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Metabolic asymmetry in the hypothalamic regulation of food-intake and reproductive processes in male and female rats.

PhD thesis

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0. List of abbreviations

Ad lib.	*ad libitum* feeding
ADP	adenosine diphosphate
AgRP	agouti gene-related protein (polypeptide)
AHA-POA	anterior hypothalamic area and preoptic area
AN	arcuate nucleus
AOX	alternative oxidases
ATP	adenosine triphosphate
CART	cocaine- and amphetamine-regulated transcript
Cast	castrated (male rats)
CNS	central nervous system
DE	diestrus
E	estrus
E2	17β-estradiol
EISP	estrogen-induced synaptic plasticity
EGTA	ethylene glycol tetraacetic acid
EP	early proestrus
ER	estrogen receptor
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FSH	follicle-stimulating hormone
GABA	gamma-aminobutyric acid
GnRH	gonadotropin-releasing hormone
GPER	G-protein coupled estrogen receptors
HPG axis	hypothalamic-pituitary-gonadal axis
LH	luteinizing hormone
LHT	lateral hypothalamus
LP	late proestrus
MCR3 and 4	melanocortin receptor 3 and 4

ME	metestrus
MSH	melanocyte-stimulating hormones
MPOA	medial preoptic area
mrr	mitochondrial respiration rate
NPY	neuropeptide Y
ovx	ovariectomized
POMC	proopiomelanocortin
PVN	paraventricular nucleus
ROS	reactive oxygen species
St1-5	mitochondrial respiratory state (type 1-5)
T	testosterone
UCP	uncoupling proteins in the mitochondria
VMH	ventromedial hypothalamus

1. Summary

The hypothalamus is the highest center and the main crossroad of numerous homeostatic regulatory pathways including reproduction and energy metabolism. Histologically, the left and right hypothalamic sides are symmetrical, still, it has been considered as an unpaired midline structure, in which the two sides regulate exactly the same biological functions. It has been known for higher, morphologically also symmetric brain areas that usually the left and right sides have distinct physiological roles providing a solution for the "ergonomic" use of brain resources. The main goal of this study was to investigate asymmetry in the hypothalamic functions such as the regulation of estrous cycle and food-intake.

In the hypothalamus, gender-specific functions seem to lie on the grounds of gender-indifferent anatomy. Therefore, it was reasonable to examine a general functional, instead of structural, parameter to test the possibility that the hypothalamus might, indeed, act in a lateralized manner in one or more of its functions. Such a parameter is the mitochondrial respiration. In order to clarify the above-mentioned goals, we investigated the metabolic asymmetry between the left and right hypothalamic sides of male and female rats by measuring mitochondrial respiration rates, a parameter that reflects the intensity of cell and tissue metabolism. In our first experiment, we used intact, normal cycling female rats sacrificed in different phases of the estrous cycle. In experiment 2 and 3, gonadectomized male and female animals were used. The effects of reproductive (estrous phase, ovariectomy, ovariectomy plus estrogen treatment) and hunger signals (*ad libitum* fed, fasted) were analyzed in various states of mitochondrial respiration.

Results revealed estrous phase-, estrogen- (females) and satiety state-dependent (males and females) metabolic differences between the two hypothalamic hemispheres of rats. It appears that in the regulation of female reproduction, predetermined, strongly estrogen-related sidedness exists with a right sided dominance. On the other hand, asymmetric, side-linked mechanisms seem to drive the feeding circuitries in males and females as well. A dynamic balance exists between the hypothalamic hemispheres in which both sides are able to react to orexigenic, as well as anorexigenic signals, but the left side dominates in orexigenic, while the right one in anorexigenic milieu. Furthermore, by comparing the metabolic profile of male and female hypothalami, we could also describe some fundamental gender-related differences showing that the hypothalamic lateralization in males is mostly related to food-intake, while in females, reproductive processes seem to have a higher impact on the hypothalamic asymmetry, at least under the present experimental conditions.

This study changes our current view on the regulation of female reproduction and food-intake, and provides new perspectives for the better understanding of these hypothalamus-driven physiological processes.

2. Introduction and literature overview

The hypothalamus, the highest center and main cross road of numerous reproductive and homeostatic regulating processes, is located at the basal part of the central nervous system (CNS). The properties through which the hypothalamus is able to orchestrate all these functions make it an anatomically “overcrowded” brain structure.

It has been known for higher brain areas that the two sides (hemispheres) usually have distinct physiological functions providing a solution for the “ergonomic” use of brain resources. Although the hypothalamus is also a morphologically symmetric brain structure, so far it has been considered as an unpaired midline structure in which the two sides regulate exactly the same biological functions.

2.1. Hypothalamic regulation of female reproduction

One of the most important, at the same time most complicated, role of the hypothalamus is the control of cyclic female reproductive functions. Pathways controlling female reproductive life converge to the left and right hypothalamic sides. Interestingly, both sides, like in the case of other hypothalamic regulatory processes, contain similar, like-named neuron populations that integrate the same peripheral and central reproductive signals. This structure renders the hypothalamus a morphologically symmetric brain area (figure 1).

The female reproduction is regulated via the so-called hypothalamic-pituitary-gonadal (HPG) axis that is the main effector of the control of cyclic follicular maturation. HPG can regulate its own activity through positive and negative feedback loops using humoral and neuronal factors, as well. The feedback signals and other metabolic factors affecting the reproduction converge to the gonadotrophin releasing hormone (GnRH) containing neurons in the hypothalamus. In rats, the vast majority of GnRH neurons are located in the medial preoptic area (MPOA) and in the medial septum (Merchenthaler et al., 1984; Malik et al., 1991), and project to the infundibulum (pituitary stalk), where GnRH is released to the portal circulation of the pituitary. After reaching the anterior lobe of the pituitary, GnRH regulates (alternating negative and positive feedbacks) the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), the two major hormones driving follicular growth and ovulation.

Estrogen (the most potent feedback signal of HPG produced by the ovaries) suppresses GnRH production (negative feedback) during most phases of the estrous cycle, and keeps GnRH and consequently the LH release on a relatively steady (pulsatile) basic level. Rising mid-cycle estrogen concentration during late proestrus (PE) turns the negative feedback to

positive feedback that accelerates GnRH production and secretion in the hypothalamus eventually leading to a GnRH peak followed by LH peak and the ovulation (GnRH-LH surge; Herbison, 1998).

Traditionally, estrogens (in rats mostly 17β-estradiol [E2]; Asarian and Geary, 2013) have been referred to as only reproductive hormones and, indeed, most of their direct actions are related to reproductive organs. Over the years, on the other hand, many other roles of E2 have been discovered, such as affecting food-intake and energy balance (see later); immune system (Cunningham and Gilkeson, 2011); neuroprotection (Bishop and Simpkins, 1994; Cordey et al., 2003); memory (Simpkins et al., 1997); regulating mitochondrial function (Klinge, 2008); *etc*. Estrogen is primarily synthesized in the developing follicles of the ovaries and influences the female reproductive system via nuclear estrogen receptors (ERs) and G-protein coupled estrogen receptors in the plasma membrane (GPERs). GnRH cells, according to the accepted view, do not express classical ERs, therefore all above of the afore-mentioned actions of E2 on GnRH production and the negative-positive feedback is likely mediated, at least in part, by other mechanisms. It seems that the E2 signal and other peripheral information are integrated and translated by a satellite system within the hypothalamus (figure 1) located in the anterior hypothalamic and preoptic areas (AHA-POA), and arcuate nucleus (AN). Major components of this satellite system include kisspeptin (AHA-POA, AN) and neuropeptide Y/agouti-related peptide neurons (AN) that project to GnRH cells (Li et al., 1994; Wojcik-Gladysz and Polkowska, 2006; Hrabovszky, 2014). These neuron populations are sensitive to metabolic factors and control feeding behavior, energy expenditure, therefore they might be a link between energy homeostasis and reproduction (Michalakis et al., 2013). On the other hand, recent studies suggest that GnRH neurons are directly responsive to E2, after all, due to a G-protein coupled membrane receptor (GPR30), and this signal might have a distinctive role in the transition of positive-negative feedback state of the cells (Lagrange et al., 1995; Noel et al., 2009), nevertheless the exact role of this specific E2 responsiveness, in the lack of results from specific inhibition studies, is not fully understood.

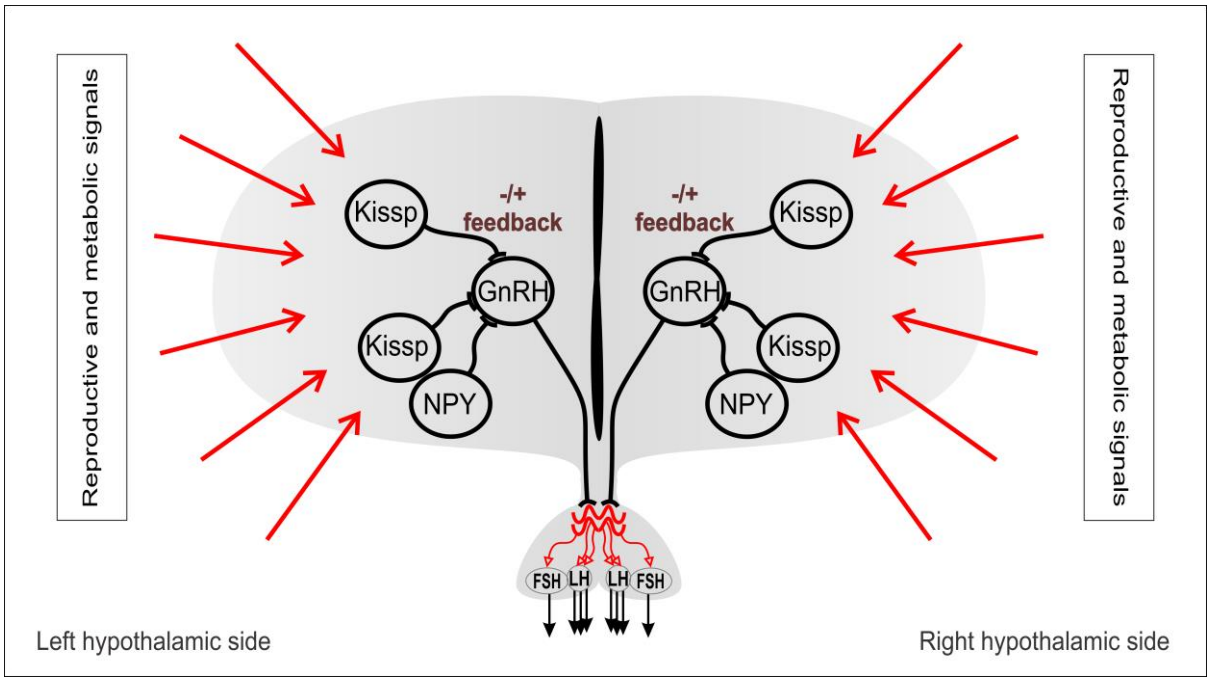

Figure 1: Schematic summary of the hypothalamic regulation of reproductive processes.

The neuroendocrine hypothalamus is a remarkable brain area in terms of the highly dynamic synaptic plasticity observed during the course of the female reproductive cycle. In a very short period of time, E2 can induce an extensive rearrangement of the microstructure to regulate the secretion and release of GnRH and, consequentially, LH release (estrogen-induced synaptic plasticity; EISP; Naftolin et al., 2007). Function and circuitry of the afore-mentioned neuron populations are eminently examined, and we possess a permanently growing body of knowledge about peripheral factors that act on these cells, about their intercellular connections and even about the orchestration of different tasks by means of their activity; however there is still only a paucity of studies (e.g. Vanetsian and Pavlova, 2004; Xavier et al., 2009, 2013; Cruz et al., 2014a, 2014b) that deal with the consequence of the paired/bilateral appearance of the hypothalamic networks in question.

It has to be noted here that the regulation of male reproductive processes are based on the very same morphological basis (HPG axis and GnRH neurons), but the main difference is that in males, due to the early masculinization of the brain, gonadal steroids have only negative feedback effects. Thus, in males the major consequence of these is the complete absence of the ability to respond to an estrogen surge with EISP in the hypothalamus and hence, with cycling in reproduction (Jin and Yang, 2014).

2.2. Hypothalamic regulation of food-intake and energy expenditure

The main neuronal populations regulating food-intake and energy homeostasis are located in the hypothalamus. Based on their mechanism of action, these neurons can be divided into two groups: orexigenic (increasing hunger and food-intake) and anorexigenic (decreasing hunger and food-intake). The orexigenic and anorexigenic neurons on both hypothalamic sides form complex circuitries, known as the melanocortin system (figure 2).

The arcuate nucleus (AN) in the mediobasal part of the hypothalamus is the main center and effector of the melanocortin system. The two major cell types in AN related to food-intake regulation are the anorexigenic proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) containing neurons together with the orexigenic neuropeptide Y (NPY) and agouti gene-related polypeptide (AgRP) co-expressing neurons. These neurons are able to sense peripheral homeostatic signals such as ghrelin and leptin directly as they express leptin and ghrelin receptors, as well (Baskin et al., 1999; Elias et al., 2000; Riediger et al., 2003; Holst and Schwartz, 2004). The AN is very close to fenestrated capillaries at the pituitary stalk, through which the metabolic and hormonal factors can easily reach the cells of the pituitary (Münzberg, 2008). On the other hand, AN neurons also receive mass projections from other brain areas, such as lateral and ventromedial hypothalamic nuclei (LHT, VMH; Elias et al., 1998; Sternson et al., 2005) that are also sensitive to peripheral satiety signals.

POMC is cleaved into melanocyte-stimulating hormone (MSH) that reaches the paraventricular nucleus (PVN) by axonal transport, and exerts its strong anorexigenic effect via melanocortin receptor 3 and 4 (MCR3 and 4; Fan et al., 1997; Biebermann et al., 2006). NPY/AgRP expressing neurons that co-express gamma-aminobutyric acid (GABA), a potent inhibitory neurotransmitter (Horvath et al., 1997), can interrupt the above-mentioned process and promote hunger by two separate mechanisms. Firstly, they send inhibitory (GABA-ergic) input to POMC cells within the AN; and they also decrease POMC effect at the action-site by antagonizing MSH action on MC receptors in the PVN (Horvath, 2005). This tonic inhibition of satiety signals promotes hunger and results in food-search and food-intake related behavior via extra-hypothalamic neuronal interactions. A very important aspect of this mechanism is that there is no projection from POMC cells to the NPY/AgRP cells, i.e. no direct feedback or inhibition exists. From an evolutionary point of view, this suggests that orexigenic signals are preferred over anorexigenic: this way the animal can eat not only if hungry but also if food is available. In nature, this histological connection was proven to be extremely beneficial, but, on the other hand, this might be one of the main reasons of obesity in modern, progressive human societies.

There are other hypothalamic areas related to the regulation of food-intake mostly by altering the activity of AN. One of the most important brain areas is the lateral hypothalamus that includes hypocretin (orexin) and melanin-concentrating hormone (MCH) containing neurons. These neurons are also key players of the melanocortin system described above, since they are able to integrate the peripheral signals and send and receive strong projections to/from AN and other brain parts (Hakansson et al., 1998; Peyron et al., 1998; Horvath et al., 1999; Zigman et al., 2006). Due to the known reciprocal innervation, the hierarchy between AN and LHT is still controversial, but according to the most recent hypotheses, the AN is considered to be the center of melanocortin system, and it receives its most important hypothalamic input from the LHT.

The activity of orexigenic and anorexigenic neurons in the afore-mentioned circuitries will result in either satiety or food-intake. Besides the peripheral signals reporting about the actual energy-status of the body, this mechanism is strongly influenced by the constantly changing neuronal inputs (synaptic remodeling) of the cells (Horvath and Diano, 2004; Pinto et al., 2004; Horvath and Gao, 2005; Sternson et al., 2005). Furthermore, recent studies also indicated the role of glial cells that, besides filtering the peripheral signals, are able to influence neuronal activity (Kim et al., 2014; Wang et al., 2015; Yang et al., 2015).

Besides regulating food-intake and energy homeostasis, the afore-mentioned neurons are also involved in several other biological mechanisms indicating the complexity of the neuroendocrine hypothalamus. For example, hypocretin neurons are critical in the regulation of sleep-awake cycles (Taheri et al., 2002), arousal, and are also involved in memory and motivation (Harris et al., 2005); while the role of AgRP neurons in stereotypic behaviors (e.g. anxiety; Dietrich et al., 2015), arousal (Yamanaka et al., 2003) and reproductive processes (Li et al., 2013) has also been described.

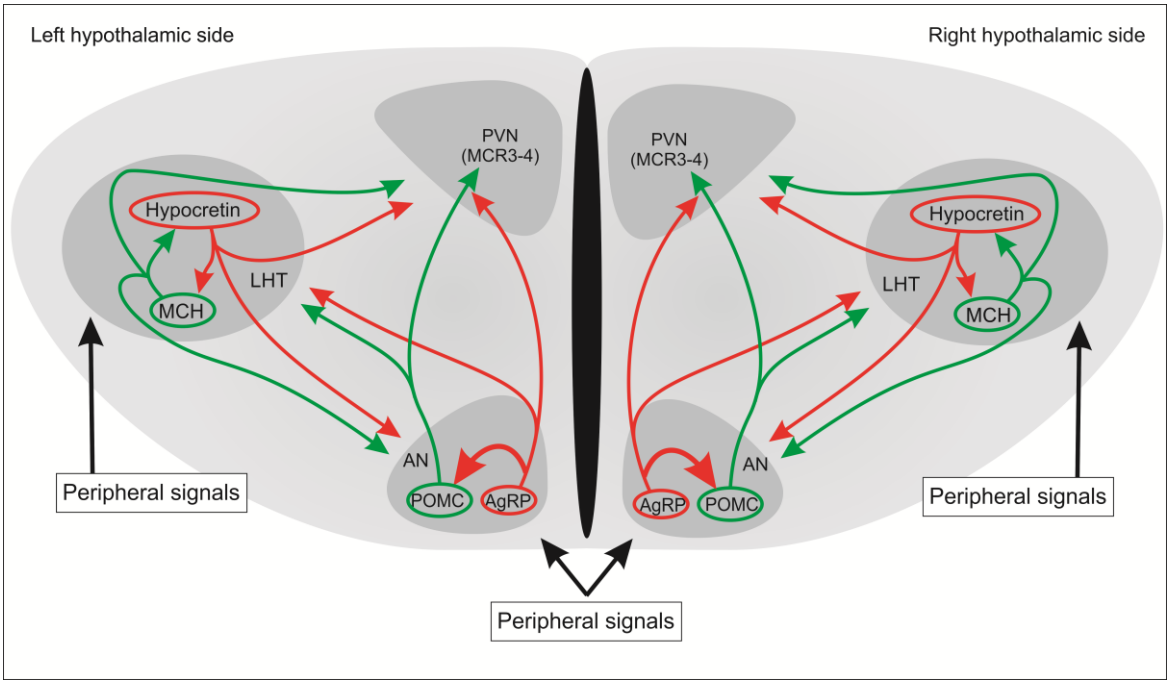

Figure 2: Summary of connections within the hypothalamus regulating food-intake and energy balance (melanocortin system). *Red arrows: orexigenic signals; green arrows: anorexigenic signals; AN: arcuate nucleus; LHT: lateral hypothalamus; PVN: paraventricular nucleus.*

Among many peripheral factors contributing to the hypothalamic regulation of food-intake and energy homeostasis, the most important hormones are the ghrelin and leptin, although many other humoral factors (e.g. estrogen, thyroid hormones, insulin, catecholamines, *etc*.) influence, directly or indirectly, the energy metabolism (Somogyi et al., 2011).

Ghrelin is an orexigenic peptide hormone described by Kojima et al. (1999). It is mostly produced by the stomach, although the hypothalamus, hypophysis, kidney and the intestines also contribute to maintain the actual level of circulating ghrelin. The major stimulus in ghrelin secretion is fasting, but the secretion pattern also depends on the ingredients of the meal (Tschop et al., 2001; le Roux et al., 2005). Besides its role in the regulation of neural circuits that underlie food-searching behaviors and feeding (Sun et al., 2007; Grouselle et al., 2008; Campa et al., 2010), ghrelin affects energy homeostasis by modulating a number of additional physiological processes, such as growth hormone production, cardiovascular functions, sleep–wake behavior, and thermogenesis (Holst et al., 2004; Broglio et al., 2005; Sun et al., 2007; Motivala et al., 2009).

Leptin (leptos [Greek]: ‘thin’) has an antagonistic role on the melanocortin system: it is a satiety hormone that inhibits excessive deposition of lipids in various tissues (Chilliard et al., 2005). In contrast to ghrelin that fluctuates in relation to food-intake, leptin has a relatively steady level in the circulation that correlates with the body fat mass: it is increased in obesity,

while decreased after fasting. Ghrelin and leptin are physiologically antagonistic hormones, and their function is closely interrelated. For example, enhanced leptin secretion has been described after prolonged ghrelin exposure, while moderate hyperleptinemia prevents the increase of plasma ghrelin during short-term energy restriction (Barazzoni et al., 2003; Giovambattista et al., 2006).

2.3. Sex differences in the hypothalamic regulation of food-intake

Numerous studies have indicated gender-related differences in the central regulation of food-intake and energy balance. These differences are due to 1) divergent development of the CNS and 2) different hormonal profiles.

1) Developmental differences of male and female rats. According to our current knowledge, gender-related differences in rats (as well as in humans) are due to the time-delayed secretion of gonadal steroid in early developmental phase (organizational effects). In male rats, androgens (mainly testosterone; T) are released into the circulation in embryonic life and early postnatal period, and it is responsible for male characteristics in brain development (masculinization; Scott et al., 2009). In female rats, however, such stimuli of circulating testosterone are absent, and on the other hand, effects of maternal estrogens are blocked by α-fetoprotein molecules. In lack of significant amount of gonadal steroids, female brain development goes on its way until α-fetoprotein levels drops from week 2 of postnatal life, and first E2-related changes start to evolve (Bakker et al., 2006). It is important to note that although the level of circulating gonadal steroids is considerably low, the locally synthetized estrogen (by aromatization of androgens or *de novo* synthetized) still have a significant role on brain development in both sexes (McCarthy, 2008). Besides the organizational effects that are responsible for long-term changes during development, gonadal steroids also have so-called activational effects to make short-term, also reversible alterations in adult life. These changes are also gender-specific and often require the properly developed organizational effects (Asarian and Geary, 2013).

The afore-mentioned differences finally lead to the well-known differences in eating and energy homeostasis between genders: e.g. male and female rats have a different “dietary” pattern (males eat longer but in a same frequency as female littermates; Funabashi et al., 2009); fat deposits of males are located mainly in the abdominal cavity, while in females subcutaneously (Lemieux et al., 1993); in females, there is an estrous-related cycle in eating (Lyons et al., 1989; Chen et al., 1995). Although the underlying organizational and activational effects of the gonadal steroids need to be further elucidated, the outcome of different hormonal profiles and interactions of gonadal steroids with other food-intake related hormones are very well studied.

2) Differences in hormonal profiles. In rats, the most important gonadal steroids affecting food-intake and energy homeostasis are 17β-estradiol (females) and testosterone (males). Male and female rats synthetize T as well as E2, and both steroids have a role in food-intake regulation in each gender; however the auxiliary hormone is mostly produced locally, and its effect is exerted therein before entering the circulation (Luu-The and Labrie, 2010).

Estrogen. E2 in female animals is cyclically produced by the developing and maturing follicles, while in males, it is generated mostly by Sertoli cells of the testes, muscles, and adipose tissue (Longcope et al., 1978; Sharpe, 1998). E2, by targeting estrogen responsive hypothalamic neurons, modulates the function of NPY/AgRP and POMC neurons, thus it plays a significant shaping role in the overall functions of the melanocortin system in order to exert its anorexigenic effect (Horvath, 2005; Gao and Horvath, 2008). Studies on knock-out mice demonstrated that ERα is more important in the mediation of the afore-mentioned anorexigenic effect of E2 than ERβ (Geary, 2001; Musatov et al., 2007). Ovariectomized rats are known to eat significantly more that can be reversed by E2 substitution (Wade, 1972). This phenomenon further strengthens the role of E2 in the regulation of food-intake and energy balance, but it does not answer the question whether it is a direct effect or E2 can modulate other hormonal signals (or both). Several studies, therefore, have been designed to answer that question, and investigated the interactions between E2 and one or more food-intake related peripheral factors. The E2-leptin and E2-ghrelin interactions are particularly interesting and deserve more attention since these are the most prominent factors in the regulation of food-intake (Somogyi et al., 2011).

E2 and leptin have similar effects on food-intake and energy homeostasis (i.e. limiting food-intake). An interesting phenomenon is that plasma leptin levels are higher in women than in men (even before puberty); and better correlate to body fat in females than males (Clegg et al., 2003). Furthermore Clegg et al. (2006), and Meli et al. (2004) also indicated that E2 modulates leptin sensitivity of hypothalamic centers. These results suggest that E2 influences leptin pathways peripherally as well as centrally in a very complex manner.

E2 and ghrelin are antagonistic in terms of food-intake regulations. Matsubara et al. (2004) found that after ovariectomy, gastric production of ghrelin have been increased that could be reversed by E2 injection. Sakata et al. (2006) further investigating the E2-ghrelin interaction found that gastric ghrelin producing cells are more responsive to locally produced E2 than the circulating E2 originated from the ovaries. Their finding is consonant with other studies on human subjects that could not indicate any significant interaction between ovarian hormones and ghrelin levels (Dafopoulos et al., 2010). Although E2 effects on peripheral ghrelin production is still questionable, much clearer results are available indicating central

interaction between the two hormones. A complex study by Clegg et al. (2007) using rats and mice proved that E2 is able to dim the orexigenic effect of ghrelin centrally, although they also claim that this effect extends to the peripheral ghrelin production, as well.

Androgens. Following orchiectomy (lack of testicular testosterone), the animals eat significantly less; this results in less food ingestion that can be reversed by T injections (Chai et al., 1999). This fundamentally different outcome of gonadectomy suggests that testosterone acts in a completely different manner than E2 does. Unfortunately, in this respect the mechanism of T action is less extensively examined than that of E2. One of the differences between males and females is that the CNS of females is more sensitive to leptin (see earlier), while in males, body fat and catabolic signals correlate much more with insulin than leptin (Clegg et al., 2006). This suggest that leptin-testosterone interactions are of lesser importance in males, however, testosterone may exert some mild central effect on leptin actions (Fan et al., 2008). On the other hand, ghrelin-T interactions seem to be more significant. For instance, it has been identified that hypogonadal men (with low T levels) also produce less ghrelin compared to healthy control subjects, furthermore this low-level ghrelin returns into the normal range upon T substitution (Pagotto et al., 2003). Also, a clear interaction has been reported by Tena-Sempere et al. (2013) as they found that ghrelin is produced in the testes, where it locally inhibits testosterone secretion. These results suggest without a doubt that ghrelin is not only a “hunger-hormone”, but also regulates the reproductive processes (at least in males); this interaction, however, has not yet been linked to the regulation of food-intake and energy balance.

Other hormones involved in the regulation of food-intake. For the sake of completeness, it should be mentioned that gender-related differences of other metabolic factors have also been indicated such as cholecystokinin, glucagon, insulin, metabolic signals (see more: Asarian and Geary, 2013). Detailed summary of these factors, however, are beyond the scope of this work.

2.4. Hypothalamic asymmetry

It has been established for some time that the left and right sides of the nervous system are specialized to the regulation of certain specific, but distinct functions. Besides the well-known assignment of the two sides of the spinal cord in the regulation and/or mediation of left or right locomotor and autonomous functions, the functional lateralization of upper brain regions has also been recognized. For example, the functional asymmetry of the two cerebral hemispheres ensures the optimal integration of different cognitive processes, such as speech, spatial relation, fine motoric movements of hands, *etc*. Also, a considerable number of studies have found asymmetry in other brain areas such as the hippocampus, habenula, and thalamus (reviewed by Harris et al., 1996; Aizawa, 2013; Hou et al., 2013).

Asymmetry of the neuroendocrine hypothalamus has also been indicated. For example, Cruz et al. (1989) and Lopez et al. (1997), found that effects of unilateral atropine (acetylcholine antagonist) and pilocarpine (acetylcholine agonist) implants into the preoptic-anterior hypothalamic areas were not only estrous phase-dependent but side-dependent as well. Even earlier, Gerendai et al. (1978) found significantly more GnRH in one side of the hypothalamus than in the other. In line with those findings, experimental manipulation of the right side of the hypothalamus-gonad axis proved to be significantly more efficient than that of the left (Nance et al., 1983; Fukuda et al., 1984). In line with these, Glick et al. (1979) provided evidence that there is more metabolic activity on the right side of the rat hypothalamus, and since E2 also modulates mitochondrial activity in the hypothalamus (Kiss et al., 2009), it is reasonable to assume that asymmetry in metabolic changes linked to reproductive functions is, at least in part, regulated by E2.

The hypothalamic regulation of reproduction and feeding is, on the one hand, based on distinct hypothalamic morphological and biochemical bases, on the other hand, however, there is a well-known overlap between the afore-mentioned two regulatory circuits in the form of neuron populations and hormones involved in the regulation of both functions. Specifically, E2, besides coordinating female reproduction, also plays a key role in the regulation of appetite and energy expenditure as an anorexigenic factor (Ainslie et al., 2001; Clegg et al., 2007; Santollo and Eckel, 2008). Together with the existence of unilateral feeding pathways that connect hypothalamic structures to brain areas with asymmetric functions (Mittleman et al., 1985; Vanetsian and Pavlova, 2004; Grundmann et al., 2005), this double role of E2 suggests that the hypothalamic regulation of food-intake may be just as lateralized as the regulation of reproductive functions.

Besides the regulation of reproduction and food-intake in females, asymmetry of other hypothalamic functions has also been indicated. For example, in male animals, Bakalkin et al. (1984) described left and right differences of GnRH levels in Wistar rats, while Inase and Machida (1992) found side-dependent changes after unilateral orchiectomy in mice. Furthermore, there are data about the asymmetry in the hypothalamic regulation of the cardiovascular system (Xavier et al., 2009, 2013); asymmetric distribution of thyroid-releasing hormone (Borson-Chazot et al., 1986); side-linked regulation of the circadian rhythm in the suprachiasmatic nucleus (Zhang and Aguilar-Roblero, 1995; de la Iglesia et al., 2000, 2003); and lateralized functions have also been found in the central regulation of the immune system (Betancur, 1991; Delrue et al., 1994). These results further strengthen the idea of a complex mechanism in the hypothalamus, by which the two sides regulate the abundance of functions that are crammed into this relatively small brain area.

2.5. Energetics of hypothalamic functions

Hypothalamus-driven homeostatic functions are considerably energy-dependent and therefore rely on mitochondrial ATP-production (Laughlin et al., 1998): mitochondrial ATP production is crucial in the supply of the hypothalamic energy needs and plays a permissive role in the regulation of the intensity of all energy consuming cellular processes.

Mitochondria are cell organelles that consist of two membrane layers: the outer membrane is similar to any other membranes in eukaryotic cells, the inner layer, on the other hand, contains higher amount of proteins than the outer. These proteins build up the so-called respiratory complexes that are responsible for energy production through several steps (see figure 3). Complex I, III and IV are proton (H^+) pumps that are able to develop an electrochemical proton gradient between the two sides of the inner mitochondrial membrane using the energy derived from the electron transport. Complex V is called ATP synthase that consists of two subunits: F0 and F1. F0 part is embedded into the inner membrane layer and it can let the protons into the mitochondrial matrix via a regulated process. This proton influx releases energy that drives the F1 subunit that resynthesizes ATP from ADP molecules using the energy freed up by the proton flow. The ATP then is used in energy-dependent cellular processes (Boyer, 1998).

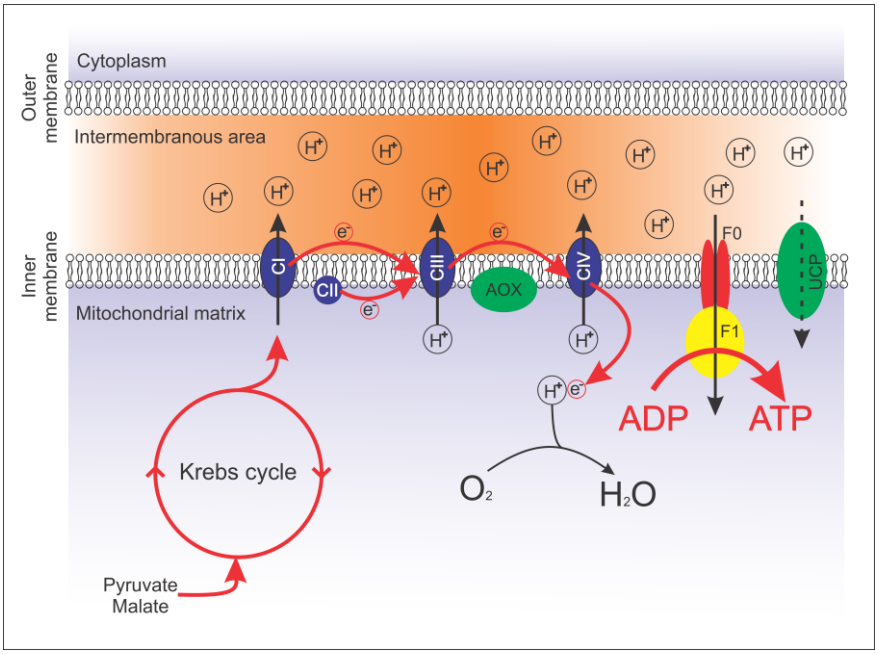

Figure 3: Schematic figure of mitochondrial ATP production. *CI-IV: Complex I, II, III and IV; F0+F1: subunits of complex V (ATP synthase); AOX: alternative oxidases; UCP: uncoupling proteins.*

The mechanism described above is strictly regulated by the actual need of the neuron (Ames, 2000; Kann and Kovacs, 2007). Several regulatory mechanisms have been discovered yet: the main regulator is the amount of the available substrates (pyruvate, NADH, ADP; the more substrate available, the faster is the energy-producing biochemical process). On the other hand, there are other regulative mechanisms: alternative oxidases (AOX), ecto-nucleoside triphosphate-diphosphohydrolases (NTPDases) enzymes, and uncoupling proteins (UCP). AOXs are located in the inner membrane to prevent the overproduction of the electrochemical gradient, i.e., the 'overcharging' of the mitochondrion. NTPDase 3 enzyme has been described in mitochondria of the neuroendocrine hypothalamus, and it is suggested that it may have an E2-dependent regulatory role in stimulatory neuronal functions such as neurotransmission (Kiss et al., 2009). UCPs are described as means for an alternative way for protons to enter into the mitochondrial matrix avoiding complex V and ATP production; this mechanism also prevents overcharging (Rousset et al., 2004). In addition, it has been shown that E2 can increase the expression of the F1 subunit of the ATP synthase enzyme (Nilsen et al., 2007), a mechanism by which the mitochondrial activity can be further fine-tuned.

The regulated mitochondrial respiration correlating with the actual cellular energy consumption offers the method of measuring mitochondrial respiration rates (*mrr*) to directly indicate the intensity (and changes in intensity) of overall functions in hypothalamic regions that are involved in the regulation of homeostatic processes. In line with this, our research group has been investigating cellular mechanisms that fine-tune the ATP-production dynamically following the actual energy needs (Kiss et al., 2009).

3. Significance and aims of the study

In our modern life, more and more people are affected by some kind of reproductive or metabolic disorder such as polycystic ovary syndrome, infertility, obesity or anorexia nervosa. These problems are usually multifactorial: various etiologies can lead to the same disorder, and sometimes seemingly the same cause results in different manifestations. Moreover, it has been known that the hypothalamic regulation of reproduction and feeding overlaps on many levels, therefore problems of metabolic origins could manifest in reproductive disorder, and *vice versa*. For instance, polycystic ovarian syndrome is a female reproductive disorder, however, in mild cases, the patients' condition can be significantly improved by low-calorie diet combined with exercise.

The hypothalamus, as the main integrator of peripheral and central reproductive and metabolic signals, plays a crucial role in keeping the physiological homeostasis that, if disturbed, results in the above diseases. Therefore numerous research groups work on deciphering hypothalamic functions, and as a result, a large amount of data is now available; still, the exact mechanisms, through which this relatively small brain area can regulate such an abundance of homeostatic processes, represent a "grey spot" for modern day researchers.

According to our hypothesis, in the morphologically symmetric hypothalamus, the like-named nuclei on the left and right sides might have different roles, a phenomenon that has long been accepted in case of higher brain areas (cortex, thalamus). The dominance of one of the hypothalamic sides over the other, in our view, could provide a much more precise and adequate adjustment to the peripheral signals by determining one single set-point in the regulation, furthermore, it could provide a solution for a more "ergonomic" use of hypothalamic resources.

In summary, this study aims to establish new perspectives, improved experimental designs, and a better understanding of these hypothalamus driven physiological processes. We believe that the understanding of the nature and exact role of asymmetry of pathways and mechanisms in the neuroendocrine hypothalamus will lead to more potent therapeutic solutions in hypothalamus-linked health conditions.

In details, the main goals of the study are to investigate the estrous cycle-related hypothalamic asymmetry of female rats, and to extend our knowledge to the regulation of food-intake and energy metabolism. We are also interested in whether hypothalami of male rats are showing similar asymmetric metabolic changes in the regulation of reproduction and food-intake. Finally, having results from male and female animals, we compare those to each other to highlight the major gender-related differences in mitochondrial differences.

Ad1

To investigate whether there is a detectable metabolic asymmetry in the hypothalamic regulation of female reproduction (1).

Ad2-3

If yes, to investigate whether the hypothalamic asymmetry related to female reproduction is E2-related (2), and whether this asymmetry also applies to those functions that are partly or entirely involved in the hypothalamic regulation of feeding (3)**.**

Ad4-5

To investigate whether there is an asymmetry in the hypothalamic regulation of reproduction (4) and food-intake (5) in male rats.

Ad6-7

To analyze the differences in the hypothalamic regulation of reproduction and food-intake between male and female rats (6), and investigate gender-related energetics of hypothalamic mitochondria (7).

4. Materials and methods

4.1. Animals

Intact and gonadectomized, Wistar rats (*Rattus norvegicus*, breed: Crl:[WI]BR) were used to examine the effects of satiety states and presence or absence of gonadal steroids. The studies were conducted at the Faculty of Veterinary Science (Szent Istvan University, Hungary) in accordance with the Directive 2010/63/EU, and was approved by the Animal Health and Animal Welfare Directorate of the National Food Chain Safety Office (Permit Number: XIV-I-001/2202-4/2012).

The animals were obtained at least five weeks before the experiments (vendor: Semmelweis University, Basic Medical Science Center; Budapest, Hungary), and were kept in groups in controlled light (12-hour-long dark and light cycles; lights on at 7 a.m.). Regular rat chow (vendor: FarmerMix Kft., Zsambek, Hungary), and tap water were *ad libitum* available.

Experiment 1: Examination of hypothalamic lateralization in intact female rats

In experiment 1, we examined normal cycling female rats. The estrous phase of animals was determined immediately after sacrifice by vaginal smears in order to avoid hormonal influence caused by mechanical stimuli of the cervix. The sacrificed animals were chosen based on their previously known estrous phase and actual behavior. The number of animals in each estrous phase is shown in table 1.

The vaginal smears were evaluated using the following principles:

Early proestrus (EP): many epithelial cells + few cornified cells

Late proestrus (LP): many epithelial cells + many cornified cells

Estrus (E): many cornified cells with or without epithelial cells

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

Diestrus (DE): few leucocytes + few epithelial cells with or without few cornified cells

Table 1: Number of animals in each estrous phase (intact females).

Estrous phase	Early proestrus	Late proestrus	Estrus	Metestrus	Diestrus
Animal number	13	8	9	24	12

Experiment 2: Examination of the role of E2- and satiety state-dependent lateralization in female rats

Surgery and treatment of animals. Seven-week-old animals were gonadectomized 3 weeks before the experiments under standard anesthesia of ketamine + xylazine combination (75 mg/kg ketamine, 0.2 mg/animal xylazine, intramuscularly). The surgery was performed through an approximately 2 cm long median incision at the back of the animal. Through the wound, the layers of the abdominal wall on the left and right sides were separated above the anatomical localization of the ovaries. After finding and ligating the blood vessels entering the gonads, the left and right ovaries were removed. Following the insertion of skin sutures, butorphanol (2.0 mg/kg) was administered for postoperative analgesia together with subcutaneous saline infusion. After gonadectomy, and in the experimental period, the animals were kept in groups of two. Before the experiment, the animals were randomly separated into two groups, one of them remained *ad libitum* fed, while the other was fasted for 24 hours before sacrifice (quick guillotine decapitation at 7:00 a.m.) with constant water supply.

Gonadectomized females were further divided to estrogen injected (17β-estradiol [E2], single dose of 23μg/100g body weight [BW], Sigma Aldrich Ltd., Hungary, water soluble), or sham injected (saline; S) subgroups. Since the most prominent effect on mitochondrial metabolism in the hypothalamus was registered between 8-10 hours after E2 treatment (Kiss et al., 2009), subcutaneous injections were performed 10 hours before sacrifice (9 p.m. on the previous day). For estrogen substitution after *ovx*, water soluble E2 is suggested due to the fact that it does not need specific binding protein for transportation (Stratton et al., 2010).

In summary (also in table 2), the experimental groups used are: E2 treated *ad libitum* fed females (E2+*ad lib.*; n=5), E2 treated fasted females (E2+fasted; n=6), sham injected *ad libitum* fed females (S+*ad lib.*; n=5), and sham injected fasted females (S+fasted; n=4).

Table 2: Number of animals in each experimental groups of female animals.

	***Ad libitum* fed**	**Fasted for 24 hours**
Presence of gonadal steroid	5	6
Absence of gonadal steroid	5	4

Experiment 3: Examination of the role of testosterone- and satiety state-dependent lateralization in male rats

Surgery and treatment of animals. Seven-week-old animals were gonadectomized 3 weeks before the experiments under standard anesthesia of ketamine + xylazine combination (75 mg/kg ketamine, 0.2 mg/animal xylazine, subcutaneously). The testes were removed through a single incision of the scrotum. After closing the wound, butorphanol (2.0 mg/kg) was administered for postoperative analgesia together with subcutaneous saline infusion. After gonadectomy, and in the experimental period, the animals were kept in groups of two. Before the experiment, the animals were randomly separated into two groups, one of them remained *ad libitum* fed, while the other was fasted for 24 hours before sacrifice (quick guillotine decapitation at 7:00 a.m.) with constant water supply.

Groups of experiment 3 are: intact *ad libitum* fed males (T+*ad lib.;* n=7); intact fasted fed males (T+*fasted;* n=7); castrated *ad libitum* fed males (cast.+*ad lib.;* n=7); castrated fasted fed males (cast.+fasted*;* n=6). Animal numbers in each experimental groups are also summarized in table 3.

Table 3: Number of animals in each experimental groups of male animals.

	***Ad libitum* fed**	**Fasted for 24 hours**
Presence of gonadal steroid	7	7
Absence of gonadal steroid	7	6

4.2. Preparation of brain synaptosomal and perikaryal mitochondria and measurement of oxygen consumption

Mitochondrial fractions (containing both perikaryal and synaptosomal mitochondria) were obtained from the separated left and right hypothalamic sides (also termed as hemispheres), then mitochondrial oxygen-consumption was measured. The experimental design of isolating and measuring mitochondria is also summarized in figure 4 and 5. The necessary reagents and equipment for the experiments are shown in appendix.

1. Dissection and tissue homogenization

After quick guillotine decapitation, the hypothalamus was extracted in ice-cold environment as follows. After removing the skin and muscles, the skull was opened as described earlier (Grossmann et al., 2013), and the cranial part of the brain was slightly lifted with a cold spatula in order to cut the optic nerve. After cutting, the brain was gently removed, and placed into an ice-cold brain matrix. The connecting tissue from the basal part of the hypothalamus was removed with a fine forceps. Then, vertical incisions were made using ice-cold blades, for a coronal section of the entire hypothalamus: an incision right behind the rostral part of the *chiasma opticum* (Bregma -0.25), and another one through the *corpus mamillare* (Bregma -5.0). The coronal sections were placed on the rostral surface, and the *piriform* and *entorhinal cortices*, then the thalamic area dorsal to the *fornices* were cut off. Finally, the hypothalamus was cut into left and right sides along the 3rd ventricle. The tissue blocks (30-35mg) were put into 750µl ice-cold isolation buffer, and stored until homogenization. Dissected brain samples were placed and further processed in ice-cold buffer starting from approximately 30 seconds after the decapitation.

The homogenization was performed in a motorized teflon-on-glass tissue homogenizer (Potter-Elvehjem, 600-800rpm) by moving the glass tube firmly up and down. After the homogenization, all buffer and foam was recollected and put into a 1.5ml Eppendorf tube. The homogenate was kept on ice until all other tissue samples were homogenized. Between the samples, the homogenizer was cleaned with isolation buffer.

2. Fractionation procedure

All fractionation steps were carried out on 4ºC. A summary of the procedure is also shown on figure 4 for better understanding.

Preparing crude mitochondrial fraction from brain tissue. Homogenized samples were spun at 1300rcf (3700rpm) for 4 minutes. The supernatant was collected in an empty Eppendorf tube, while the pellet was resuspended in 750µl isolation buffer (with EGTA), then it was spun again with the same settings in order to release mitochondria from large cell debris. After the second spin, the supernatant was put together with the former supernatant collected from the first centrifugation step, and the pellet was discarded. Next, the two supernatants collected in one tube were spun together at 13000rcf (11800rpm) for 11 minutes. The mitochondria-containing pellet was saved and resuspended in 500µl isolation buffer. This stage is called “crude mitochondrial fraction” that still contains contaminating particles (cell organelles, myelin, cell debris, *etc.*).

Percoll gradient fractionation procedure. For further purification, we used a simplified discontinuous Percoll gradient that merely consists of a 15% and a 0% Percoll layer (filtered Percoll stock solution is diluted to 15% with isolation buffer). Using a gradient centrifugation step, mitochondria and synaptosomes were separated from other, non-useful elements. The crude mitochondrial fraction was layered on 500µl of 15% Percoll solution in a special “Percoll tube” (2ml, conical shape). The Percoll gradient containing tubes were gently put into the centrifuge and spun at 22000rcf (15400rpm) for 7 minutes 40 seconds. In order to save the layers during the centrifugation, we used the lowest possible acceleration and the break was turned off. The two layers at the bottom (somal and synaptosomal mitochondria) were collected before the last steps, while the top layer (cell membrane and myelin debris) was discarded. Percoll, although considered as a harmless compound, was cleared off the sample. In order to obtain uninjured, coupled, viable mitochondria, the following cleaning steps were used before the final utilization: entirely filled tubes (of the resuspended sample) were spun at 22000rcf (15400rpm) for 11 minutes (full acceleration and break). After the centrifugation, the supernatant was carefully poured off. As the last step, the remaining, minimal amount of Percoll and the EGTA was removed by diluting it with 1ml of isolation buffer without EGTA. The tubes were centrifuged at 13000rcf (11800rpm) for 11 minutes, and the samples were stored as pellet in isolation buffer (without EGTA) on 4°C until the measurements.

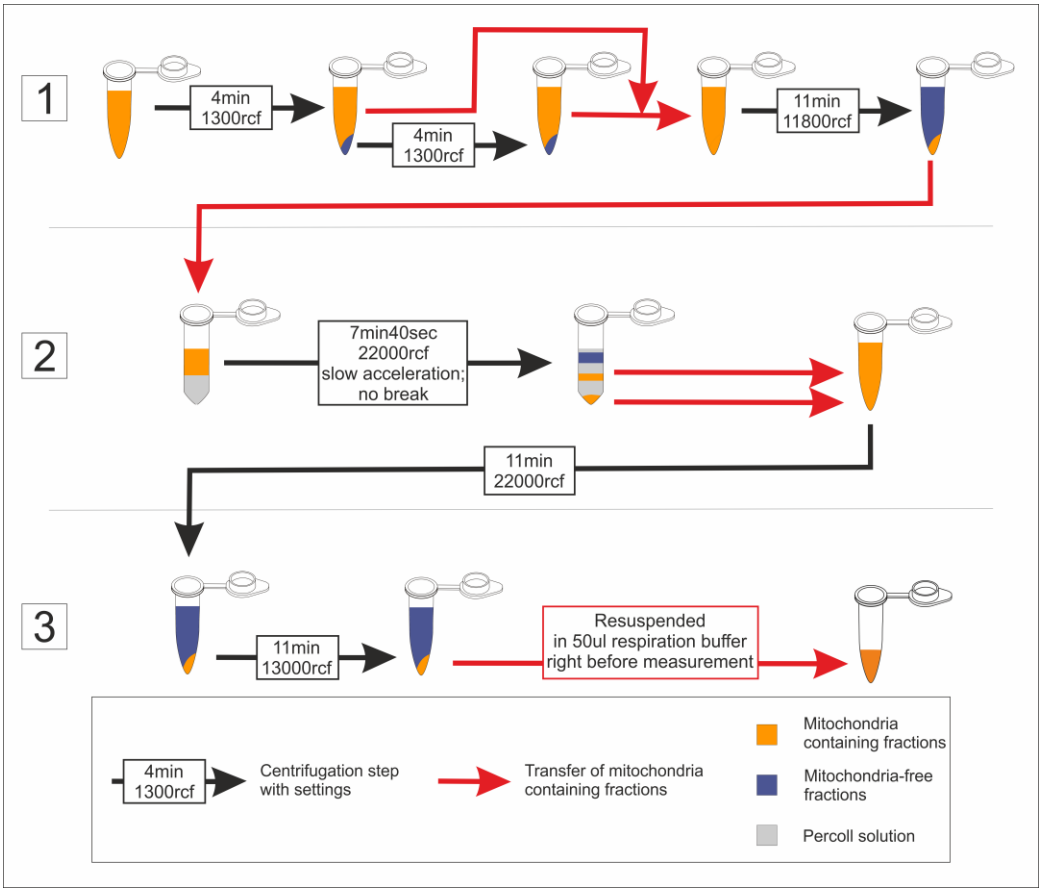

Figure 4: Fractionation protocol for synaptosomal and non-synaptosomal mitochondria

1) Preparation of so called 'crude mitochondrial fraction' 2) Purification by Percoll gradient 3) Clearing off the Percoll from the mitochondrial samples.

3. Mitochondrial respiration rate measurements

The mitochondria-containing fraction was transferred into respiration buffer and put into a Clark-type oxygen electrode chamber (Hansatech Instruments, Norfolk, UK) to measure mitochondrial respiration on 37°C. The electrode groove was filled with potassium chloride to form the electrode bridge between cathode and anode. Calibration was fulfilled by air saturated, deionized distilled water in order to establish the air line, while sodium dithionite for zero oxygen line. We measured the oxygen consumption by consecutively adding 5µl pyruvate together with 2.5µl malate, 2.5µl ADP, 1µl oligomycin and 2.5µl carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone to 50µl of resuspended samples diluted with 450µl respiration buffer in the electrode chamber.

The oxygen consumption was measured real time, and the results are expressed as consumed oxygen per minute (nmol O_2/ml). Five stages (each measured for 60 seconds) were distinguished according to the subsequently added respiration modifiers (Brand and Nicholls, 2011). Since the terminology of the mitochondrial respiration states varies in the relevant literature, below we define the 5 states that we measured and evaluated.

State 1 (St1): mitochondrial oxygen consumption in respiration buffer only, without the addition of any substrates that may affect mitochondrial activity. The measured *mrr* depends on the actual metabolic state of the mitochondria.

State 2 (St2): Mitochondrial function in the presence of oxidative substrates (pyruvate and malate in a final concentration of 5mM and 2.5mM, respectively) of the Krebs' cycle, but in lack of added substrate for the ATP synthase (Clark and Nicklas, 1970). Under such conditions, the Krebs' cycle intensifies and oxygen consumption increases due to consequential facilitation of the terminal oxidation and oxidative phosphorylation if the down-regulating mechanisms are not active. Mitochondrial respiratory rate measured in St2 is limited by the amount of ADP present in the mitochondria at the time of sacrifice.

State 3 (St3): State 3 is initiated by adding ADP in a final concentration of 130µM. Being the substrate for ATP synthase, ADP is a major up-regulator of mitochondrial respiration. Under such conditions, *mrr* increases if prior fuel supply of the hypothalamic tissue was sufficient. Therefore, if excess amount of ADP is added to the sample (Krebs' cycle is already fueled up), *oxidative phosphorylation* is limited exclusively by the activity of ATP synthase.

State 4 (St4): In state 4, oligomycin (2.5µM in the final concentration) is used to block the ATP synthase activity, therefore the oxidative phosphorylation, however the steps of terminal oxidation continues (Dennis and Clark, 1978). Under such conditions, oxygen consumption depends on the actual uncoupled stage and the activity of alternative oxidases of the mitochondria. Under physiological conditions, uncoupling and alternative oxidation play important roles in transient down-regulation of ATP biosynthesis when cellular energy needs drop. In case of fully viable mitochondria, oligomycin results in remarkably reduced *mrr* compared to that observed in state 3. It is to note that improper purification of the sample may easily lead to elevated state 4 *mrr*, and thereby renders these data unreliable.

State 5 (St5): At last, FCCP [carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone] is added to the sample in a final concentration of 5µM. FCCP is a cyanide derivative, therefore, by binding to and blocking cytochrome C oxidase, depletes all remaining oxygen from the sample (also acts as uncoupler; Kalckar et al., 1979; Villa, 1981). Decrease of oxygen level under such conditions depends on the initial (*in vivo*) metabolic state of the sampled tissue, and the amount of oxygen consumed during states 1-4 respiration. Therefore, this experimental setup is also known as total mitochondrial respiratory capacity.

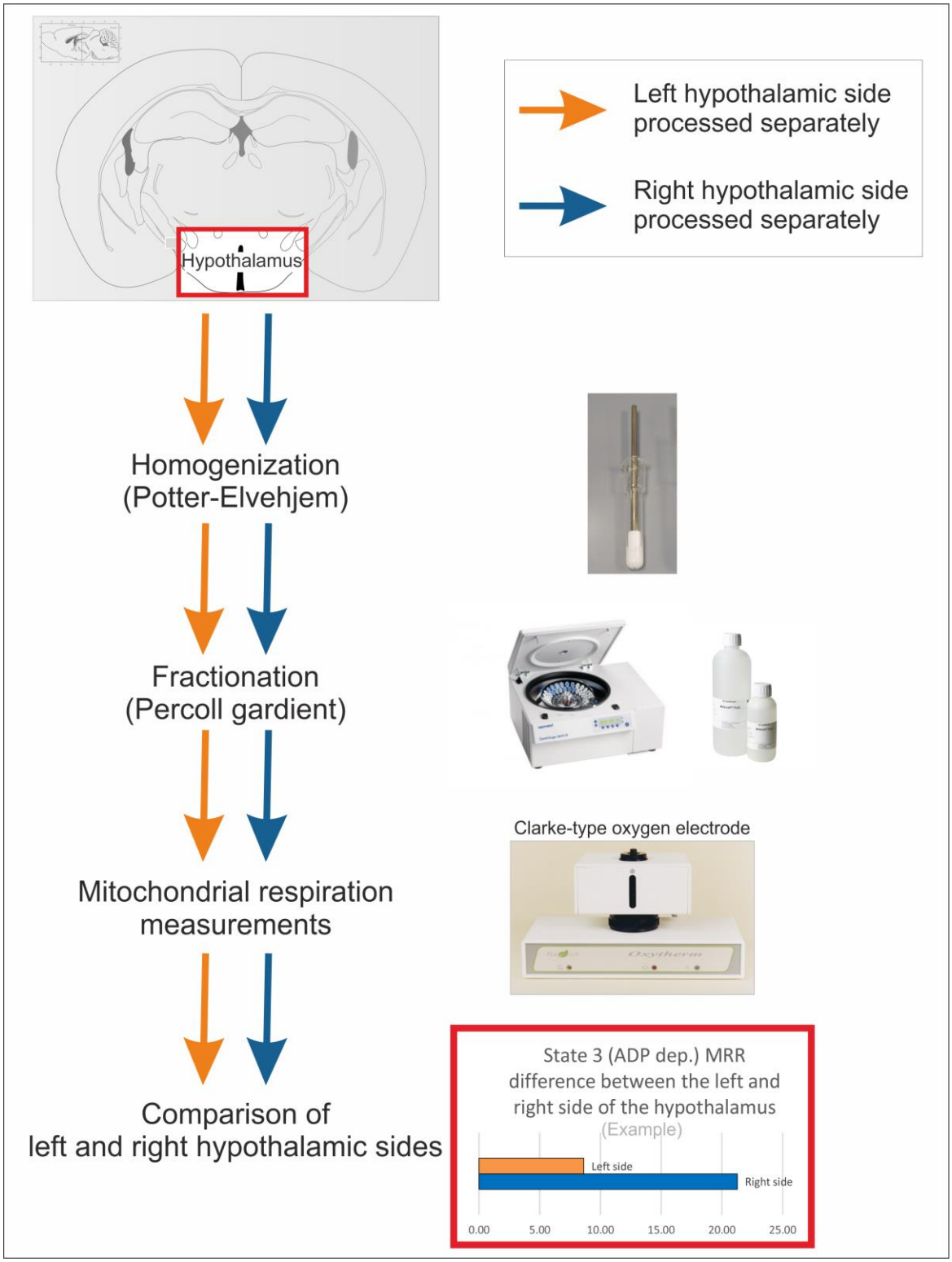

Figure 5: Experimental design. *Mitochondrial respiration rates (mrr) were measured on isolated hypothalamic synaptosomal and mitochondrial fractions. Left and right hypothalamic sides were homogenized, centrifuged, and measured separately.*

4.3. Data analysis

Although all mitochondrial respiration states were evaluated, St3 and St4 mitochondrial respiration *mrr* data were analyzed to determine functional sidedness. St3 gives a plausible insight into mitochondrial metabolism since ADP/ATP ratio potently regulates mitochondrial activity (Brand and Nicholls, 2011); and St4 that indicates uncoupling and alternative oxidation play important roles in transient down-regulation of ATP biosynthesis when cellular energy needs drop. The other mitochondrial respiration states were used as internal controls to monitor the suitability of the samples for analysis.

St3 and St4 *mrr* data were analyzed from two aspects. Firstly, we compared the left and right hypothalamic sides of individuals and the results gained from the comparison were used to shed light on the degree of the asymmetry. After comparison, we also detected the more active side of the individual based on St3 values. In St3, ADP is provided for the mitochondria (fuel is already present from St2), thus abolishing the limiting factors imposed by the lack of these substances in mitochondrial respiration; therefore values of St3 represent the maximal respiratory rate (maximal metabolic activity) at which the mitochondria can run their metabolism (not to be confused with the “total respiratory capacity” usually interpreted as St5 values after the treatment of the samples with FCCP).

We also compared the absolute *mrr* values collected from the active sides (as a data group) vs all less active sides (as another data group). This analysis of “population sidedness” highlights the metabolic activity itself on each side of the hypothalamus, instead of evaluating the differences between the two hemispheres. Thus, this approach provides insight into the case of metabolically balanced sides (i.e. minimal *mrr* differences), i.e., whether the metabolism of the two hemispheres are equally high or equally low under a given experimental conditions.

Because of the internal error of the described protocol (*cca* 10% in St3 in case of a 30mg hypothalamic block), we set up a strict requirement, and sidedness as a term was only used if the difference between left and right hypothalamic sides of the individual was 20% or higher.

As statistical analyses, Fisher’s exact test was applied to evaluate sidedness, and two-way ANOVA with Bonferroni posttests to compare degree of asymmetry between groups by Prism 5 (GraphPad Software Inc., San Diego, CA).

5. Results

5.1. Experiment 1: Examination of hypothalamic lateralization in intact female rats

Mitochondrial respiration rates were measured separately in the left and right sides of female rat hypothalami. The left-right results of the individuals were compared to each other and expressed as fold differences between hypothalamic sides.

The extent of hypothalamic asymmetry (proportion of sided and not-sided animals) yields basic information about how reproductive and satiety states influence the metabolic conditions in the two sides of female hypothalami (figure 6). The hypothalamic metabolism (in St3) of intact female animals show a fluctuating asymmetry throughout the estrous cycle. The highest extent of asymmetry was observed in early and late proestrus, when circulating E2 rises, and interestingly, this extent was clearly reduced in the estrus phase (p=0.0301). Later in metestrus and diestrus, the extent increased again, and around 50% of the animals showed some kind of hypothalamic sidedness. In order to elucidate the share of left and right sides in hypothalamic sidedness, we determined the dominant side (i.e. more active side) based on St3 values. This analysis revealed that the higher metabolic rate was detected mostly on right hypothalamic side (i.e. right sided dominance; figure 7).

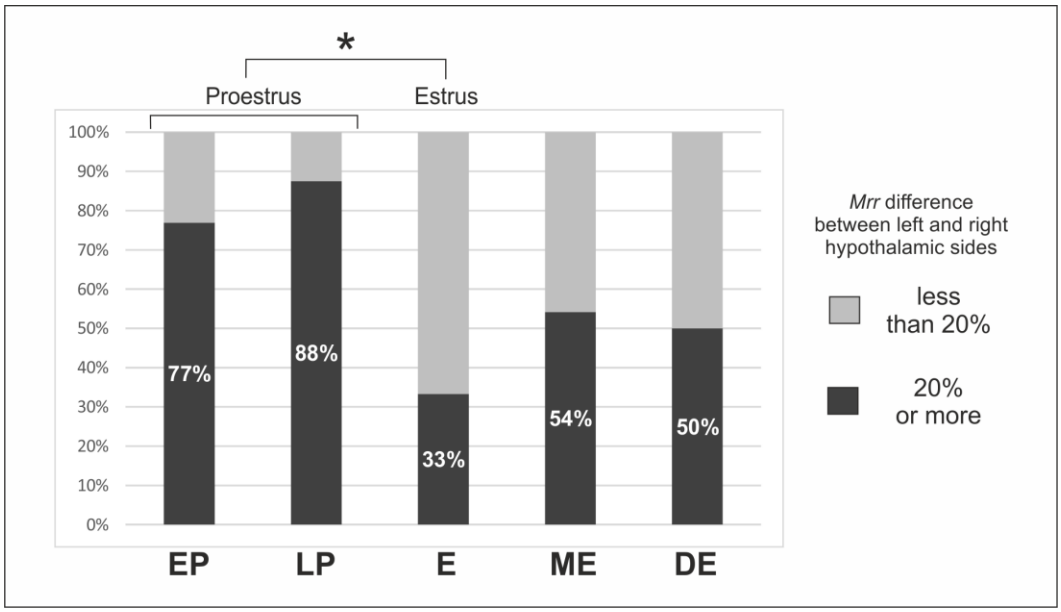

Figure 6: Percentage of animals with hypothalamic asymmetry in state 3 mitochondrial respiration. *The highest extent of asymmetry was observed in EP and LP followed by a strongly reduced value in E phase. ME and DE, around the 50% of the animals showed metabolic sidedness. (Fisher's exact test, *: p=0.0301; EP- early proestrus [n=13], LP- late proestrus [n=8], E- estrus [n=9], ME- metestrus [n=24], DE- diestrus [n=12])*

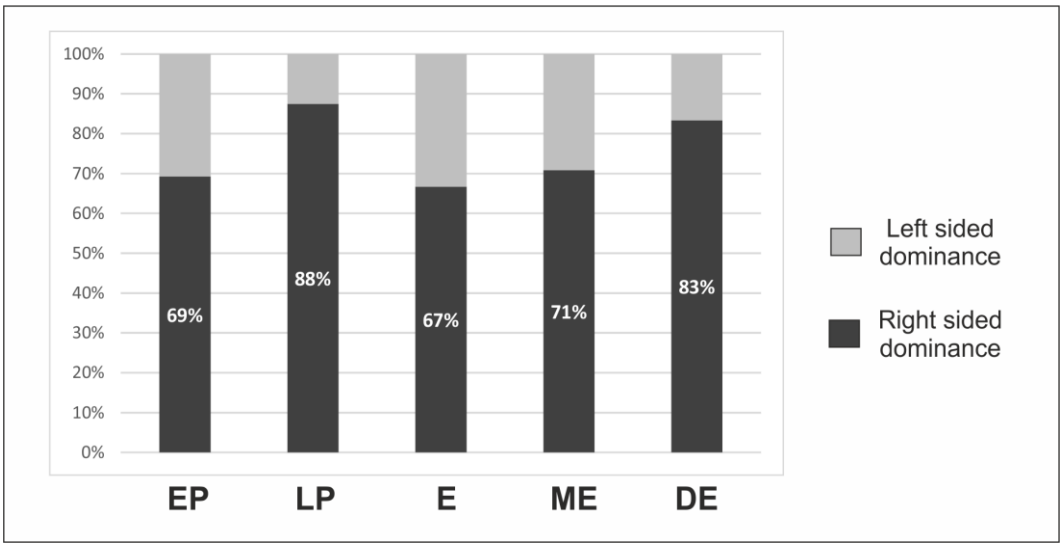

Figure 7: Share of left and right side dominance in intact normal cycling female rats. *In all phases of the estrous cycle, a right sided dominance can be observed in most of the animals. (EP- early proestrus [n=13], LP- late proestrus [n=8], E- estrus [n=9], ME- metestrus [n=24], DE- diestrus [n=12])*

After having determined the proportion of left- and right-sided animals in the examined population, we also analyzed the degree of metabolic lateralization by the fold differences in mitochondrial metabolism between the left and right hypothalamic sides (St3 and St4). Our results (figure 8) revealed a clear fluctuation throughout the estrous cycle, in which the two peaks were detected in the early proestrus and metestrus. The slowly decreasing degree of asymmetry after early proestrus reached its minimum in estrus. In St3 and St4, a similar pattern of degree can be observed. Absolute *mrr* values in St3 were constrained between a minimal (around 8nmol/ml/minute) and maximal activity (around 50nmol/ml/minute) levels.

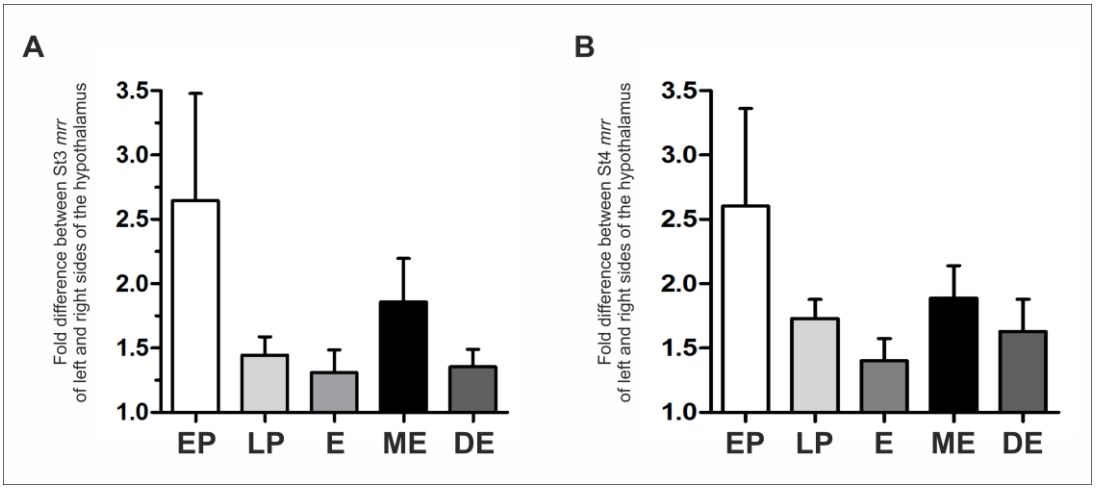

Figure 8: Degree of hypothalamic asymmetry in St3 (A) and St4 (B) in female rats. *In St3 and St4, a similar pattern of degree can be observed with two peaks (EP, ME). (EP- early proestrus [n=13], LP- late proestrus [n=8], E- estrus [n=9], ME- metestrus [n=24], DE- diestrus [n=12])*

5.2. Experiment 2: Examination of the role of E2- and satiety state-dependent lateralization in female rats

Firstly, we examined the extent of hypothalamic asymmetry (proportion of sided and not-sided animals) on *ovx* and *ovx*+E2 treated females as well (figure 9). The most prominent result is that E2 treatment, regardless of the satiety state, caused remarkably higher proportion of sided animals (p=0.0152). Interestingly, E2+*ad lib.* group showed right sided dominance, while in the E2+fasted group, the left and right sidedness was well balanced. In the absence of the masking effect of E2, we found that 24 hours of food restriction reduced the proportion (number) of animals with sided hypothalamic metabolic intensities.

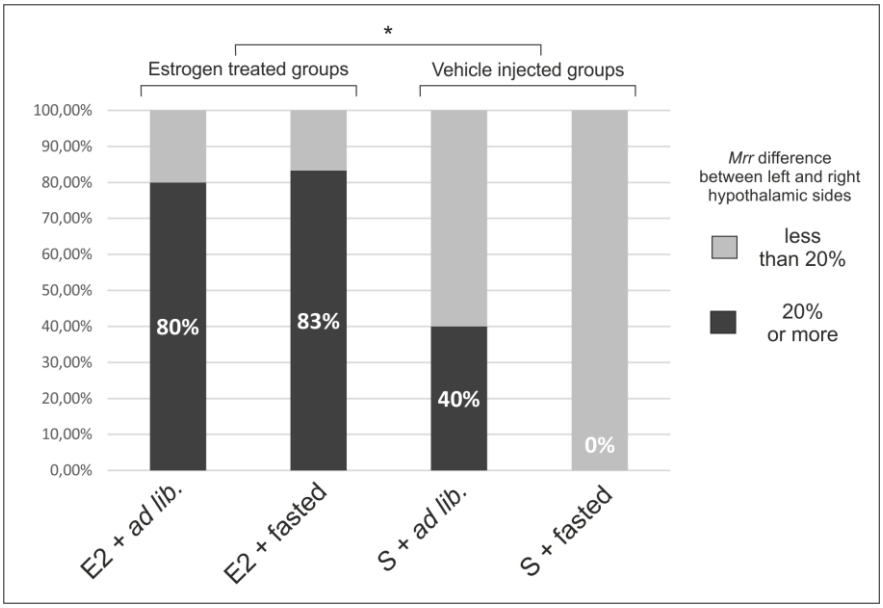

Figure 9: Percentage of animals with hypothalamic asymmetry in state 3 mitochondrial respiration. *E2 treatment, regardless of satiety states, caused significantly higher proportion of sided animals (Fisher's exact test, *: p=0.0152). In absence of E2, 24 hours food restriction further reduced the extent of hypothalamic asymmetry. (Sidedness was considered if the difference between the left and right hypothalamic sides of the individual was 20% or higher. E2+ad lib: n=5; E2+fasted: n=5; S+ad lib: n=6; S+fasted: n=4)*

By analyzing the fold differences between left and right hypothalamic sides of individuals (figure 10), we found that in St3, the presence of high E2 levels combined with *ad libitum* feeding provoked the most striking sidedness (E2+*ad lib.* group); E2+fasted animals show a somewhat less remarkable sidedness, while gonadectomy nearly abolishes the differences between hypothalamic hemispheres (vehicle injected groups, p=0.0036). St4 results did not depend on the effect of E2 and satiety states, down-regulating processes show similar activities among experimental groups.

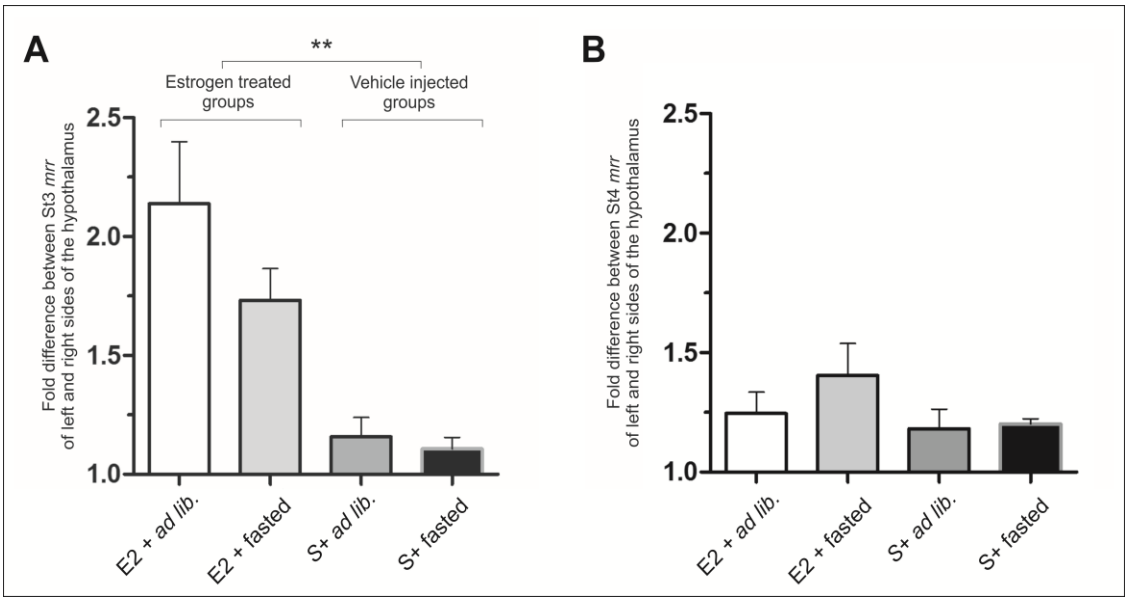

Figure 10: Degree of hypothalamic asymmetry in St3 (A) and St4 (B) in ovariectomized female rats. *The presence of high E2 levels combined with ad libitum feeding provoked the most striking sidedness; E2+fasted animals show a little less remarkable sidedness; and gonadectomy (sham injection) nearly abolishes the differences between hypothalamic hemispheres (vehicle injected groups);such prominent differences are completely absent in St4 results. (Two-way ANOVA with Bonferroni posttest, **: p=0.0036; E2+ad lib: n=5; E2+fasted: n=5; S+ad lib: n=6; S+fasted: n=4)*

As the last aspect of our analysis (figure 11), we also compared absolute St3 *mrr* values collected from the more active sides (as a data group; in this sense dominant sides) vs all less active sides (as another data group; in this sense silent sides). The comparison reinforces our previous finding showing that E2 treatment causes the highest sidedness, whereas gonadectomy reduces the differences between the sides. Furthermore, we also see that 24 hours of fasting lowered the *mrr* values, however, *mrr* could be unilaterally increased by single E2 injection (E2+fasted group). Considering the lowest and highest *mrr* data, it appears that under the experimental conditions applied, hypothalamic hemispheres may have a minimal and maximal level of metabolic activity.

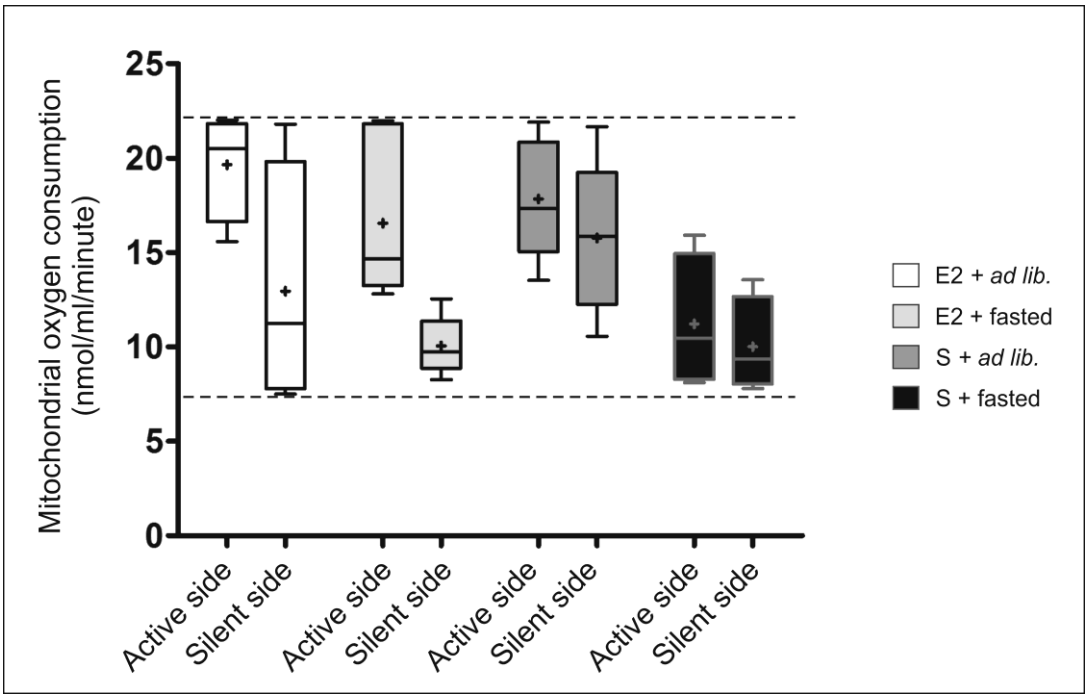

Figure 11: Boxplot diagram of "population sidedness" in state 3 mitochondrial respiration comparing the less active hypothalamic sides (silent sides) to the more active hypothalamic sides (active sides). *E2 treatment causes high differences between the silent and active sides. 24 hours of fasting lowered the mrr values that could be increased unilaterally by single E2 injection (E2+fasted group). Under our experimental circumstances, hypothalamic halves have a minimal and maximal activity (indicated with the broken lines; E2+ad lib: n=5; E2+fasted: n=5; S+ad lib: n=6; S+fasted: n=4).*

5.3. Experiment 3: Examination of the role of testosterone- and satiety state-dependent lateralization in male rats

For male groups, we used the same aspects of analysis that we applied earlier for female groups; therefore, firstly, we determined the proportion of animals with hypothalamic metabolic sidedness in each group (i.e. left-right difference is 20% or higher; results are presented on figure 12). Unlike females, where gonadectomy nearly abolished hypothalamic sidedness, male animals showed similar proportion of sided and not sided hypothalamic phenotype among experimental groups. Only 24 hours of food deprivation together with fasting could lower to some degree the extent of hypothalamic lateralization.

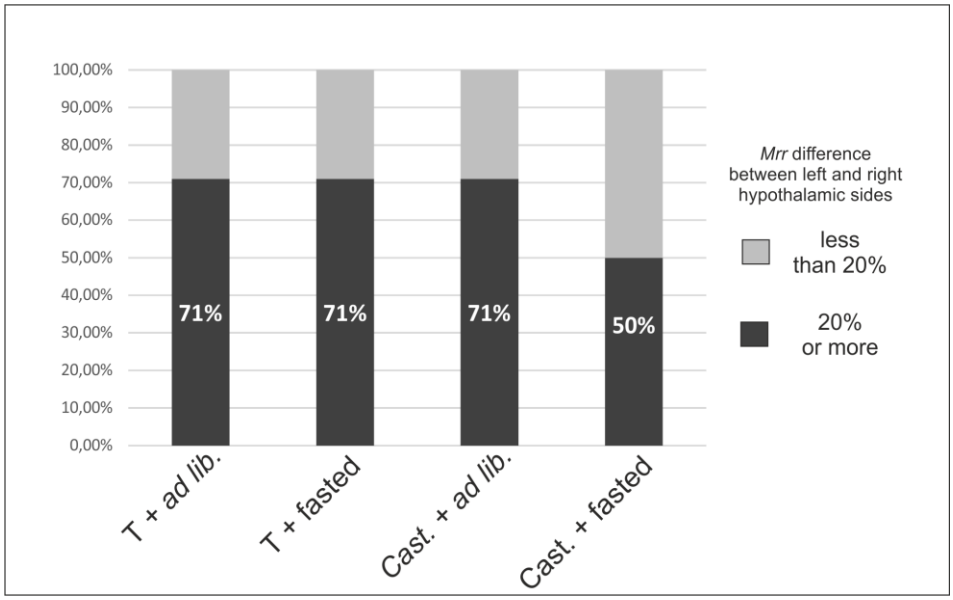

Figure 12: Percentage of animals with hypothalamic asymmetry in state 3 mitochondrial respiration. *Male animals showed similar proportion of sided and not sided hypothalamic phenotype among experimental group, except cast.+fasted group. (Sidedness was considered if the difference between the left and right hypothalamic sides of the individual was 20% or higher; T+ad lib: n=7; T+fasted: n=7; Cast.+ad lib: n=7; Cast.+fasted: n=6)*

In order to elucidate the share of left and right sides in hypothalamic sidedness in males, we determined the dominant side (i.e. more active side) based on St3 values (the result is plotted on figure 13). In case of *ad libitum* animals, the higher metabolic rate was detected exclusively on right hypothalamic side, regardless of the reproductive state (castrated/intact). In contrast, in fasted animals sidedness vs symmetrical was nearly balanced, as only around 40-60% of the individuals were right-sided (satiety state effect: p=0.0058).

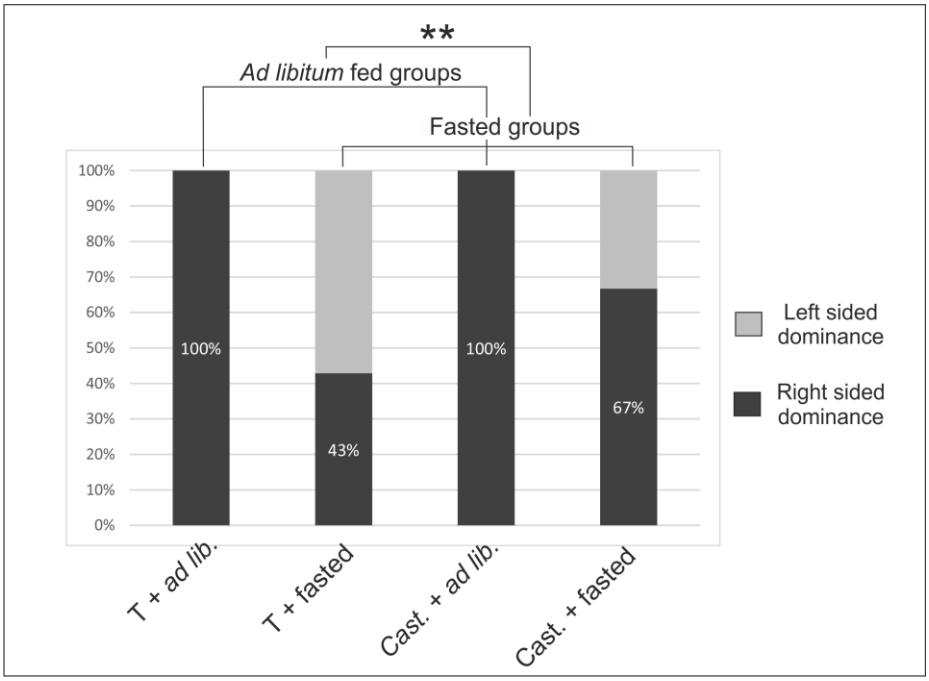

Figure 13: Share of left and right side dominance in male rats. *Animals in ad libitum fed groups showed right sided dominance; while in fasted animals left sided dominance could also develop in approximately 50% of the examined individuals. (Fisher's exact test, **: p=0.0058; T+ad lib: n=7; T+fasted: n=7; Cast.+ad lib: n=7; Cast.+fasted: n=6)*

We also analyzed the degree of metabolic lateralization by the fold differences in mitochondrial metabolism between the left and right hypothalamic sides (St3 and St4). Our results show that sidedness was remarkably higher in *ad libitum* animals, and it even reached a significant level in St4 (p=0.0384). In contrast to this, orchiectomy, regardless of the satiety state, led to only slight alteration of metabolic differences between the two sides (figure 14). It has to be noted that in *ad libitum* groups, regardless of the reproductive state, there were a few animals showing balanced metabolic phenotype (i.e. L-R difference is around 1) in St3 and St4, as well.

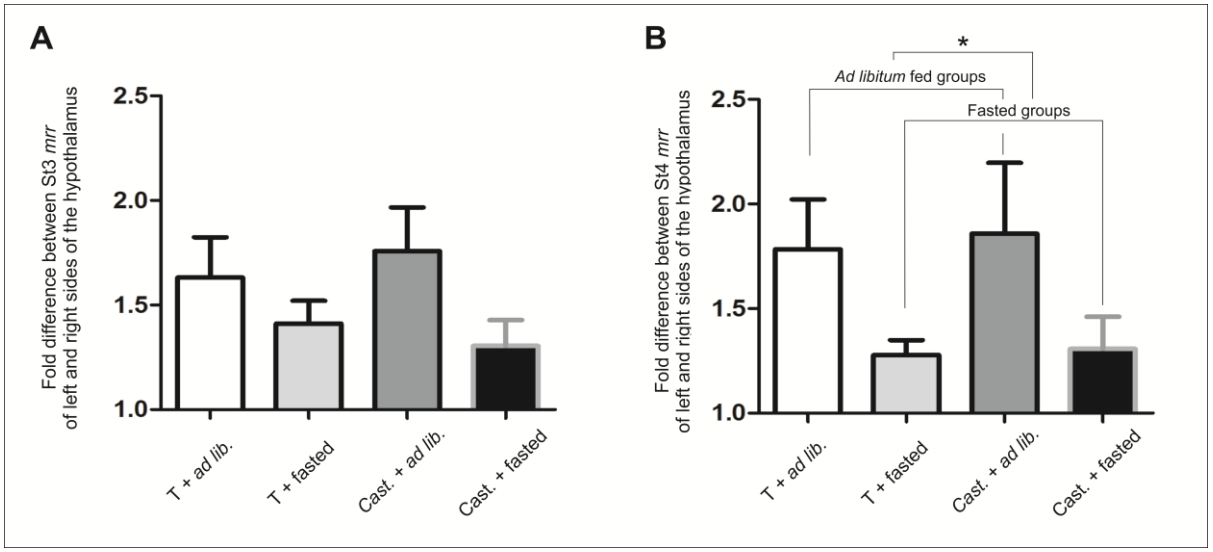

Figure 14: Degree of hypothalamic asymmetry in St3 and St4 in male rats. *Sidedness was remarkably higher in ad libitum fed animals in both St3 and St4. (Two-way ANOVA with Bonferroni posttests, *: p=0.0384; T+ad lib: n=7; T+fasted: n=7; Cast.+ad lib: n=7; Cast.+fasted: n=6)*

As the last aspect of our analysis, we also compared absolute *mrr* values collected from the left (as a data group) vs right sides (as another data group) of the hypothalamus. The analysis reinforced our previous results in all aspects, furthermore we also determined the range of St3 *mrr* values that, similarly to the female animals, constrained between a minimal (around 8nmol/ml/minute) and maximal (around 22nmol/ml/minute) activity levels, regardless of the experimental conditions.

5.4. Summary and comparison of *mrr* data collected from female and male hypothalami

Results (fold differences between the left and right sides of individuals) collected from experiment 2 and 3 are summarized on figure 15. The figure clearly demonstrates the most prominent, fundamental differences between the lateralization of male and female hypothalamic metabolism in the experimental conditions used.

On figure 15 1A-2A, left-right metabolic differences are shown in St2-St5. The comparison of the curves reveals basic dissimilarities between metabolic profiles of male and female animals. The most prominent result is that the hypothalamic asymmetry in females seems to be strongly E2-dependent, while male gonadal steroids are less determinant in hypothalamic lateralization. In terms of the degree of sidedness, on the other hand, asymmetric functioning in females was little influenced by the satiety state compared to males, i.e. instead of the right sided dominance, in fasted group left sidedness could also developed. Similarly to females, fasting also caused left sided dominance in 50% of the animals, but this phenomenon is accompanied with higher degree of asymmetry.

Another difference between males and females emerges by analyzing St3 and St4 (figure 15 1B,C-2B,C). In females, E2 increased hypothalamic metabolism unilaterally without significantly affecting St4 (i.e. down-regulating processes). On the other hand, in case of male animals, asymmetric changes in St3 that were mainly related to the satiety-state of individuals were further and significantly increased in state 4 *mrr*.

As the last aspect, we also compared minimal and maximal *mrr* data in all experimental groups. No significant difference was registered between males and ovariectomized females: intact and gonadectomized males, as well as all gonadectomized females had a minimal hemispheric activity (average 8nmol/ml/minute) running up to a maximal value (22nmol/ml/minute) depending on the experimental conditions. It is noteworthy that we registered significantly higher St3 *mrr* in intact females, where the maximal values could reach even 50nmol/ml/minute.

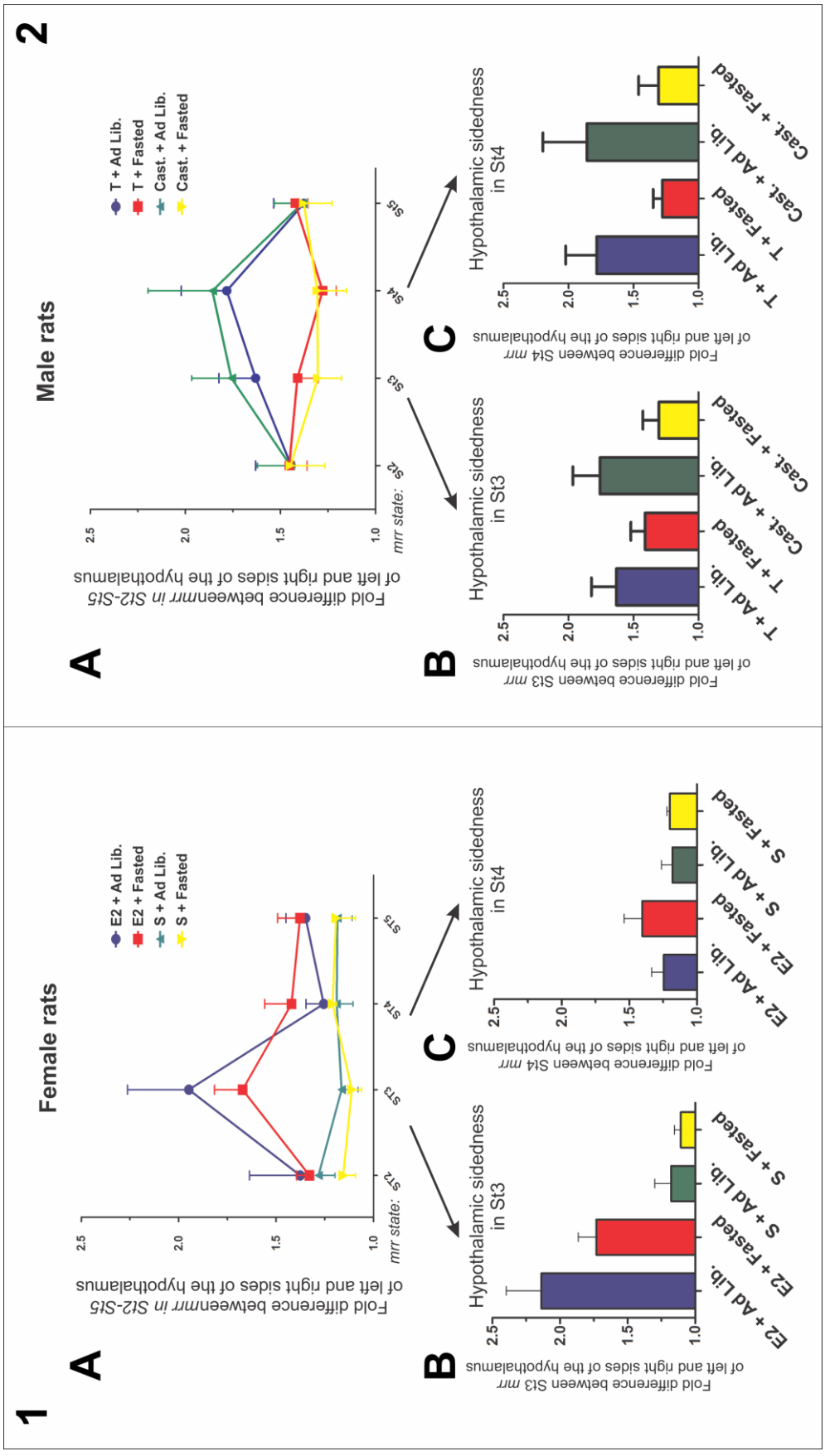

Figure 15: Summary and comparison of the results gained from male and female hypothalami.

6. Discussion

The first findings that indicated hypothalamic sidedness were published more than 40 years ago (in the 1970's); however, those studies seem to have been discontinued and hence, the exact nature and function of this phenomenon is still unknown. Here, we demonstrate new results indicating a complex mechanism by which the left and right hypothalamic sides are able to regulate different homeostatic and reproductive processes in an asymmetric manner.

6.1. Asymmetry in female hypothalami

In our experiments on intact, normal cycling rats (experiment 1) and *ovx* rats (experiment 2), we found that an estrous phase- and estrogen-dependent metabolic lateralization exists in the cyclic regulation of female reproduction (figure 16). Furthermore, we could also describe a lateralized functioning of the hypothalamic feeding centers, and that this lateralization is influenced by E2.

Firstly, we examined the effects of the estrous cycle on hypothalamic metabolism in normal cycling female rats. We found higher metabolic activity (St3) in the right hypothalamic sides during proestrus. Due to the fact that circulating E2 concentration begins to rise at the very end of diestrus and beginning of early proestrus (Butcher et al., 2013), it is reasonable to assume that E2, as the most potent feedback factor of female reproduction, plays a crucial role in the lateralization of female hypothalami. The E2 effect is probably mediated by ERα, one of the most important E2 receptors that is known to be synthetized in an asymmetric-manner in the left and right hypothalamic sides in proestrus (Arteaga-Lopez et al., 2003); we note at this point that receptor-ligand (ERα-E2) interaction significantly increases mitochondrial activity (Klinge, 2008; Razmara et al., 2008; Kiss et al., 2009). In estrus phase, circulating E2 levels drop that results in a reduced metabolic asymmetry in the hypothalamus. Interestingly, in the following phases (ME, DE), the metabolic lateralization slightly increases again that might also be a result of ERα-E2 interaction. According to Arteaga-Lopez et al. (2003), ERα mRNA levels in the right hypothalamic hemisphere has a second peak in metestrus therefore baseline concentration of circulating E2 and locally synthetized E2 might be able to increase the metabolism in the right hypothalamic side. It should to be noted here that ERβ decreases mitochondrial activity (Manente et al., 2013), and since its level is elevated in DE (Arteaga-Lopez et al., 2003), it could also contribute to the metabolic asymmetry by lowering the hypothalamic metabolism of the contralateral side.

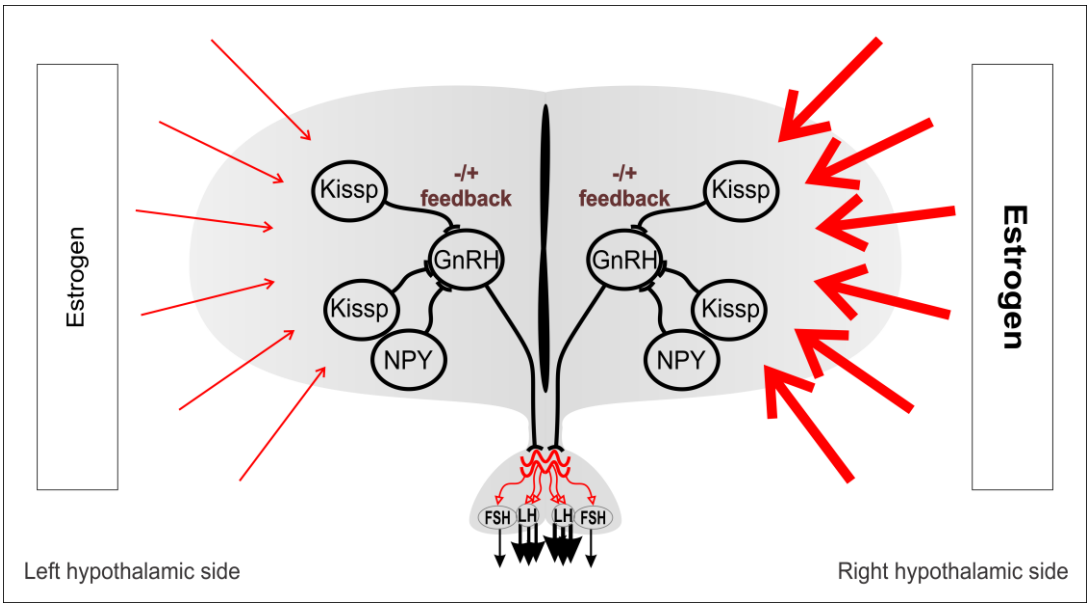

Figure 16: Schematic summary of hypothalamic regulation of female reproduction. *Estrogen causes right sided metabolic dominance by affecting the central part of HPG axis, this phenomenon might have a role in the earlier described negative-positive feedback change and the GnRH peak.*

The above described mechanism of E2-ER interaction clearly indicates the role of E2 in the estrous cycle-related metabolic lateralization. Therefore in experiment 2, by the single dose E2 injection of *ovx* animals, we intended to mimic the acute rise of E2 concentration during the estrous cycle. As a result, we found early proestrus-like changes in the intensity of mitochondrial metabolism, supporting our conclusion that the estrous phase-dependent metabolic asymmetry is linked, at least in part, to the fluctuation in plasma E2 levels.

E2 treatment, regardless of satiety states, caused remarkably higher proportion of sided animals meaning that after bilateral ovariectomy, we could observe a hypothalamic state of low metabolic asymmetry, in which the left and right sides' capability to react to E2 seem to significantly differ. Similar effects have been described on asymmetric GnRH levels by Gerendai et al. (1978) suggesting that the underlying mechanisms of GnRH secretion may be modulated by the E2-related unilateral metabolic responses. Other reports that focused on the rapid behavioral and endocrine changes associated with the process of ovulation are also consonant with the above observations (Gerendai et al., 1978; King and Nance, 1985; Roy and Lynn, 1987; Arteaga-Lopez et al., 2003; Cruz et al., 2014b). While the phenomenon of the metabolic asymmetry seems to lie on the grounds of side-linked unequal potentials within the hypothalamus, further studies are needed to clarify whether it is based on genomic or non-genomic differences between the hypothalamic hemispheres. Moreover, since an overall sidedness exists in the female reproductive system (Nance et al., 1983, 1984), the putative role of genetic coding in sidedness should be investigated outside of the hypothalamus as well.

Interestingly, the changes described above were not detectable in St4 meaning that E2 could increase the metabolic activity of the mitochondria unilaterally, without affecting the activity of the alternative oxidases and the uncoupled-state of the mitochondria (these down-regulating processes have a very important role when cellular energy needs drop, and mitochondrial readiness is still at high level, but ATP production decreases dramatically). It is also known that E2 increases mitochondrial activity and, at the same time, reduces the production of reactive oxygen species and other harmful byproducts in mitochondria (Razmara et al., 2007; Guevara et al., 2011). This phenomenon together with our findings suggests that E2 effects on mitochondrial activity is restricted to the ATP producing capacity without increasing the inherited mitochondrial safety mechanisms, nevertheless activates other, yet unidentified mechanisms to exert its protective functions. It has to be noted that in intact females, unlike *ovx* groups, St3 and St4 showed a similar pattern throughout the estrous cycle indicating the role of other factors that might attenuate the E2-induced neuroprotection. The neuroprotective role of E2, however, still incontrovertible, especially if one also considers our results gained form male rats (i.e. differences in St4 were increased compared to St3).

Taken together, the afore-mentioned results regarding the role of E2 in reproductive functions, we found strongly E2-dependent asymmetric metabolic changes in the hypothalamus that might well be attributed to E2-driven rapid events leading to short-term neuronal plasticity-related to GnRH peak (Naftolin et al., 2007; Kelly and Ronnekleiv, 2008). This idea is supported by findings showing that rapid and non-genomic E2 effects include alterations in mitochondrial structure and function (reviewed by Arnold et al., 2012) such us ATP synthesis (Zheng and Ramirez, 1999a, 1999b).

As the last aspect of our analysis, we also compared absolute *mrr* values collected from the more active sides (as a data group; meaning: dominant sides) vs all less active sides (as another data group). The comparison reinforces our findings showing that E2 treatment causes the highest sidedness, whereas gonadectomy reduces the differences between the sides. Furthermore, 24 hours of fasting lead to lowered *mrr* values; and also, as a subsidiary, albeit interesting outcome is that mitochondrial oxygen consumption rates are constrained into a range of a minimal (around 8nmol/ml/minute) and maximal (around 22nmol/ml/minute) activity regardless of experimental conditions. Taken into consideration the corresponding data of normal cycling female rats, where this range falls between 8 and 50nmol/ml/minute, ovariectomy causes a drastic decline in the metabolic range that remains irreversible even by E2 treatment.

Besides the E2-dependent metabolic sidedness in female rats, the present results brought at least two interesting additions to those observations: 1) sidedness in late proestrus and estrus was linked exclusively to the right side of the hypothalamus, while without intact

ovaries and after a single injection of E2 left sidedness could also develop; 2) in the absence of the masking effect of E2, we found that 24 hours of food restriction reduced the proportion (number) of animals with sided hypothalamic metabolic intensities. These phenomena may be explained by considering that full reproductive effects of E2 are only exerted in the presence of all other ovarian hormones (i.e. progesterone, *etc*.), and without those hormones the anorexigenic effects of E2 (Clegg et al., 2007; Santollo and Eckel, 2008) may develop more prominently (satiety hormone-like effects are more dominant). In summary, the afore-mentioned results, together with the existence of unilateral feeding pathways connecting hypothalamic structures to asymmetric brain areas (Mittleman et al., 1985; Vanetsian and Pavlova, 2004; Grundmann et al., 2005), further support the idea that the food-intake related hypothalamic functions show a lateralized distribution between the two hypothalamic hemispheres in female rats, but the effects are less remarkable than the changes caused by the estrous cycle and E2.

As stated above, 24 hours of food deprivation led to lowered *mrr* values. This obviously raises questions if one considers the recently accepted concept stating that food deprivation enhances metabolic activity (i.e. mitochondrial ATP synthesis) of those neuron populations in the medial part of hypothalamus (arcuate nucleus) that are prominently involved in the regulation of food-intake, as it was published by Cakir et al. (2009). It is to note, however, that they worked exclusively on male rats. This raises the possibility that food deprivation alters the mitochondrial activity of the medial hypothalamus in a gender-specific manner. Therefore, in the lack of confirming-supporting data in the literature, deeper understanding of our present data requires further region-specific, if not neuron-specific investigations of mitochondrial responses in the presently applied conditions.

6.2. Asymmetry in male hypothalami

In male animals, we also examined the left and right hypothalamic sides separately in order to further analyze food-intake related metabolic activities and, at the same time, to compare it with the results from female animals. This way, we found that the function of hypothalamic feeding centers in males is also lateralized, however changes in the reproductive state of the animal did not result in notable alteration in the ratio of sided and not sided individuals.

As in the case of female animals, first we determined the proportion of animals with hypothalamic metabolic sidedness in each group. In male animals, there was no change in the proportion of sided vs not sided animals in St3, except of cast.+fasted animals where only 50% of the individuals were metabolically imbalanced. While in female rats the proportion of sided animals changed significantly depending on the presence or absence of gonadal steroid, and it was modified by satiety states, in males neither bilateral orchiectomy nor food deprivation resulted in notable alteration in the ratio of sided and not sided individuals. Although this phenomenon would suggest an asymmetric hypothalamic metabolism that does not change upon experimental manipulation, further analyzing the metabolic lateralization, the proportion of left-right dominance and degree of asymmetry (figure 13-14) clearly reveals a dynamically changing sidedness between the left and right hypothalamic sides that seems to be related to the food-intake, at least under the experimental conditions used.

In *ad libitum* conditions the right hypothalamic hemisphere was metabolically more active, while after 24 hours of food deprivation the left side takes over the metabolic dominance in almost half of the individuals. This is fully in line with our findings in female rats, where fasting balanced the proportion of left and right sided dominance in St3 as well, even if satiety state exerted less impact on the overall hypothalamic metabolism compared to the effect of gonadectomy. The left-sided dominance in rats after food-deprivation is consistent with findings in cats and rabbits (Pavlova and Mats, 1996; Vanetsian and Pavlova, 2004) suggesting that this kind of bias in food-searching and food-intake related motivation and behavior might be a general phenomenon in many mammals.

As a summary, it seems that fasting abolished right-sided dominance (at least in case of the applied condition), likely by activating the orexigenic neuronal populations on the contralateral hypothalamic side (figure 17). This idea is further supported by the degree of metabolic asymmetry observed in male animals: in *ad libitum* fed animals (in both St3 and St4), we detected higher degree of hypothalamic asymmetry of the individuals. These results taken together may imply that the well-studied dynamic interactions between orexigenic and anorexigenic hypothalamic centers might differ on the left and right sides as follows:

1) During satiety (*ad libitum* feeding), animals have low levels of the circulating orexigenic factors such as ghrelin (Ariyasu et al., 2001). In this state, POMC cells are liberated from the AgRP tonic inhibitory effect that results in the activation of MCR3 and 4 melanocortin receptors in PVN (Horvath, 2005). Although this kind of neuronal activation possibly happens on both hypothalamic sides, our results of mitochondrial respiration indicate a stronger activation on the right hypothalamic side versus the left, suggesting that the satiety state (feeling) of the animal primarily depends on the right hypothalamic side.

2) On the other hand, in case of food-deprivation, the hunger signals cause an elevated ghrelin level in the circulation (Ariyasu et al., 2001). Ghrelin activates AgRP and hypocretin neurons, while POMC neurons are inhibited that results in an orexigenic activation of the melanocortin system even if the animal has eaten before ghrelin administration (Horvath et al., 2001; Toshinai et al., 2003; Seoane et al., 2013). In our experiment, we described that fasting caused left-sided metabolic dominance in 50% of the animals; this strongly suggests that (ghrelin-induced) orexigenic activation of the melanocortin system dominates on the left hypothalamic side (however, probably it happens on both sides). This orexigenic activation, due to the dynamic interaction and reciprocal innervation with anorexigenic neurons, slowly inhibits the POMC neurons that leads to hunger and food-intake related changes in behavior (Horvath, 2005).

The above data, together with the current results, raise the idea of a dynamically changing balance between the left and right hypothalamic hemispheres, where the orexigenic activation that is stronger on the left side firstly decreases the left-right metabolic differences, and later causes a left sided metabolic dominance (figure 18). This would also explain that in *ad libitum* fed groups, we found animals with high left-right *mrr* difference (St3 and St4) as well as animals with balanced hypothalamic metabolic phenotype. *Ad libitum* fed animals eat several times a day if they are hungry, bored or due to other stereotypic behavior. Therefore, in *ad libitum* fed group, we probably had animals before (getting hungry) and after eating (sated), as well, causing a bigger variation of data. In summary, it is very likely that animals in our experimental groups showing big hypothalamic difference either ate before the sacrifice or had their last meal long before the morning experiments.

Based on our results obtained from female animals, i.e. the share of left and right dominance changed between *ad libitum* fed and fasted groups, the above-described asymmetric regulation of food-intake might apply to female animals as well, but under the experimental conditions used, it was blunted by the strong effect of reproductive signals, and remained in the shadow of the estrogen-induced events.

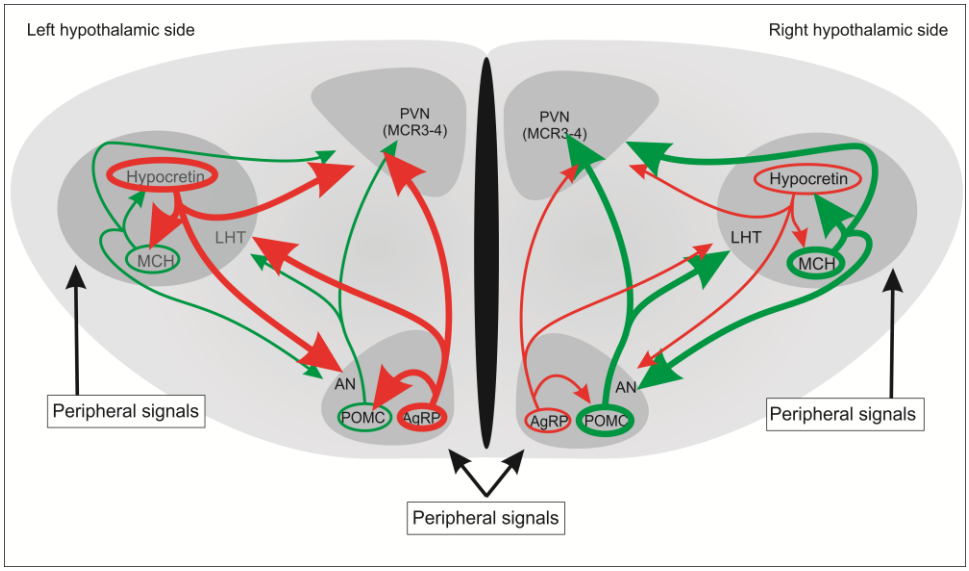

Figure 17: Schematic summary of the activity of melanocortin system on the left and right hypothalamic sides. *Orexigenic neurons seem to have a higher activity on the left side (red arrows), while anorexigenic neurons dominate on the right side of the hypothalamus (green arrows).*

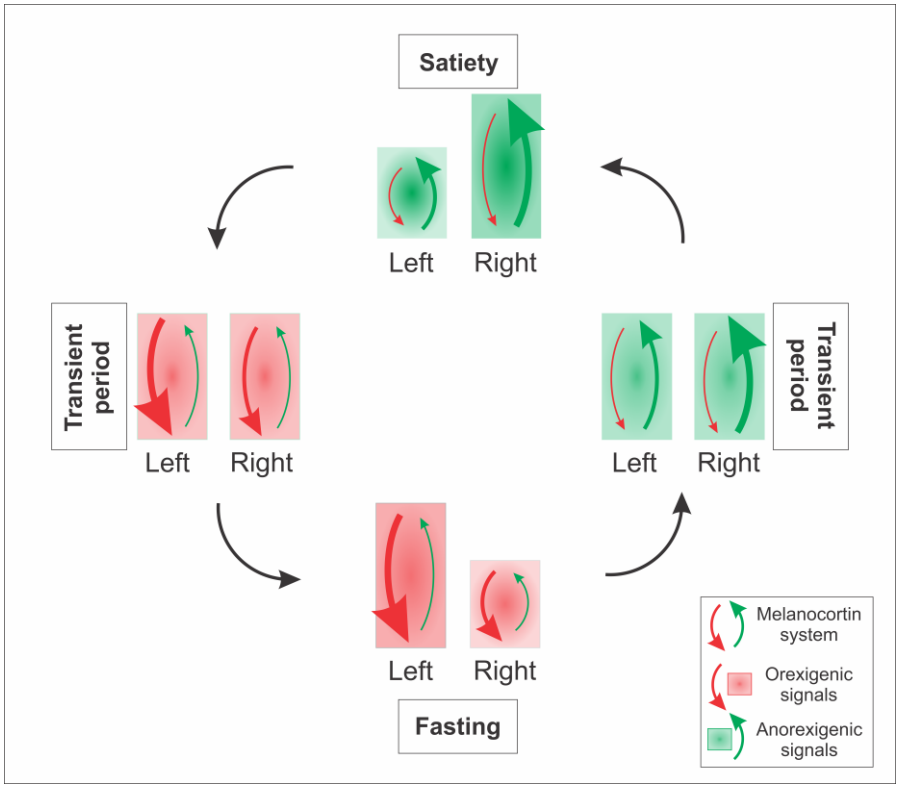

Figure 18: Dynamic metabolic changes in food-intake regulation on the left and right hypothalamic sides. *In fasting, orexigenic activation is stronger on the left side causing decreased left-right metabolic differences, and later a left sided metabolic dominance. In satiety, anorexigenic signals increase the metabolic activity of the right side that is resulted in a right sided metabolic dominance. Height of columns indicates the metabolic activity of the hypothalamic sides.*

Although, in the present work, the food-intake and energy expenditure was considered to be regulated through the same mechanisms, it has to be noted that the effector cells might be different. Studies on human subject examining weight loss and diets have also indicated a divergent pathway in energy expenditure (Leibel et al., 1995). As it is suggested by a complex study of Balthasar et al. (2005), AgRP/NPY and POMC neurons are integrating the peripheral and central information of the actual satiety status of the animal (as described earlier), however, they projection onto PVN and amygdala neurons having MCR3 or 4 regulates only food-intake and food-search related behavior, while centers of energy balance are located somewhere else probably scattered in several locations. These raise the possibility of an even more complicated interaction between hypothalamic hemispheres in which the sides are activated slightly differently depending on whether the animal is able to eat or fasting is permanent.

6.3. Gender-related differences in hypothalamic metabolism

The last and most interesting aspect of this study is the comparison of the results gained from female versus male animals. This comparison revealed fundamental gender-related differences in hypothalamic metabolic profiles. According to the present results, hypothalamic asymmetry of females seems to be strongly E2-dependent, while male gonadal steroids do not seem to be strong determinants of the hypothalamic lateralization. In contrast to this, in males, it is the regulation of food-intake that elicits higher levels in metabolic changes in the hypothalamus, and influences sided metabolic alterations compared to the effects of heavy changes in gonadal factors; nevertheless, food deprivation resulted in a detectable decrease in the fold difference between the two hypothalamic hemispheres also in E2-treated females. This observation suggests that food deprivation elicits similar effects on the metabolism of the left side, however in females this action is, at least in part, masked by the overwhelming effect of gonadal steroids. These two observations suggest that the hypothalamic regulation in males are mostly satiety state-dependent, while in females it is more about the complex and cyclic events of the reproductive functions (estrous).

In summary, it is obvious that similar experimental conditions (i.e. gonadectomy; food-deprivation) caused basically different metabolic changes in males and females that most probably evolved due the different hormonal profiles, and therefore different hormonal interactions. For example, leptin induces rapid synaptic remodeling in the hypothalamus (Pinto et al., 2004; Horvath, 2006), and E2 increases leptin sensitivity of the hypothalamic centers regulating food-intake and energy expenditure (Meli et al., 2004; Clegg et al., 2006). These results suggest that the interaction between leptin and E2 (or the lack of the interaction in case of ovariectomized individuals) seems to have the most significant effect in satiety when both

hormones are present, and they have a higher impact on the right hypothalamic side together. Interestingly, we could not determine any significant interactions between E2 and hunger (ghrelin?), i.e. even fasting and E2 injection could increase the mitochondrial metabolism unilaterally, in lack of E2, fasting on its own (ghrelin?) in S+fasted group was not able to induce significant changes in mitochondrial metabolism in any of the hypothalamic sides. This phenomenon is consonant with other findings claiming that, unlike E2-leptin, no significant interaction exists between E2 and ghrelin (Sakata et al., 2006; Dafopoulos et al., 2010).

In male rats, although the relevant literature that is far incomplete in this respect, describes some interaction between T and food-intake related hormones (ghrelin, leptin, insulin; Chai et al., 1999; Pagotto et al., 2003; Clegg et al., 2006), our experiments could not reveal any detectable effect of T on hypothalamic lateralization in male animals. This result suggests that the testosterone-underproduction or orchiectomy caused decrease in food-intake in male rats (Chai et al., 1999) might be due to an equal impact on the left and right hypothalamic sides.

Besides the differences mentioned above between male and female hypothalami, our experiments revealed some other diverse characteristics: E2 could increase the metabolism unilaterally (St3), but the higher mitochondrial activity was not followed by elevated downregulation of biochemical processes (St4). This interesting phenomenon might be related to the neuroprotective effect of the E2 (Bishop and Simpkins, 1994; Cordey et al., 2003) implying an alternative, less harmful way of activating mitochondria. In contrast to the female animals, males showed a further elevated St4 *mrr* in our experiments suggesting that in lack of the neuroprotective E2 the mitochondrion has to adapt itself to the elevated ATP producing activity.

It is a scientific fact that females of many mammalian species live longer among the same laboratory conditions than males do (Borras, 2007), and also mitochondrial functions have been indicated as key players in the aging processes (Miquel et al., 1980; Guevara et al., 2011). In line with these findings, it is reasonable to assume that elevated downregulating mechanisms (UCP, AOX) are not sufficient to keep the optimal function and over time the high metabolism burns the mitochondria out that finally leads to rapid aging and death and/or disease on cellular and entire body levels as well. Female mitochondria are more differentiated, and show higher efficiency (Justo et al., 2005a, 2005b; Guevara et al., 2009), and this phenomenon is probably due to E2-related alternative ways to increase ATP production that seem to be more effective in terms of long term activity. One of these alternative ways might be that E2 is able to decrease the reactive oxygen species production in mitochondria (Razmara et al., 2007) since this reduction of cellular oxidative stress is proven to be protective against mitochondrial dysfunction, and therefore it can delay neuronal aging

processes (Cui et al., 2012). Further strengthening the role of E2 in the above-mentioned process, Razmara et al. (2007) indicated that E2 treatment exerts its neuroprotective role under physiologic conditions not only in female but in male animals as well.

6.4. Conclusions

The hypothalamus is an anatomically “overcrowded” brain structure that regulates reproduction and food-intake among many other homeostatic processes. Although it is a morphologically symmetric brain structure, it has been referred to as an unpaired midline structure with the two sides equally and simultaneously regulating the same biological functions.

In this study, we demonstrated that the like-named nuclei on the left and right sides of the hypothalamus might have different roles, as it has been discovered and accepted long ago with regard to higher brain areas (cortex, thalamus). It seems that the left and right hypothalamic sides, even though they are able to regulate the same functions, might act on different activity levels to react to homeostatic stimuli that results in a side-linked dominance. Our results suggest that the functions of CNS from the spinal cord to the cerebral cortex are more and more specified to certain functions, and functions show lateralization to different degrees. This evolutionary process of lateralization would provide a much more effective use of brain resources. Based on the functional lateralization that we presented here it seems to be rightful to re-name the hypothalamic sides to hypothalamic hemispheres.

An obvious shortcoming of the metabolic screening method that we used is that hypothalamic sidedness was determined *post mortem*. Therefore, measurements cannot be repeated in the same individual nor the data can be used for the prediction of the exact type (right or left) of sidedness. We believe that further improvement of the technical methods to determine hypothalamic sidedness *in vivo* (i.e., the adoption of functional MRI or other methods) will help find answers to a plethora of questions that arise from the matter of hypothalamic asymmetry (such as whether or not the hypothalamic hemispheres are able to overtake each other’s functions), and would definitely help in the understanding of some forms of medical conditions with hypothalamic origin.

This study changes our current view on the regulation of female reproduction and food-intake, and may provide new perspectives for the better understanding of these hypothalamus driven physiological processes. Also, disturbances of lateralized functions may take part in the pathogenesis of hypothalamus-linked health conditions (infertility, obesity, anorexia nervosa, *etc*.), as it is already indicated in the case of other brain areas (Ribolsi et al., 2014).

7. New scientific results

Ad1-2

A predetermined functional sidedness exists in the hypothalamic regulation of female reproduction. This lateralization is showing a right sided dominance, it is strongly estrogen-dependent, and it could be detected by our metabolic screening method.

Ad3

Food-intake related hypothalamic functions are also lateralized in female rats, although it has a milder impact on the hypothalamic sidedness than the reproductive functions.

Ad4

Testosterone (or lack of testosterone) in male animals is not contributing significantly to the hypothalamic asymmetry.

Ad5

Male animal show a strong lateralization in food-intake related hypothalamic regulatory functions. Right side seem to dominate in satiety, while left side dominates in food-deprived (fasting) stages.

Ad6

Female and male hypothalamic regulation of reproduction and food-intake (and probably other functions) show fundamental gender-related differences. Hypothalamic lateralization in males is mostly food-intake related, while in females, reproductive processes seem to have higher impact on the hypothalamic asymmetry, at least under the present experimental conditions

Ad7

Mitochondrial energetics of female and male rats differ radically: in females, E2-induced elevated mitochondrial activity is not followed by elevated downregulating mechanisms (St4), however, in males, the downregulating processes are further increased after mitochondrial activation.

8. References

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10. The author's scientific publications

10.1. Publications related to the topic of the present dissertation

Full text papers in peer-reviewed journal

Toth I, Kiss DS, Jocsak G, Somogyi V, Toronyi E, Bartha T, Frenyo LV, Horvath TL, Zsarnovszky A (2015) Estrogen- and Satiety State-Dependent Metabolic Lateralization in the Hypothalamus of Female Rats PLOS One 10(9):e0137462 doi: 10.1371/journal.pone.0137462

Toth I, Kiss DS, Goszleth G, Bartha T, Frenyo LV, Naftolin F, Horvath TL, Zsarnovszky A (2014) Hypothalamic Sidedness in Mitochondrial Metabolism: New Perspectives. Reprod Sci 21:1492–1498.

Toth I, Goszleth G, Frenyo LV (2012) A táplálékfölvétel fő szabályzó molekulái: a grelin, a leptin és azok kölcsönhatása: Irodalmi összefoglaló Magyar Állatorvosok Lapja 134:(8) 504-512

Poster and oral presentations on international conferences:

Toth I, Szabo Cs, Jocsak G, Somogyi V, Kiss DS, Bartha T, Frenyo LV, Zsarnovszky A (2015) Impact of satiety state and testosterone on the metabolic lateralization in the male rat hypothalamus - MITT (2015) Budapest (poster presentation)

Toth I, Kiss DS, Jocsak G, Bartha T, Frenyo LV, Zsarnovszky A (2014) A possible way to decrease "crowdedness" through functional asymmetry in the hypothalamus FEPS Budapest (poster presentation)

Toth I, Kiss DS, Jocsak G, Bartha T, Frenyo LV, Zsarnovszky A (2014) Metabolic lateralization in the hypothalamus: possible mechanism for cramming multiple functions into a small place FENS Milano (poster presentation)

Kiss DS, **Toth I**, Jocsak G, Bartha T, Frenyo LV, Zsarnovszky A (2014) Mitochondrial metabolic lateralization in the rat hypothalamus MITT-IBRO Debrecen (poster presentation)

Zsarnovszky A, **Toth I**, Scalise TJ, Somogyi V, Kiss DS, Gyorffy A, Goszleth G, Bartha T, Frenyo LV (2012) Possible hypothalamic laterality in the central regulation of GnRH release: thoughts that might lead to a novel approach in hypothalamic studies IBRO Szeged (poster presentation)

Oral presentations on Hungarian national conferences:

Kiss DS, **Toth I**, Jocsak G, Goszleth G, Bartha T, Frenyo LV, Zsarnovszky A (2015) A jóllakottság és tesztoszteron hatása a hypothalamus metabolikus féloldaliságára hím patkányban Akadémiai beszámoló (oral presentation)

Toth I, Kiss DS, Bartha T, Frenyo LV, Zsarnovszky A (2014) Tápláltság-függő mitokondriális metabolikus aszimmetria patkányok hypothalamus-féltekéinek sejtjeiben. Akadémiai beszámoló (oral presentation)

Toth I, Somogyi V, Gyorffy A, Goszleth G, Zsarnovszky A (2013) Biszfenol A, arzén és zearalenon hatása fejlődő patkány kisagysejtjeinek ösztrogén- és pajzsmirigyhormon receptor mRNS-ének expressziójára. Akadémiai beszámoló(oral presentation)

Kiss DS, **Toth I**, Goszleth G, Somogyi V, Bartha T, Frenyo LV, Zsarnovszky A (2013) A mitokondriális metabolizmus hypothalamicus aszimmetriája. Akadémiai beszámoló (oral presentation)

Toth I, Kiss DS, Somogyi V, Goszleth G, Bartha T, Frenyo LV, Zsarnovszky A (2012) A hipotalamikus GnRH szekréció unilaterális szabályozása. Akadémiai beszámoló (oral presentation)

10.2. Publications not related to the topic of the present dissertation

Somogyi V, Horvath TL, **Toth I**, Bartha T, Frenyo LV, Kiss DS, Jocsak G, Kerti A, Naftolin F, Zsarnovszky A (submitted) Influence of bisphenol A on thyroid hormone receptors in rat cerebellar cell culture. J Expo Sci Environ Epidemiol

Somogyi V, Horvath TL, **Toth I**, Bartha T, Frenyo LV, Kiss DS, Jocsak G, Kerti A, Zsarnovszky A (2015) Glial modulation of estrogen receptor beta mRNA expression is influenced by individual and combined effects of bisphenol A, zearalenone, arsenic and camphor in the developing rat cerebellum. - MITT (2015) Budapest (poster)

Somogyi V, Jocsak G, **Toth I**, Kiss DS, Goszleth G, Bartha T, Frenyo LV, Zsarnovszky A, Sterczer A (2015) A hepaticus encephalopathia hatása a fejlődő idegrendszerre: az ösztrogén- és pajzsmirigyhormon-receptorok mrns expressziójának vizsgálata kisagyi sejttenyészeten Akadémiai beszámoló (oral presentation)

Mandoki M, Jocsak G, Somogyi V, Kiss DS, **Toth I,** Bartha T (2014) Use of virtual patients in teaching veterinary physiology at the Faculty of Veterinary Science, Szent István University, Budapest FEPS Budapest (oral presentation)

Jocsak G, **Toth I**, Somogyi V, Kiss DS, Bartha T, Frenyo LV, Zsarnovszky A (2014) Identification of a likely mechanism for endocrine disruption: Effects of bisphenol A on the expression level of estrogen- and thyroid hormone receptors in the developing cerebellum FENS (2014) Milano (poster presentation)

Toth I, Kiss DS, Jocsak G, Bartha T, Frenyo LV, Zsarnovszky A (2014) Effects of Bisphenol A on the regulation of estrogen- and thyroid hormone receptor expression in presence of estrogen and/or thyroid hormones on the developing cerebellum IBRO Budapest – (poster presentation)

Scalise TJ, Gyorffy A, **Toth I**, Kiss DS, Somogyi V, Goszleth G, Bartha T, Frenyo LV, Zsarnovszky A (2012) Ligand-induced changes in oestrogen and thyroid hormone receptor expression in the developing rat cerebellum: a comparative quantitative PCR and Western blot study. Acta Veterinaria Hungarica 60:(2) 263-284. (Peer reviewed article)

Zsarnovszky A, **Toth I**, Scalise TJ, Somogyi V, Gyorffy A, Kiss DS, Goszleth G, Bartha T, Frenyo LV (2012) Analysis of ligand-dependent changes in estrogen receptor- and thyroid hormone receptor mRNA and protein expression in the developing rat cerebellum MÉT (2011) Pecs (poster presentation)

Toth I, Scalise TJ, Gyorffy A, Kiss DS, Somogyi V, Goszleth G, Bartha T, Frenyo LV, Zsarnovszky A (2011) Comparative analysis and functional implications of ligand dependent changes in estrogen- and thyroid hormone receptor expression in the developing cerebellum IBRO, Firenze (poster presentation)

10.3. Supervising of DVM theses

Knyihar V: Kisméretű agyszövet-minták mitokondriális metabolizmusának vizsgálati módszere és annak gyakorlati jelentősége, TDK dolgozat, 2012, Supervisors: Kiss DS, **Toth I**

Nyitrai Berdin B.: A hypothalamus funkcionális aszimmetriája, TDK dolgozat, scheduled time: 2015, Supervisors: **Toth I**, Kiss DS

Pope H: Functional asymmetry of the hypothalamus, a literature review, DVM thesis, scheduled time: 2015, Kiss DS, **Toth I**

11. Appendix

(Reagent and equipment used in the experiments)

11.1. Reagents

Reagents for preparation of buffers and for the fractionation

BSA	bovine serum albumin; fatty acid free (!); Sigma, cat. no. A7511
EGTA	ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid, $C_{14}H_{24}N_2O_{10}$; RNA, DNA free (!); Sigma, cat. no. E3889
HEPES	HEPES potassium salt, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acidpotassium salt, $C_8H_{17}KN_2O_4S$; Sigma, cat. no. H0527
HCl	hydrochloric acid 37%; Carlo Erba, cat. no. 403871
KH_2PO_4	potassium phosphate monobasic, H_2KO_4P; Sigma, cat. no. P5655
KOH	potassium hydroxide; Sigma, cat. no. P1767
Mannitol	D-Mannitol, $C_6H_{14}O_6$; Sigma, cat. no. M9546
$MgCl_2$	magnesium chloride solution; Sigma, cat. no. M1028
Sucrose	α-D-glucopyranosyl β-D-fructofuranoside; $C_{12}H_{22}O_{11}$; Sigma, cat. no. S7903
Percoll	GE Healthcare, cat. no. 17-0891-01

Buffer solutions

To set pH 7.2 5M KOH and the 37% HCl solutions were used. The buffers were stored in a reagent bottle at 4°C for not more than a couple days.

Isolation buffer with EGTA	Isolation buffer without EGTA	Respiration buffer
215mM Mannitol	215mM Mannitol	215mM Mannitol
75mM Sucrose	75mM Sucrose	75mM Sucrose
0.1% BSA	0.1% BSA	0.1% BSA
1mM EGTA	20mM HEPES	2mM MgCl
20mM HEPES		2.5mM KH_2PO_4
		20mM HEPES

11.2. Equipment

- Filtration apparatus for preparation of Percoll solution applied with 90mm diameter Millipore AP15 prefilter (glass fiber filter; Millipore, Billerica, MA, USA; cat. no. AP1509000)
- pH meter (e.g. SevenEasy S20; Mettler Toledo; Schwerzenbach, Switzerland)
- Rodent guillotine (DCAP; Kent Scientific, Kent, UK)
- Nylon mesh (pore size 0.45µm, diam. 90mm; Sigma, cat. no. Z290785)
- Brain matrix (rat 175-300g, 0.5mm coronal, stainless steel; World Precision Instruments, Sarasota, FL, USA; cat. no. RBMS-300C)
- 10-15ml teflon-glass tissue grinder of type Potter-Elvehjem applied with a motor driven (capable of 500 and 800rpm.) pestle of 0.1–0.15mm clearance. (e.g. Wheaton (Millville, NJ, USA; distributed by Thermo Fisher Scientific, Waltham, MA, USA)
- 1.5ml transparent microcentrifuge tubes (Eppendorf tubes, natural; VWR International, Radnor, PA, USA; cat. no. 700-5239, 3810X)
- 2.0ml transparent conical microcentrifuge tubes (Eppendorf tubes, natural; Deltalab, Barcelona, Spain; cat. no. 4092.6N)
- Bench-top centrifuge capable of running a Beckman J2M1, J2-21, JA-20, Avanti J-26XPI or similar fixed angle rotor. We used a Hettich Universal 32 centrifuge (Hettich Instruments, Beverly, MA, USA) applied with a rotor no. 1689 (30 sleeve, fixed 45° rotor.

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