is possible that the mechanism through which E2 facilitates wakefulness involves increased NTPDase3-activity. On the other hand, LHN hypocretin-orexin (plus NTPDase3-IR) neurons are not only targets of E2, but also that of the gastric hormone ghrelin; at the same time, these neurons also represent the major excitatory input of AN neuropeptide Y/Agouti-related protein-containing cells whose activity is responsible for the initiation of food intake. Changes in the functional intensity of this circuit also involve synaptic remodeling (Horvath, 2005). It is therefore possible that in response to E2-treatment, LHN NTPDase3-IR hypocretin-orexin-containing neurons intensify their action on AN neuropeptide Y/Agouti-related protein-containing cells, thereby leading to increased NTPDase3 levels in both (medial and lateral) parts of the hypothalamus. If this was the case, one could speculate that the anorexigenic effect of E2 may in some way involve the action of NTPDase3.

III.3.2. Demonstration of NTPDase3 activity in hypothalamic mitochondria

We provided immunohistochemical, light and electron microscopic evidence that NTPDase3 is expressed in putative stimulatory neurons of the hypothalamus and that this enzyme is present in the mitochondrial matrix. Additionally, we also demonstrated that the hypothalamic

expression level of NTPDase3 is estrogen-dependent. To further characterize the role of this enzyme in the hypothalamus, we aimed to seek functional evidence for the mitochondrial presence of NTPDase3. We demonstrated the ATP-hydrolyzing function of NTPDase3 in mitochondria by measuring ADP-dependent state 3 mitochondrial respiration in hypothalamic samples of rats. Since earlier we found that changes in estrogen concentration influence NTPDase3 expression, it was reasonable to demonstrate mitochondrial NTPDase3 function in male animals at this time. NTPDase3 activity was monitored with and without the experimental inhibition of NTPDase3.

III.3.2.1. Materials and Methods

Preparation of hypothalamic synaptosomal fractions was carried out by minor modification of the protocol described in chapter II.4.1. Briefly, male animals (n=8) were used for the determination of mitochondrial respiration rates. Animals were anesthetized with isoflurane and brains were removed after quick decapitation. Hypothalami were dissected and cut into two halves. One side was always used as control (incubated in vehicle), while the contralateral side was used to determine the effects of the NTPDase inhibitor suramin (Calbiochem, San Diego, CA). The ability of suramin to block the enzymatic function of NTPDases has been previously reported (Muller *et al.*, 2006). Samples were homogenized in isolation buffer (pH 7.2; 215 mM mannitol, 75 mM sucrose, 0.1 % bovine serum albumin, 1 mM EGTA, 20 mM HEPES). The homogenate was spun at 1300 x *g* for 3 min, the supernatant was removed, and the pellet was resuspended with isolation buffer and spun again at 1300 x *g* for 3 min. The two sets of supernatants from each sample were topped off with isolation buffer and spun at 13,000 x *g* for 10 min. The supernatant was discarded, and the step was repeated. After this second spin at 13,000 x *g*, the supernatant was discarded, and the pellets were resuspended with isolation buffer without EGTA and spun at 10,000 x *g* for 10 min. The final synaptosomal pellet was resuspended with 50 µl of isolation buffer without EGTA (Coppola *et al.*, 2007; Andrews *et al.*, 2008). Protein concentration of mitochondrial suspensions was determined with a BCA protein assay kit (Pierce, Rockford, IL). As mentioned above, one half of the samples collected (i.e., the right side of the hypothalamus) was treated with suramin at a final concentration of 20 µM (diluted in respiration buffer) followed by a 30 minutes incubation of all samples at 37 °C just before the measurements. Control sides were incubated for 30 minutes at 37 °C in vehicle (respiration buffer only). Mitochondrial respirations were assessed using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK) at 37 °C with pyruvate and malate (5 and 2.5 mM; State 2 respiration) as oxidative substrates in respiration buffer (215 mM mannitol, 75 mM sucrose, 0.1% fatty acid-free BSA, 20 mM HEPES, 2 mM MgCl, 2.5 mM KH₂PO₄, pH

adjusted to 7.2 with KOH). For analysis of ADP-dependent respiration (state 3 respiration), ADP was added after the addition of oxidative substrates. After the addition of oligomycin (state 4 respiration), mitochondrial respiration was measured as increased fatty acid-induced respiration (Palmitate 150 μ M). Total uncoupled respiration was also measured after the addition of the protonophore FCCP (carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone, 1 μ M). The results are expressed as nmols of O₂ consumed per minute per mg protein.

Statistical analyses

Statistical analyses were conducted with a Student's t test or by one-way ANOVA with Tukey's Multiple Comparison Test as appropriate. Data were analyzed with Excel (Microsoft) and GraphPad Prism version 4 (GraphPad Software, San Diego, CA).

III.3.2.2. Results and discussion

To study the functional link between NTPDase3 and mitochondria, we investigated the effect of the NTPDase inhibitor, suramin, on mitochondrial functions. There are two major concerns against the use of suramin for NTPDase3 inhibition:

1) To the best of our knowledge, there is no selective NTPDase (including NTPDase3) inhibitor available on the market. Muller *et al.* (2006) compared and characterized the ability of polyoxometalates (the best-known NTPDase inhibitors) to block the functions of the different NTPDases (and purinergic receptors). Based on those results, we chose to use suramin, which was described as the most potent NTPDase3 inhibitor among the compounds examined, however, it also inhibits other NTPDases. Additionally, in a review summary by Langer *et al.* (2008) of the present knowledge of the distribution of different NTPDases in the brain, the authors arrived to the conclusion that in the lack of considerable amounts of other NTPDases, much of the ATPase activity in the hypothalamus is attributable to NTPDase3. Therefore, in the present study the effects of suramin treatments are considered as general inhibitory effects on NTPDases, including NTPDase3.

2) Since suramin is a non-selective NTPDase- and purinoceptor inhibitor, the question might arise that administration of suramin to hypothalamic samples may mask our results by the simultaneous inhibition of the extracellular ATPase domains of NTPDases. However, it should be pointed out that: **a)** we have worked on synaptosomal/mitochondrial fractions with no natural extracellular environment; **b)** changes in extracellular ATP-hydrolysis would not affect directly the mitochondrial respiration; and **c)** previous studies carried out on synaptosomes from several brain regions suggested that the ecto-ATPase activity detectable

under such experimental conditions is most likely the result of NTPDase3-, rather than any of the two other (NTPDase1 or NTPDase2) NTPDases present in the CNS (James *et al.*, 1993; Cunha, 2001).

Inhibition of NTPDases by suramin had a significant inhibitory effect on state 3 mitochondrial respiration (Figure 20A.) (45.05 ± 4.9 nmol O₂/mg protein/min with suramin versus 65.1 ± 6.6 of control; $P < 0.05$) indicating that a decrease in ATP-ADP conversion occurred in mitochondria after NTPDase inhibition. The total mitochondrial respiratory capacity decreased as well (Figure 20B.) (87.8 ± 5.5 nmol O₂/mg protein/min in control synaptosomes versus 57.7 ± 6.5 nmol O₂/mg protein/min ($P < 0.05$) in synaptosomes incubated with suramin), suggesting that mitochondrial NTPDase3 activity likely influences oxygen-consuming biochemical processes in the mitochondrial matrix.

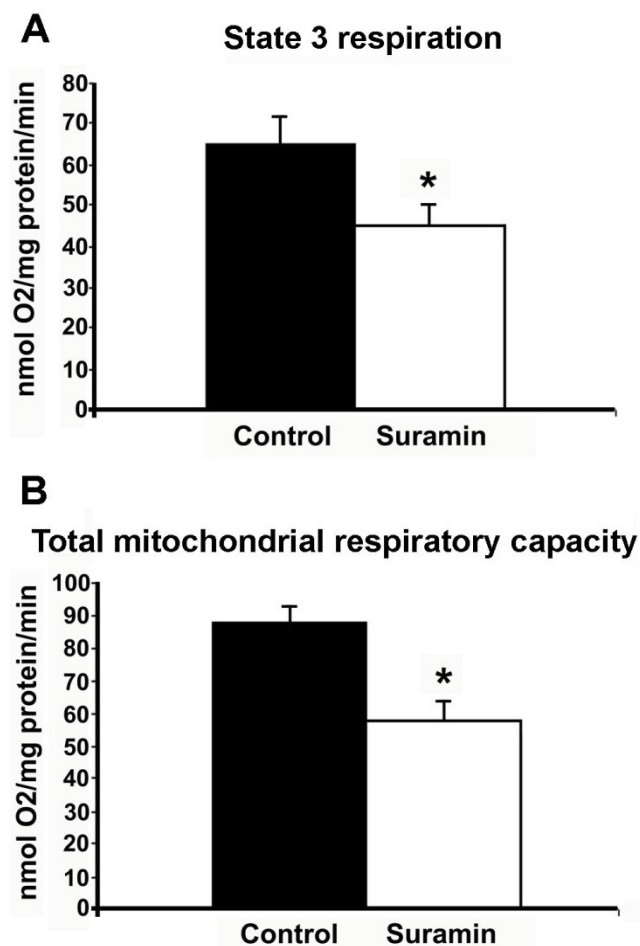


Figure 20. Determination of mitochondrial respiration rates in hypothalamic synaptosomes. Inhibition of ecto-nucleoside triphosphate diphosphohydrolase activity by Suramin decreased oxygen consumption during state 3 mitochondrial respiration (**A**), and also decreased the total mitochondrial respiratory capacity (**B**).

It has been shown that interneuronal signaling is a highly ATP-dependent, energy-demanding process (Laughlin *et al.*, 1998). To supply the energy needs of neurotransmission, ATP is produced and maintained in neuronal mitochondria in a regulated fashion. It has been previously proposed (Horvath *et al.*, 1999) that one potential mechanism

down-regulating mitochondrial ATP production may involve uncoupling proteins (UCPs), specifically UCP2, however, UCP2 was only found in inhibitory neurons of the hypothalamus. The specific mechanism involved in the regulation of mitochondrial ATP levels in excitatory hypothalamic neurons is currently less known. Therefore, the identification of NTPDase3 in mitochondria in synaptic or perikaryal sites of excitatory hypothalamic neurons might be the most novel and intriguing finding of this study, and warrants further experiments to elucidate the exact functional role of mitochondrial NTPDase3 in neurotransmission. Suramin blockade of NTPDase3 reduced mitochondrial O₂ consumption in state 3 mitochondrial respiration by 30%, and also decreased the total mitochondrial respiratory capacity by 34%. Thus, the yet unidentified endogenous regulation of intramitochondrial NTPDase3 activity is a likely candidate mechanism for the energetic regulation of excitatory neurotransmission.

Based on the present findings, it is tempting to speculate that an increase in the activity level of NTPDase3 may result in the exhaustion of mitochondria (and the parent cell), whereas partial inhibition of NTPDase3 may be neuroprotective. Ongoing studies in our laboratory test this hypothesis. The reported pharmacological neuroprotective effects of polyoxometalates, such as suramin, seem to support this idea. For example, some data show that NTPDase inhibition is also antidiabetic (Hillaire-Buys *et al.*, 1994), although the exact mechanisms through which the beneficial effects of NTPDase inhibitors act are unknown. Therefore, the present results rise the possibility that in the pancreas, inhibition of (potentially intramitochondrial) NTPDases (NTPDase3?) may protect the insulin-producing beta cells from overt ATP consumption and consequential exhaustion.

III.4. Effects of estrogen on ADP-dependent state 3 mitochondrial respiration in ovariectomized rats

We have shown that mitochondrial metabolism is asymmetric in the rat hypothalamus and that ADP-dependent state 3 mitochondrial respiration (St3) is regulated by NTPDase3 in this CNS region. We also found that temporal changes in medial hypothalamic NTPDase3 expression after a single subcutaneous injection of E2 are distinct from those found in the lateral hypothalamus. This phenomenon is most likely the result of the functional separation of neuronal circuits that regulate ovarian cyclicity (medial hypothalamus, MHT) and feed-intake (mostly the lateral part of the hypothalamus, LHN). Some overlap between these circuits exists, nevertheless. Most importantly, lateral hypothalamic NTPDase3-IR neurons that are also orexinergic, are the major targets of the gastric hormone ghrelin (known as the 'hunger hormone'). These NTPDase3/orexin cells represent, in turn, the major stimulatory input to medial hypothalamic neuropeptideY/agouti-related protein- (NPY/AgRP) containing neurons, latter which are the initiators of feed-intake. Purinergic interneuronal signaling between the aforementioned two cell types plays a key role in the hypothalamic initiation of feed-intake. Based on the data above, we aimed to clarify the differential effects of E2 and hunger-satiety on St3 in the lateral-medial parts of the hypothalamus.

III.4.1. Estrogen's differential effects on St3 in the lateral-medial parts of the hypothalamus

III.4.1.1. Materials and Methods

Female Sprague-Dawley rats were used in this study (n=6 per group). Animals were ovariectomized and E2-treated as described in chapter III.3.1.1. Since previously (please see chapter III.3.1.2.) the strongest estrogen effects on NTPDase3 protein expression were found 10 hours after the single subcutaneous injection of E2, mitochondrial respiration rates at this time were also measured 10 hrs after E2-treatment. Preparation of hypothalamic synaptosomal fractions was carried out as described in chapter II.4.1. Please note that sample homogenates were prepared through the mixture of both left and right medial and both left and right lateral parts of the hypothalami.

III.4.1.2. Results and discussion

Ten hours after a single subcutaneous injection of E2, St3 increased by 63 % (LHT) and 43 % (MHT), respectively (Figure 21.). Since E2 also modulated NTPDase3 expression and NTPDase3-inhibition decreased St3, it is suggested that E2 differentially regulates NTPDase3-activity in the LHT and MHT. The differences between NTPDase3-expression and enzyme activities in respective hypothalamic areas suggest that additional mechanisms may exist that, besides the regulation of E2-dependent NTPDase3-expression, might be involved in the regulation of NTPDase-activity. It is worthy of note that the facilitatory effects of E2 on St3 values might have only happened in either the left or the right hypothalamic hemisphere only, since we found a high degree of metabolic asymmetry, including the St3 when the two hypothalamic hemispheres were examined separately from each other.

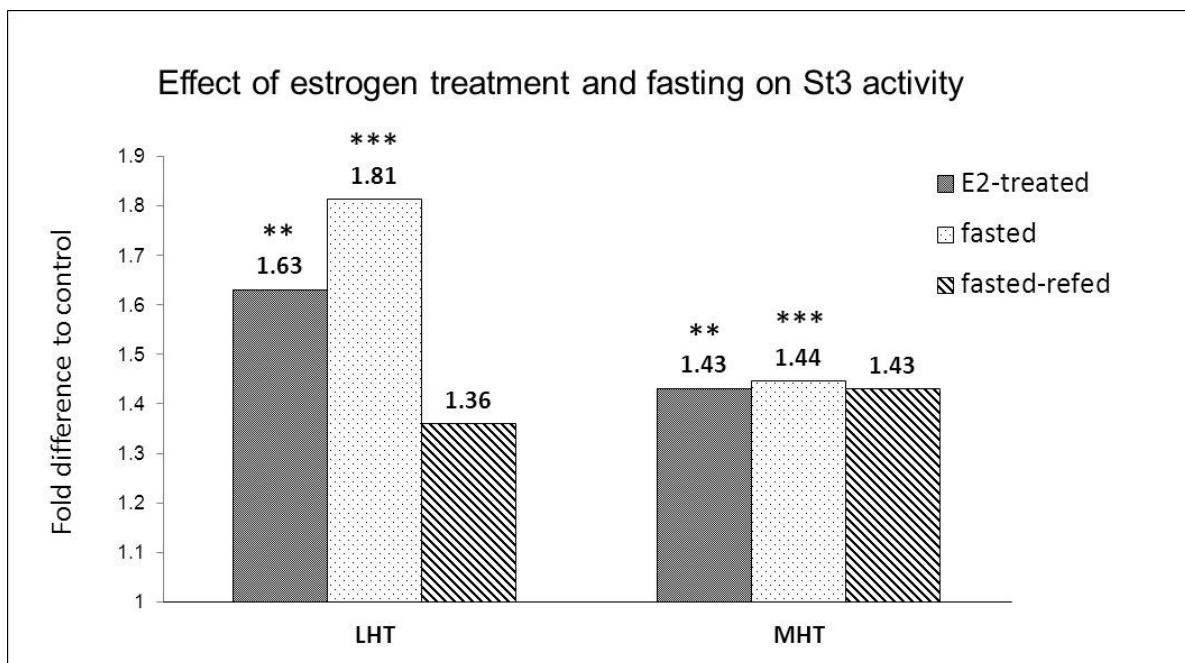


Figure 21. Impact of gonadal steroids and hunger state on NTPDase3 activity observed through ADP-dependent (St3) mitochondrial respiration. Y axis indicates fold difference to control levels. Significance was tested by one-way ANOVA.

III.4.2. Effects of fasting versus fasting/re-feeding on ADP-dependent state 3 mitochondrial respiration in ovariectomized rats

III.4.2.1. Materials and Methods

Ovariectomized Sprague-Dawley rats were used in this study (n=6 per group). The OVX animals were fasted for 24 hours before the sample collection. A group of the fasted animals was re-fed *ad libitum* for 4 hours after 24 hours of fasting. Control animals were OVX rats with *ad libitum* feed and water supply. Preparation of hypothalamic synaptosomal fractions was carried out as described in chapter II.4.1. The sample homogenates were prepared through the mixture of both left and right medial and both left and right lateral parts of the hypothalamus as mentioned in chapter II.4.1.1.

III.4.2.2. Results and discussion

Effects of 24 hrs fasting in E2-deprived (OVX) animals increased St3 by 81 % in the LHT as compared to the 44 % increase in the MHT (Figure 21.). In rats that were re-fed, the elevation in LHT St3 values changed towards reverting to the control value (total reversion would have probably needed more than 4 hrs). Results are in line with our previous notion that LHT NTPDase3/orexin neurons (the latter neurons project to the MHT NPY/AgRP cells) are primary targets of the 'hunger hormone' ghrelin, as fasting-induced ghrelin signaling leads to increased stimulatory input on these neurons (Horvath, 2005). The dominance of LHT over the MHT in the regulation of feed-intake is also reflected in that 4 hrs after re-feeding no considerable difference was found between the MHT St3 values of the fasted and fasted/re-fed animals. The observation that E2-treatment resulted in changes comparable to those evoked by fasting in the LHT but had no such effect in the MHT indicates that, in terms of mitochondrial respiration (at least with the timing applied, i.e., 4 hrs after E2-treatment), E2's early effects on the regulation of feed-intake target LHT neurons rather than MHT cells.

IV. Summary and Conclusion

The neuroendocrine hypothalamus is a central target of major peripheral hormones, such as estrogens, thyroid hormones, ghrelin, leptin etc. These hormones convey signals from peripheral organs to the entire CNS about their actual functional and metabolic status. The CNS, according to its functionally differentiated specific regions, elaborate on the incoming peripheral signals and converge their responses to the specific effector regions, such as the hypothalamus, to regulate functionally relevant functions and homeostasis by adequate central 'commands'. The brain is an organ with symmetric tissue organization, with either paired regions or unpaired midline structures. Cerebral regions are specialized to distinct functions, i.e., each of the cerebral hemispheres dominate in certain specific functions. Histologically, the hypothalamus comprises like-named nuclei with symmetrical localization on the two sides of the third ventricle. Although early studies indicated the possibility of functional lateralization of the hypothalamus, those findings have been ignored by successive hypothalamic research that continued to consider the hypothalamus as an unpaired midline structure. If the hypothalamus was indeed functionally asymmetric, it would raise several interesting and important questions that, if answered, would have the potential to revolutionize future hypothalamic research and its clinical applications. Few of these questions are as follows: How and when does the hypothalamic asymmetry develop during the ontogenesis? Could the development of the hypothalamic asymmetry be experimentally/therapeutically influenced, and if so, what would be the consequences? Is the hypothalamic asymmetry bound to a hypothalamic hemisphere in a given individual or the functional lateralization is alternating from cycle to cycle? Is the functional asymmetry age-bound, or is/are there one or more switch(es) between the hemispheres during the reproductive lifespan of the animal? If there is a unilateral functional dominance in hypothalamic reproductive centers, does the 'silent' hemisphere possess the potentials to overtake the regulatory function and thus carry the potential of cyclicity and that of a 'second' reproductive life?

The above questions well demonstrate the significance of the matter and prompted us to search for evidence proving the existence of hypothalamic laterality. As a first step of this endeavor, 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 (called the 'active' side). The contralateral side of the hypothalamus did not show such fluctuation in the examined

metabolic aspect (called the '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 phases, it is most likely related to the regulation of GnRH-secretion/release.

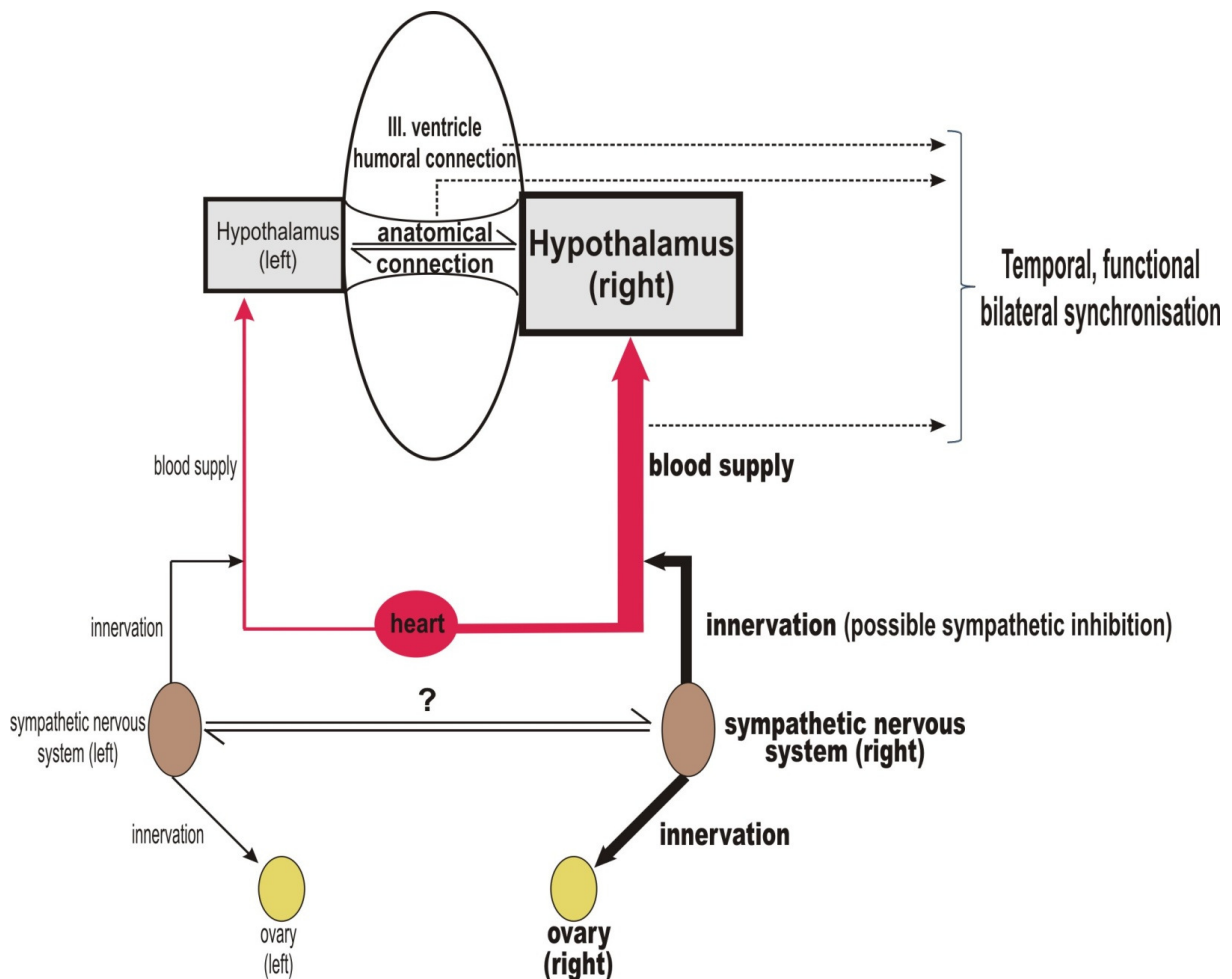


Figure 22. Structural elements of temporal functional bilateral synchronization: 1) direct anatomical connections between the two sides of the hypothalamus (e.g.: interthalamic adhesion, infundibular region); 2) the third ventricle and its lateral evaginations create humoral contact; 3) sidedness in the sympathetic tonus system, which obviously establishes an asymmetry in the blood and therefore O_2 -supply between the hypothalamic hemispheres.

The hypothalami of nearly all females showed asymmetry in mitochondrial metabolism. The intensity of metabolism and thus, the degree of laterality dynamically changed depending on the phase of the estrous cycle. These dynamic changes suggest that there must be mechanisms that ensure the dominance of the „active” hypothalamic side and the simultaneous inhibition of the „silent” side, while a given hypothalamic function is regulated based on the synchronous activity of the two hypothalamic hemispheres. This hypothesized phenomenon we termed temporal functional bilateral synchronization (TFBS) (Figure 22.). The TFBS may lie on three structural bases: 1) direct anatomical interconnection between the two hypothalamic hemispheres, most probably through the adhesio interthalamica and/or

the hypophyseal infundible (median eminence); 2) humoral interconnection through the third ventricle and its evaginations into the matter of the ventrobasal hypothalamus; 3) the sided function of the sympathetic nervous system that results in asymmetric blood flow/oxygen supply that can lead to both oxygen- and thermo-gradients between the two hemispheres, thereby influencing the intensity of biochemical reactions.

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 (considered as the 'micro-batteries' of cells) are constantly adjusted to the actual physiological needs of the relevant cells/neurons. Based on its ATP-hydrolyzing function, one of the likely candidates to regulate mitochondrial energy (ATP) levels was the NTPDase3. Therefore, after our research group mapped its distribution in the brain, here we aimed to determine whether or not NTPDase3 belongs to the stimulatory or inhibitory or both types of hypothalamic neural circuits. To this end, we co-localized NTPDase3 and GAD (the key limiting enzyme of the GABA biosynthesis), and found that NTPDase3 is not expressed in GABAergic neurons. Since GABA is the most ubiquitous inhibitory neurotransmitter in the brain, we concluded that NTPDase3 expression is highly restricted to excitatory neurons. Electron microscopic studies confirmed and supported this inference. NTPDase3 was identified 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 state 3 mitochondrial respiration. 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-secretion/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.

In search for the functional link between the E2-regulated hypothalamic functions and NTPDase3, we carried out experiments on OVX and OVX plus E2-primed rats and examined the possible changes in the hypothalamic expression level of NTPDase3. Results demonstrated that E2 increased the hypothalamic expression level of NTPDase3, however, the temporal pattern of NTPDase3-expression was different between the medial (double-peaked curve) and lateral parts (single-peaked curve) of the hypothalamus, suggesting that E2 may regulate neuronal ATP-hydrolysis in these two parts of the hypothalamus in a

functionally relevant manner (according to the specific function of the hypothalamic region). This finding was consonant with results from lateralization experiments, where ADP-dependent state 3 respiration appeared strongly estrous phase dependent. However, to further support the aforementioned consonance, we also examined how ADP-dependent state 3 mitochondrial respiration, that in an earlier experiment proved to be NTPDase3-regulated, changes after the administration of E2 to OVX animals. Results clearly showed an increase in St3 MRR values in E2-treated animals, further implying that an E2-caused increase in NTPDase3-expression manifests in increased NTPDase3-activity.

The hypothalamus is the regulatory center of more homeostatic processes, such as reproduction and feed-intake. Accordingly, there is a functional distinction between the medial and lateral parts of the hypothalamus: The LHN hosts NTPDase3/orexin neurons that are the primary hypothalamic targets of the gastric 'hunger' hormone ghrelin, and these cells represent the major stimulatory input to the AN (in the medial part of the hypothalamus) neurons that are the effector cells of the initiation of feed-intake. Thus, neural circuits that directly participate in the regulation of feeding are present in both the medial and lateral parts of the hypothalamus, whereas, as far as current knowledge goes, the LHN is only indirectly involved in the regulation of GnRH-secretion. Estrogen, however, influences neuronal functions in both hypothalamic regions. Therefore, it seemed reasonable to examine and compare the effects of E2 and hunger on St3 separately and differentially 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 (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 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 E2 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.

V. 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 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 St3 ADP-dependent 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.

VI. References

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