The role of estrogen in hypothalamic regulation of hypothalamus-pituitary-adrenal axis activity, energy homeostasis and bone metabolism

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Thesis, University of Amsterdam, The Netherlands

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The role of estrogen in hypothalamic regulation of hypothalamus-pituitary-adrenal axis activity, energy homeostasis and bone metabolism

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Chapter 1

General introduction
1. The hypothalamus-pituitary-gonadal axis

1.1 The HPG axis and synthesis of estrogen

Gonadotropin-releasing hormone (GnRH) is a neuropeptide stimulating the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland. In rodents, GnRH neurons are found primarily within the septal region, preoptic area, and anterior hypothalamus [1]. These neurons secrete GnRH within the median eminence into the portal blood, which is the vehicle for GnRH to reach the anterior pituitary. LH enters the systemic blood stream and finally stimulates estrogen release by the ovaries [2, 3]. Estrogens are key hormones in female reproduction. There are three subtypes of endogenous estrogens, i.e., 17β-estradiol (E2), estrone (E1) and estriol (E3), of which E2 is the predominant and biologically most active hormone. E2 is produced from testosterone by the aromatase enzyme cytochrome P45019 A1, encoded by the CYP19 gene [4]. This conversion occurs mainly, but not exclusively, in the ovary. Other organs, including the adrenal gland, liver, adipose tissue and mammary also produce small amounts of estrogen. In postmenopausal women, the contribution from these sources may become more important.

In women, plasma estrogen levels show profound fluctuations during the estrous cycle until endogenous estrogen levels decline to a much lower level after menopause. In rodents, the estrous cycle is divided into four stages: proestrus, estrus, diestrous and metestrous, and –like in women- plasma estrogen concentrations vary during the estrus cycle [5] (figure 1.1).

Aromatase cytochrome P450 is the enzyme that catalyzes the last and rate-limiting step of estrogen biosynthesis, that is, the irreversible aromatization of androgens to estrogens.

![Figure 1.1](image.png)

**Figure 1.1** Schematic illustration of fluctuation of plasma estradiol and progesterone concentrations in female rats during the estrous cycle. Estradiol concentrations are highest in proestrus, whereas progesterone levels are lowest.
Chapter 1

This enzyme was identified in the 1980s and its crystal structure was finally revealed in 2009 [6]. Aromatase is primarily expressed in granulose cells and in the corpus luteum of the ovary [7] in pre-menopausal women. In addition, extragonadal tissues such as adipose tissue [8], skin fibroblasts [9], bone osteoblasts and osteoclasts [10], skeletal and smooth muscle [11] and vascular endothelium [12] express aromatase. Generally, estrogen receptors are expressed in the same tissues that express aromatase. Recently aromatase was found to be abundantly expressed in the central nervous system, i.e. in the limbic system, preoptic nucleus, sexually dimorphic nucleus, bed nucleus of the stria terminalis, hippocampus, cerebellum [13], and in the pituitary [18] [14]. In the fetal hypothalamus, male rats express higher levels of aromatase than female rats [15].

1.2 Estrogen signaling

The genomic actions of estrogen are mediated by two distinct intracellular receptors that function as ligand-activated transcription factors. These are termed estrogen receptor alpha (ER\textsubscript{\alpha}) and beta (ER\textsubscript{\beta}), both belonging to the nuclear receptor family of transcription factors [16, 17]. Although ER\textsubscript{\alpha} and ER\textsubscript{\beta} are encoded by two different genes located on chromosomes 6 and 14, respectively, they share similar DNA-binding domains (96% homology) and similar ligand-binding domains (56% homology) and bind to the same hormone response element on DNA [17].

Both forms of ER contain five distinct functional domains, i.e. one ligand-binding domain, one transactivation domain, two DNA-binding domains and one variable domain. The binding of estrogen to ER results in receptor dimerization, binding to specific DNA sites in gene promoter regions known as estrogen response elements (ERE) and subsequent modulation of gene transcription [18]. Like other steroids, estrogen is passively absorbed by the cell, subsequently liganding and activating ERs. The binding of the complex of E2 and ERs to an ERE can affect >100 different types of gene transcription, the majority being direct targets of E2 [19]. The biological functions of E2 are largely determined by ER expression in target cells, since E2 can enter into most cell types. Both receptors are expressed in a wide range of tissues and cell types throughout the body. Tissues showing the highest expression are ovary, uterus and breast.

It is now widely accepted that some of the rapid functions of estrogen cannot be attributed to the classical nuclear-initiated steroid signaling of ER\textsubscript{\alpha} or ER\textsubscript{\beta}. An orphan G-protein-coupled receptor, GPR30, has been found to bind estrogen as well, and exhibits binding and signaling characteristics of a membrane ER [20, 21]. In breast cancer cells transfected with GPR30, estrogen activates the mitogen-activated protein kinases (MAPK), ERK1 and ERK2, and these actions are independent of ER\textsubscript{\alpha} or ER\textsubscript{\beta} [22, 23]. Based on these studies, Qiu et al. purified a membrane estrogen receptor (mER) dependent receptor: STX [24, 25]. STX (and E2) selectively target a Gq-coupled phospholipase C-protein kinase which can activate the Gq signaling pathway in mice lacking both ER\textsubscript{\alpha} and ER\textsubscript{\beta}[25].
1.3 Estrogen function domains

Estrogen exerts a wide range of functions in its target cells. It is, therefore, not surprising that estrogen is associated with a variety of pathophysiological conditions as well. Some of these will be shortly discussed in the following subparagraphs.

1.3.1 Estrogen and HPA-axis

The hypothalamus-pituitary-adrenal (HPA) axis is the final common pathway in the response to stress. The hypothalamus releases corticotropin-releasing hormone (CRH) in response to a stressor. CRH acts on the pituitary gland, triggering the release of adrenocorticotropin (ACTH) into the bloodstream, which subsequently causes the hormonal end product of the HPA-axis, i.e., corticosterone (cortisol in humans), to be released from the adrenal cortex. Sex hormones and the HPA axis interact at several levels [26]. First, the HPA axis shows a pronounced sex-difference in many species, both in the basal state [27-29] and during activation of the HPA-axis [30-32]. Second, the female menstrual cycle is a modulator of HPA-axis activity as the marked menstrual cycle-dependent differences in HPA activity are partly attributable to circulating sex steroid hormones [33-36]. Third, many studies showed that ovarian steroids increase HPA-axis activity [36-38], although other reports indicated that ovarian hormones may decrease certain aspects of the HPA-axis stress response [39, 40]. Finally, estrogen was reported to modulate CRH expression directly, while corticosterone was reported to inhibit GnRH release [26].

1.3.2 Estrogen and metabolism

The metabolic syndrome (MS) is characterized by obesity, dyslipidemia, hypertension, insulin resistance, and a prothrombotic and proinflammatory state. MS increases the risk for cardiovascular disease and type 2 diabetes mellitus (T2DM) [41]. The pathogenesis of MS is multi-factorial and may involve estrogen on the basis of its role in the regulation of appetite, energy expenditure, body weight and adipose tissue distribution [42, 43].

A few studies have shown a correlation between estradiol and several aspects of the MS [44]. Food intake in females varies across the menstrual cycle, with daily food intake being lowest during the peri-ovulatory period when estrogen levels are at a maximum [45]. Menopausal women tend to gain body fat, which appears to be a consequence of the decline in endogenous estrogens [46-48]. In animal models, ovariectomy induces an increase in food intake while decreasing ambulatory and wheel running activities, which can be reversed with estrogen replacement [49-52]. Therefore, hypo-estrogenic states are associated with decreased activity and increased body weight in females.

The development of specific knock-out mice for the ERs and aromatase has further substantiated the involvement of estrogens in aspects of MS. ERα null mice develop obesity and decreased energy expenditure [53]. Female aromatase knockout (ArKO) mice show reduced glucose oxidation and increased adiposity and insulin levels [54]. Male ArKO mice
develop glucose intolerance and insulin resistance which can be reversed by E2 treatment [55]. Men lacking aromatase also present with hyperinsulinemia [56].

1.3.3 Estrogen and bone

Osteoporosis is associated with an increased risk of fracture. In osteoporosis, bone mineral density (BMD) is reduced, bone microarchitecture deteriorates, and the content and variety of proteins in bone is altered. Multiple factors are involved in the pathophysiology of osteoporosis [57-59]. The reduction of endogenous estrogen levels after menopause is a major contributing factor to the accelerated bone loss and increased risk for osteoporotic fractures in postmenopausal women [60-63]. In female rats, OVX induces higher bone resorption, which is only partly compensated by higher bone formation, leading to an overall loss of cancellous bone [64, 65]. Some studies suggest that estrogen replacement therapy prevents osteopenia both in postmenopausal women [63, 66] and OVX rats [65, 67] by decreasing bone resorption, and to a lesser extent bone formation, with an overall improvement in bone balance. Other investigations indicate that estrogen treatment increases bone measurements related to bone formation in OVX [68] and intact rats [69].

2. Hypothalamic estrogen

The hypothalamus is located in the ventral part of the diencephalon [70]. Three rostral to caudal regions can be distinguished in the hypothalamus, which correspond to three prominent features on its ventral surface, i.e., the supraoptic region (anterior region), the tuberal region (middle region) and the mamillary region (posterior region).

The supraoptic region includes two prominent nuclei: the supraoptic and paraventricular nuclei (SON and PVN, respectively). The neurons in these nuclei synthesize many neuropeptides including vasopressin, oxytocin and CRH. CRH, produced in the PVN, reaches the portal system by axonal transport to the median eminence, and acts on the corticotrophes in the anterior pituitary to stimulate ACTH release into the general circulation. Vasopressin and oxytocin, released from the magnocellular neurons in the PVN and SON, reach the general circulation via axonal projections to the posterior pituitary. Vasopressin is responsible for regulating serum osmolality between narrow limits via variations in water reabsorption in the collecting ducts in the kidney. Oxytocin is best known for its roles in lactation and reproduction.

The tuberal region contains at least 3 important nuclei, the lateral hypothalamic area (LH), the ventromedial nucleus (VMH) and the arcuate nucleus (ARC). One important function of the VMH is the control of eating behavior. Bilateral lesions of the VMH in animals and probably humans as well, result in overeating (hyperphagia) and extreme obesity. On the other hand, bilateral lesions of the LH result in anorexia (lack of appetite). Thus, the VMH can be considered to be a satiety center and the LH to be a feeding center. Together
these two opposing centers define a “set point” for body weight. The ARC contains neuropeptide Y (NPY) and agouti-related protein (AGRP) containing neurons which promote food intake and pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) containing neurons that inhibit food intake. In addition, the ARC contains many neurons controlling neuroendocrine functions of the adenohypophysis. These neurons do not directly release their neuropeptides into the general circulation like the vasopressin and oxytocin neurons in the supraoptic region, but rather secrete releasing or release-inhibiting factors into the portal circulation that regulate the release of hormones produced by the adenohypophysis. The posterior part of hypothalamus is involved in many functions as well, including arousal and thermoregulation. Unlike the other hypothalamic nuclei, the nuclei in the posterior part of the hypothalamus do not appear to be closely related to autonomic and endocrine functions. The tuberomammillary nuclei (TMN) play a role in arousal and memory. Neuronal histamine shows a clear diurnal rhythm in rodents and plays a major role in the maintenance of vigilance. The diurnal fluctuation of histidine decarboxylase-mRNA expression in human TMN supports a role for neuronal histamine in regulating day-night rhythms [71]. Thus, the hypothalamus is a functionally heterogeneous structure and critical for various vital functions. Hypothalamic estrogen signaling can, therefore, interact with functionally diverse hypothalamic output, i.e. the HPA axis (PVN area), body weight regulation and adipose tissue metabolism (ARC area), glucose metabolism (PVN and VMH area) and insulin resistance (PVN, VMH and ARC).

2.1.1 Hypothalamus and HPA axis
The HPA axis is the final common pathway in the mediation of the stress response. As mentioned above the PVN contains corticotrophin-releasing hormone (CRH) producing neurons which, in response to a stressor, release CRH in the median eminence. Via a negative feedback, corticosterone inhibits CRH expression via glucocorticoid receptors (GR) located in PVN. Estrogen receptor β immunoreactivity (ERβ-IR) is colocalized with CRH-IR in neurons within the medial parvocellular PVN, and ERβ mRNA is found in CRH-IR neurons of the caudolateral PVN [72, 73]. In the human brain, however, ERα colocalises with CRH neurons in PVN. Moreover, an up-regulation of CRH and nuclear ERα was observed in mood disorders, both in men and women [74]. Five perfect half-palindromic estrogen responsive elements were found in the human CRH promoter, which indicates that estrogen can regulate the transcription activity of CRH. Indeed, in vitro evidence indicated a direct estrogenic regulation of CRH gene expression through those EREs [75]. By implanting E2 and ERα agonist 4,4’,4”-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) containing pellets into the PVN, stress induced c-fos mRNA expression was increased in OVX female rats, whereas it impaired the normal dexamethasone-induced suppression of neuronal activation in the PVN. These data indicated that in addition to a direct effect of
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estradiol on CRH expression [65], estradiol may also affect HPA axis activity via modulation of the local negative feedback actions of corticosterone.

2.1.2 Hypothalamus and metabolism

In the ARC, the NPY/AgRP neurons stimulate an orexigenic (appetite-stimulating) pathway which is suppressed by leptin and/or insulin signaling during a positive energy balance [76-78]. The PVN is the hypothalamic nucleus where multiple signals from the LH, VMH, DMH and ARC converge to control energy homeostasis. The PVN consists predominantly of four classes of cells. They produce: CRH, thyrotropin-releasing hormone (TRH), oxytocin (OXT) and vasopressin (AVP) which exert considerable influence over blood pressure and reproductive functions [79, 80]. Previous studies in our group have shown that a disinhibition of neuronal activity within the PVN, by local infusion of a GABA-antagonist, increases plasma glucose concentrations, indicating direct modulation of glucose metabolism by the PVN [81]. Further studies showed that these effects of PVN activation are mediated via the sympathetic innervation to the liver, ultimately resulting in an increased hepatic glucose production [81]. In line, CRH and TRH are known to be implicated in the control of energy homeostasis and feeding behavior [82, 83]. In another set of experiments we showed that local administration of T3 in the PVN increases hepatic glucose production via the autonomic nervous system [84]. The VMH is glucose sensitive and has effects on the regulation of glucose uptake. Electrical stimulation of the VMH in female rats induced a high glucose uptake in skeletal muscle, which was at least partly mediated by autonomic nervous system [85]. Also the direct administration of insulin within the VMH increased glucose uptake into skeletal muscle [86] while the administration of T3 activated brown adipose tissue by increasing sympathetic BAT input [87]. Moreover, the deletion of ERα signaling in the VMH induced obesity in rats [88]. Further studies found that female mice lacking ERα in the hypothalamic steroidogenic factor-1 (SF-1)-containing neurons display hypometabolism and abdominal obesity. This suggests that estrogen signaling in the VMH regulates energy metabolism via SF1 neurons [89].

2.1.3 Hypothalamus and bone metabolism

Estrogens exert their effects on bone metabolism via direct interactions with osteoblasts within the bone compartment. The osteoblast specific overexpression of aromatase increases bone mass without increasing plasma estrogen concentrations [90]. However, a novel and perhaps modulatory role for the CNS in the control of bone metabolism was recently proposed by Karsenty et al [91].

Currently it is well accepted that the crosstalk between the brain and bone comprises two distinct routes. The brain pathway consists of a number of neuro-endocrine signals arising from neuroendocrine neurons of the hypothalamus and subsequently processed within the pituitary. The second pathway comprises the hypothalamus-autonomic nervous system route. Interestingly, the neuropeptides that regulate energy metabolism are also heavily
involved in the control of bone metabolism (Figure 1.2). Leptin-deficient ob/ob mice show increased cancellous bone mass, which can be partly reduced by peripheral as well as central injections of leptin [92]. Gold thioglucose (GTG) lesions of the neurons in the VMH abrogate the effect of central leptin administration [93]. Further studies suggested that leptin affects bone resorption by increasing the expression of CART in the hypothalamus [94, 95]. Central NPY overexpression results in a reduction of bone mass, which is a remarkable contrast with the ob/ob mice that also show an increased NPY expression [81]. NPY null mice demonstrate a generalized bone anabolic phenotype [96], which can be partly corrected by hypothalamic NPY replacement.

### 2.2 Hypothalamic estrogen signaling

The human hypothalamus shows abundant aromatase expression [97]. In rats, however, there is a lack of experiments investigating the central presence and distribution of aromatase expression, although local fluctuations of estrogen in the parabrachial nucleus have been reported during stroke [98, 99]. Additional evidence does support brain derived estrogen release independent from the systemic circulation [100, 101]. As an example, Remage-Healey et al showed an acute inhibition of estrogen production within a cortical-like region involved in complex auditory processing in songbirds [100]. The same group later found that rapid local estrogen production is regulated by the neurotransmitters glutamate and GABA [25]. The local change of hypothalamic estrogen in songbirds and rats may sort many effects via the two classical receptors (ERα and ERβ). Both are expressed throughout the rostral-caudal extent of the brain and spinal cord of rats, but striking local differences.
in the expression pattern can be found in certain brain areas. For instance, in rats, the ventromedial hypothalamic nucleus (VMH) and subfornical organ only ERα is expressed. In contrast, neurons of the olfactory bulb, SON, PVN and suprachiasmatic nucleus (SCN) contain almost exclusively ERβ, although some ERα expression has also been found in the periPVN. Finally, both receptors are expressed in the ARC, but the relative expression of ERα is much higher than that of ERβ [72, 102-105] (Figure 1.3).

Figure 1.3 The expression of ERs in the rodent hypothalamus. Estrogen receptor α is abundantly expressed in both ARC and VMH, but shows a low expression in the periPVN. ERβ on the other hand is mainly detected in the PVN and ARC, but not in the VMH.

3 Scope of thesis

The main question we want to answer in this thesis is whether hypothalamic estrogen can modulate physiological responses independently from circulating estrogen levels, and -if so- what is the neuro-anatomical substrate for these actions. We specifically focused on four physiological systems: the HPA axis response to stress, glucose metabolism, body fat distribution and bone mass.

First, we investigated whether acute stress affects local hypothalamic estrogen concentrations. In chapter 2, we used the microdialysis technique to measure estrogen concentrations locally in the PVN, both in intact and OVX animals during acute restraint stress. Estrogen related gene expressions in the PVN were investigated using qPCR. The finding of altered local estrogen concentrations during stress, resulted in the study described in chapter 3. We now aimed to investigate whether, conversely, local changes in estrogen concentration in the hypothalamus modulate HPA axis activity. In the second part of the thesis, we investigated the effects of hypothalamic estrogen on energy
metabolism. In chapter 4, we investigated the effects of hypothalamic estrogen on glucose metabolism by local administration of estrogen in specific hypothalamic nuclei combined with euglycemic hyperinsulinemic clamps, and selective hepatic autonomic denervations. In chapter 5, we investigated the effects of central estrogen on the distribution of body fat. We used MRI scanning to monitor the effects of peripheral and central estrogen treatment on body fat distribution in OVX rats. In addition, we investigated the effects of local hypothalamic estrogen administration on gene expression in adipose tissue. In part three of the thesis, we investigated the effects of central estrogen on bone metabolism. In chapter 6, OVX animals were treated with estrogen, either i.c.v or subcutaneously, and immunohistochemistry and Q-PCR was used to investigate effects on bone osteoblasts and bone resorption.
Reference


Chapter 1


General introduction


Chapter 1


Acute Restraint Stress Increases Intrahypothalamic Oestradiol Concentrations in Conjunction with Increased Hypothalamic Oestrogen Receptor β and Aromatase mRNA Expression in Female Rats
Acute Restraint Stress Increases Intrahypothalamic Oestradiol Concentrations in Conjunction with Increased Hypothalamic Oestrogen Receptor β and Aromatase mRNA Expression in Female Rats

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Activation of the hypothalamic-pituitary-adrenal axis is considered to be one of the key physiological responses to stress and, interestingly, shows a marked sex difference. Oestradiol plays an important role in this sex difference. The present study investigated the systemic and intrahypothalamic oestradiol response to physical restraint stress in female rats. We used jugular catheterization and intrahypothalamic microdialysis to simultaneously measure plasma oestradiol and local oestradiol concentrations in the paraventricular nucleus (PVN) of the hypothalamus. We also assessed corticotrophin-releasing hormone (CRH), aromatase, and oestrogen receptor (ER) α and β mRNA expression in the PVN by quantitative polymerase chain reaction immediately after the acute stress period. As expected, PVN CRH mRNA and plasma corticosterone were significantly increased after acute stress. Interestingly, the local oestradiol concentration in the PVN also increased during the 1-h stress period in pro-oestrus and in ovariectomised (OVX) animals. Aromatase mRNA expression in the PVN was increased markedly in pro-oestrus but only modestly in oestrus. PVN ERβ but not ERα mRNA expression was significantly elevated in prooestrous animals. In addition, plasma oestradiol levels increased 10 min after stress, both during pro-oestrus and oestrus but not in OVX animals. To conclude, we report an intra-hypothalamic oestradiol response to restraint stress. The rising hypothalamic oestradiol concentration together with increased ERβ gene expression indicates a positive feedback of hypothalamic oestradiol signaling during acute stress in rats.

Key words: oestradiol, stress, HPA axis, oestrogen receptor, microdialysis.
Introduction

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is considered to be a characteristic physiological response to stress. Interestingly, the activity of the HPA axis shows a pronounced sex difference in many species, both in the basal state (1–3) and during activation of the HPA axis by a variety of stressors (4–6). Moreover, the female menstrual cycle is a modulator of HPA axis activity. In spite of similar baseline adrenocorticotrophic hormone and cortisol plasma levels in the early follicular and mid-luteal phase of the menstrual cycle, there is a pronounced difference in HPA axis responsivity across the menstrual cycle after stress exposure (7,8).

The pronounced gender- and menstrual cycle-dependent differences in HPA activity are partly attributable to circulating sex steroid hormones (9–12). Oestradiol and androgen can alter HPA axis activity directly (13,14) or indirectly (15–17). There is a great amount of experimental data showing that ovarian steroids increase HPA axis activity (12,18,19), although there are other reports indicating that ovarian hormones may decrease certain aspects of the HPA axis stress response (20,21).

Conversely, the HPA axis affects the hypothalamic-pituitary-gonadal (HPG) axis. Corticotrophin-releasing hormone (CRH) fibres from the paraventricular nucleus in the hypothalamus not only project to the portal capillaries, but also to gonadotrophin-releasing hormone (GnRH) neurones in the preoptic area (22). It is well established that HPG axis activity is inhibited by various components of the HPA axis (23–25). Physiological experiments have shown that stress exposure decreases plasma oestradiol in female rats (26). Others have reported that sex steroid levels are elevated during stress exposure (27).

Although plasma oestradiol can increase HPA axis activity, it is unknown at present how local oestradiol bioavailability in the brain responds to acute stress. In addition, it is unknown whether local oestradiol bioavailability is a determinant of altered HPA axis activity. To explore this, we used the microdialysis technique to investigate local hypothalamic changes in oestradiol concentration in the extracellular fluid surrounding the paraventricular nucleus (PVN) of the hypothalamus in response to stress. We also analysed the hypothalamic expression of oestrogen receptor (ER) and aromatase mRNA upon stress exposure. Our findings reveal the PVN as a potential site for the integration of stress-induced oestradiol effects on HPA axis activity.

Materials and methods

Animals

Adult female Sprague–Dawley rats (8–10 weeks) (SLAC Laboratory Animal, Shanghai, China) were housed under a 12 : 12 h light/dark cycle (lights on 07.00 h) at 23±2 °C. Animals were allowed to adapt to the new environment for 1 week before beginning
the experiments. Animals were housed four to six per cage until 1 week before surgery, at which time they were transferred to individual cages (25×25×50 cm). Food and water were available ad lib. Animals weighed 250–300 g at the time of the experiments. Rats were allowed 1-week of post-operative recovery before the start of the experiment. All experiments were performed between 13.00 h and 15.00 h. To minimize the animals’ suffering, all experimental procedures were carried out in accordance with the approval of the Animal Care Committee of the University of Science and Technology of China and in accordance with legislation.

Surgical procedures

Experiment 1 was aimed at investigating the effect of stress on local oestradiol concentrations in the PVN and included a surgical procedure to place the jugular catheter and microdialysis probes. For Experiment 2, only a jugular catheterisation was needed to measure plasma oestradiol during stress. Experiment 3 was designed to investigate mRNA levels in the PVN and did not include any surgery. Experiment 4 was designed to investigate the local hypothalamic and systemic plasma oestradiol changes during stress exposure in ovariectomised (OVX) rats, and again included the surgical placement of a jugular vein catheter and microdialysis probes.

Experimental animals destined to undergo infusion and blood-sampling studies were fitted with bilateral microdialysis probes and / or an intra-atrial silicone catheter through the right jugular vein when their body weight reached 250-300 g (28). Rats were anesthetised using chloral hydrate (7%, 1 ml / 100 g body weight). During experiments, animals were permanently connected to blood-sampling and microdialysis lines, which were attached to a metal collar and kept out of reach from the rats by means of a counterbalanced arm. This allowed all manipulations to be performed outside the cages without handling the animals. The metal collars were attached at least 12 h before the actual experiment. To familiarise the animals with the experimental procedures, they were handled and sham blood was sampled (i.e. blood was withdrawn and immediately returned) regularly in the week before the first experiment. The bilateral microdialysis probes were stereotactically implanted directly lateral to the PVN (-1.8 mm anterioposterior, +2.0 mm lateral, angled at 10° and -8.1 mm ventral from the dura). The loop of the microdialysis probe was positioned in the rostrocaudal direction. The probe location was checked after sacrificing the animals (Figure 2.1). Jugular catheterisation was performed according to the method of Steffens (29). We used dental cement to secure the probes and the atrial outlet to two stainless-steel screws inserted into the skull.

Physiological experiments

To identify the phase of the oestrous cycle, we used the vaginal smear method. A precise description of the different subphases is provided by Thung (30). Briefly, pro-oestrous
Stress increases hypothalamic estrogen

Smears contained only nucleated and cornified cells, oestrous smears contained exclusively cornified cells, and metoestrous smears contained large numbers of leukocytes in clumps or dispersed in smeary mucous with nucleated epithelial cells and some cornified cells. During dioestrus, leukocytes were the predominant type of cells, although they were less abundant in the smears than during metoestrus and they were accompanied by a few epithelial nucleated and cornified cells and by pleomorphic cells with large dark stained nuclei.

The input port of the dialysis probe was connected to a remote syringe via a fluid swivel (375/22; Instech Laboratories, Plymouth Meeting, PA, USA) interconnected with polyethylene tubing. The syringe contained Ringer’s solution. Perfusion of the microdialysis probes was performed by a syringe pump (Harvard Apparatus, Holliston, MA, USA). Ringer’s perfusion (2 µl/min) began 1 h before the start of the stress period, lasting another 1 h during the period of stress. The outlet port of the dialysis probe was connected to polyethylene tubing ending in a 1-ml Eppendorf. Two separate dialysis samples were collected, one before stress and one during stress (each 1 h). Blood samples (0.4 ml) were taken before stress and 60 min (Experiment 1) or 10, 30 and 60 min (Experiment 2) after the onset of the stress period. In Experiment 3, animals were sacrificed at t = 0 or 30 and 60 min after the onset of the restrain stress. For Experiment 3, only oestrous and prooestrous animals were used. The restrain stress was performed in a rat fixed tubular gantry (Huaibei Zhenghua Bioinstrumentation, Anhui, China).

Microdialysis probes
Dialysis probes were constructed according to procedures described previously (28). Thin platinum wire (diameter 0.05 mm) was inserted into a piece of microdialysis tubing (molecular weight cut-off, 6000 kDa) and bent; each end was then inserted into one of two pieces of 25-gauge hypodermic tubing that had been soldered together. The dialysis tubing was then secured with epoxy. The U-shaped tip of the microdialysis probe was 1.5 mm long, 0.7 mm wide and 0.2 mm thick.
Chapter 2

Hormone assays

Blood samples were immediately chilled on ice in tubes containing a 5-μl solution of heparin and centrifuged (at 4000 rpm, 15 min) at 4 °C. Plasma was then stored at -80 °C until additional analysis. Dialysis samples were collected and immediately stored at -80 °C. Dialysis and plasma oestradiol concentrations were determined using an oestradiol enzyme-linked immunosorbent kit (sensitivity range: 5 ± 2–1000 pg/ml, inter-assay variation: 6.0%, intra-assay variation: 3.9%) (BioSource, Nivelles, Belgium). Plasma corticosterone concentrations were determined by a radioimmunoassay kit (sensitivity range: 10–9000 ng/ml, inter-assay variation: 6.5%, intra-assay variation: 3.4%) (MP Biomedicals, Eschwege, Germany).

Quantitative-polymerase chain reaction (Q-PCR)

Rats were anaesthetised and then decapitated 30 or 60 min after onset of the restraint stress, or without stress, and their brains were removed quickly. Hypothalamic parts were dissected according to Yasin et al. (31) using the boundaries: anterior border of the optic chiasm, anterior border of the mammillary bodies, the lateral hypothalamic sulci, and the dorsal apex of the third ventricle. The thickness of the hypothalamic slice was approximately 3 mm. In a first experiment, we performed Q-PCRs on the whole hypothalamic block (see Supporting Information, Fig. S1). In a second experiment, we confined our Q-PCR analysis to the PVN tissue that had been obtained by a punch from the hypothalamic block with a hollow needle (20 gauge; BD Microlance; Becton Dickinson, Fraga, Spain) (32). The punched tissue was quickly frozen in liquid nitrogen and preserved at -80 °C. Each frozen PVN was homogenised, and total RNA was isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesised using reverse transcriptase (Promega, Madison, WI, USA). Q-PCR was performed using SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 7000 Sequence Detector system in 30 μl volume (15 s at 95 °C and 60 s at 64 °C, 40 cycles, 95 °C 5 min, 60 °C 5 min was allowed for the melt curve). Primer sequences for amplification are indicated in Table 2.1. The PCR product was run on 1.5% agarose gel electrophoresis to check the specific amplification. The relative amount of target gene in relation to the housekeeping gene was calculated using the 2[−delta delta C(T)] method as described by Livak and Schmittgen (33). The relative amplification efficiencies of the primers were tested and shown to be similar.

Table 2.1. The sequence of amplify primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta actin</td>
<td>TTGCTGACAGGATGCAGAA</td>
<td>ACCAATCCACACAGATCTACCTT</td>
</tr>
<tr>
<td>CRH</td>
<td>CAGAACAAAAAGGCGGGCTCA</td>
<td>AAGGCAGACAGGGCGACAGAG</td>
</tr>
<tr>
<td>Aromatase</td>
<td>TCTTCGGATCTGGAATTTGTG</td>
<td>GCGCCGATTCCACAGACA</td>
</tr>
<tr>
<td>ER α</td>
<td>CCAAGGCCCTGGGAATTGG</td>
<td>AGCTCGGGGGGCTAGAG</td>
</tr>
<tr>
<td>ER β</td>
<td>TTGCTGGAACAGATCATCAGAG</td>
<td>AACAGGGCTGGGCACAACTG</td>
</tr>
</tbody>
</table>
Statistical analysis
For statistical analysis, we used SPSS, version 17.0. (SPSS Inc., Chicago, IL, USA). All results were initially analysed by ANOVA with repeated measures or by two-way ANOVA. If appropriate, further post-hoc tests were performed with one-way ANOVA, and paired or unpaired Student’s t-tests. P < 0.05 level was considered statistically significant. Data are expressed as the mean±SEM.

Results
Local oestradiol concentration in the PVN during a 1-hr restraint stress
Stress significantly affected local oestradiol concentrations in the PVN (stress, P = 0.002) (Figure 2.2). The stimulatory effect of stress was independent of the oestrous cycle because there were no significant effects of Cycle (P = 0.11) or the Cycle×Stress interaction (P = 0.314). However, when zooming in on the different oestrous stages, only the increase during pro-oestrus reached significance (P = 0.02), whereas, during oestrus, the increase just missed significance (P = 0.06). In metoestrus and dioestrus rats, the increase in the local PVN levels of oestradiol did not reach significance (P = 0.36 in metoestrus and P = 0.29 in dioestrus).

![Figure 2.2 Increased PVN estradiol concentrations during 1hr restraint stress in intact rats. The open bars indicate estradiol release in the 1-h period before the onset of the stress exposure. Black bars indicate estradiol release during the 1-h restraint exposure. Stress: F(1,16)=13.1, P=0.002; Cycle: F(3,16)=2.3, P=0.110; Stress x Cycle: F(3,16)=1.28, P=0.314. Post-hoc analysis was performed with paired t-tests. Data represent mean±S.E.M. (n=5 each group).](image)

Plasma oestradiol concentrations during 1-h restraint stress
Plasma oestradiol concentrations changed significantly when stress exposure occurred during oestrus and pro-oestrus (in both groups the effect of Time (P < 0.001)) (Figure 2.3A) but not during metoestrus and dioestrus (P = 0.838 and P = 0.844) (Figure 2.3B). The significant effects of Cycle (P < 0.001) and Cycle× Time (P < 0.001) are also evidenced by the different responses to stress depending on cycle stage. Moreover, in pro-oestrus
rats, plasma oestradiol concentrations increased 10 min after the start of the restraint stress (P = 0.003), and decreased again afterwards (P = 0.003 when comparing t = 10 min and t = 30 min) (Figure 2.3A). On the other hand, in oestrous rats, plasma oestradiol concentrations increased after 10 min of restraint stress (P < 0.001) but remained elevated afterwards (Figure 2.3A).

Figure 2.3 Plasma estradiol response to stress in intact animals. Time: F(3,18)=19.13, P<0.001; Cycle: F(3,20)=79.32, P<0.001; Time x Cycle: F(9,60)=19.21, P<0.001. Post-hoc analysis was performed with paired t-tests. Data represent mean±S.E.M. (n=6 each group) The Time effect in detail: Proestrus: F(3,3)=13.85, P=0.029; Estrus: F(3,3)=23.3, P=0.014; Metestrus: F(3,3)=0.777, P=0.58; Diestrus: F(3,3)=0.195, P=0.893.

Plasma corticosterone and hypothalamic CRH mRNA in response to restraint stress
Plasma corticosterone concentrations changed significantly during stress exposure (P < 0.001) (Figure 2.4). Again, these changes differed depending on the stage of the oestrous cycles reflected by significant effects of Cycle (P < 0.001) and the Cycle×Time interaction (P < 0.001). Post-hoc analysis showed that plasma corticosterone concentrations were significantly different between groups during the basal condition (P = 0.033), as well as 10 and 30 min after stress (P = 0.046, P = 0.029) but not 60 min after stress (P = 0.091). CRH mRNA expression in the PVN showed a significant increase during stress exposure (P < 0.001). This increase did not depend on the stage of the oestrous cycle (P = 0.108). The
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Stress-induced expression of CRH mRNA was significant in both oestrous and pro-oestrous animals (P = 0.006 in pro-oestrus and P = 0.007 in oestrus). Post-hoc analysis showed that already 30 min after the onset of stress CRH mRNA had increased significantly both in pro-oestrus and oestrous rats compared to the nonstressed control groups (P = 0.014 in pro-oestrus and P = 0.046 in oestrus) (Figure 2.5A).

Aromatase mRNA levels are increased after stress

PVN aromatase mRNA expression changed significantly after stress (P = 0.018). ANOVA did not show a significant effect of Cycle (P = 0.869) or the Cycle×Time interaction (P = 0.317). However, separate analysis of the two oestrous stages by ANOVA revealed a significant effect of Time in the pro-oestrous group (P < 0.001) but not in the oestrous group (P = 0.235). Furthermore, post-hoc analysis showed that, in the pro-oestrous group, the increase of PVN aromatase mRNA expression reached significance after 1 h of restraint stress (P = 0.002) but not yet after 30 min (Figure 2.5B).
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ER mRNA levels

PVN ERb mRNA expression changed significantly during stress exposure (P = 0.004) but no significant effects of Cycle (P = 0.067) or the Cycle×Time interaction (P = 0.437) were detected. ANOVA analysis of the separate stages revealed a significant effect of Time in pro-oestrous (P = 0.001) but not in oestrous (P = 0.189) animals. Pro-oestrous rats showed a significant increase in the ERb mRNA level, both after 30 min (P = 0.015) and 60 min (P = 0.001) of restraint stress (Figure 2.5C). ERa expression in the PVN was below detection levels.

**Figure 2.5** Stress-induced changes in PVN mRNA expression during proestrus and estrus.

A. CRH mRNA expression: Time: F(2,9)=25.41, P<0.001; Cycle: F(1,10)=3.11, P=0.108; Time x Cycle: F(2,20)=0.642, P=0.537.

B. Aromatase mRNA: Time: F(2,9)=6.532, P=0.018; Cycle: F(1,10)=0.029, P=0.869; Time x Cycle: F(2,20)=1.217, P=0.317.

C. ER-β mRNA expression: Time: F(2,9)=10.74, P=0.004; Cycle: F(1,10)=4.24, P=0.067; Time x Cycle: F(2,20)=0.862, P=437. Post-hoc analysis was performed with one way ANOVA. Data represent mean±S.E.M. (n=6 for each group).
Oestradiol concentration changes locally in the PVN but not in plasma during stress exposure of OVX animals.

The local PVN oestradiol concentration was significantly increased ($P = 0.002$) by the 1-h stress exposure (Figure 2.6A) but no significant changes in plasma oestradiol were detected ($P = 0.386$) (Figure 2.6B). Restraint stress induced a significant elevation of plasma corticosterone ($P < 0.001$) (Figure 2.6C).

**Figure 2.6** Increased estradiol concentration in the PVN, but not in systemic plasma by stress exposure.

A. Local PVN estradiol concentration before and during restraint stress. Student $t$-tests (paired) shows a significant increase in local PVN estradiol concentration during stress exposure ($P=0.002$) ($n=6$).

B. Plasma estradiol concentration during stress exposure. ANOVA showed no significant effect of Time ($F(3,20)=1.065$, $P=0.386$). Data represent mean±S.E.M. ($n=6$).

C. Plasma corticosterone concentration during stress exposure. ANOVA showed a significant effect of Time ($F(3,19)=31.34$, $P<0.001$) ($n=6$).
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Discussion

The key findings of the present study are that: (i) the local bioavailability of oestradiol in the PVN is increased during restraint stress; (ii) relevant molecular components for oestradiol action in the PVN, such as ER\(\beta\) and aromatase mRNA, are up-regulated during restraint stress; and (iii) these stress-induced changes are oestrous cycle-dependent.

One of the major confounders in studies on sex differences in the response of the HPA axis is the plasma concentrations of sex hormones, and possibly sex hormone bioavailability at the level of the hypothalamus. Microdialysis enabled us to investigate the local changes of extracellular oestradiol concentrations in the PVN. We found that stress had a stimulatory effect on local PVN oestradiol levels, although the increase only reached statistical significance in pro-oestrous female rats. After a short-lived increase, peripheral oestradiol concentrations decreased 30 min after stress onset in pro-oestrous animals, which is the opposite of the changes we found in the PVN. These data indicate that differential effects of stress on oestradiol concentrations are present for the central nervous system and the circulation. We found significant changes in local PVN oestradiol concentrations in pro-oestrous animals. In the intact animal, however, oestradiol is produced in abundance by the ovaries and can pass readily through the blood–brain barrier. To further differentiate the effects of stress on central and peripheral oestradiol concentrations, we repeated the stress exposure in OVX animals. Again, in the OVX animals, local PVN oestradiol concentrations showed a significant rise, whereas plasma oestradiol concentrations did not change significantly during the stress exposure. In addition, although plasma oestradiol concentrations decreased by > 50% after OVX, local PVN concentrations of oestradiol in OVX animals were comparable to those of intact animals during metoestrus. These results thus support an independent role for local PVN oestradiol as opposed to oestradiol from the general circulation.

Remage-Healey et al. (34) reported that local oestradiol concentrations in the cortex of zebra finches fluctuate rapidly during social interactions. Our findings are the first to show rapid changes of oestradiol concentrations in a localised mammalian brain area in the female rat during stress exposure (i.e. the PVN). The changes in oestradiol concentration in the PVN were accompanied by increased aromatase mRNA, further supporting that increased extracellular oestradiol concentrations in the PVN result from a local increase in oestradiol production. Aromatase expression and activity in the hypothalamus are mediated by a number of factors, such as androgens (35), glucocorticoids (36–38), GnRH (39), insulin (40) and excitatory amino acid transmitter-dependent pathways (41). Because the stress response includes a complex ensemble of hormone releases and neuronal activation, it is difficult to determine the exact mechanism responsible for the increased expression of aromatase during stress exposure. However, there are three main candidates in the hypothalamus. First, recently, Yague et al. (42) reported that dexamethasone up-regulates aromatase transcription in glioma cells. Thus, the increased circulating...
concentrations of corticosteroids during stress are a possible candidate to mediate the observed up-regulation of aromatase expression. Second, Liang et al. (39) reported that GnRH can regulate aromatase activity. Thus, the increased expression of GnRH (see Supporting Information, Fig. S2) in our experiments could contribute to the increased expression of aromatase. Finally, it is well known that stress activates different populations of glutamatergic neurones, and the increased release of excitatory amino acid transmitters could contribute to the increased aromatase expression (41).

Previously, our group showed that the nuclear ER is co-localised with CRH neurones in the human hypothalamic PVN. In addition, an up-regulation of CRH and nuclear ERa was observed in mood disorders (43). The results obtained in the present study show an up-regulation of ERb mRNA during a 1-h stress exposure in pro-oestrous animals. In the rat PVN, ERb (44,45) expression is more pronounced than ERa expression (46). In vivo animal experiments have shown that the administration of oestradiol and ERa selective agonists increase HPA axis activity, whereas ERb decreases its activity (47). It has therefore been suggested that the balance between the activity of ERa and ERb pathways may be important for the regulation of HPA axis activity during stress (48). Although the expression level of ERb in neuroendocrine CRH neurones is relatively low in rodents, there are many ERb-expressing neurones that co-express arginine vasopressin or oxytocine, both of which are involved in the regulation of HPA axis activity (49,50).

In addition to hypothalamic oestradiol, peripheral oestradiol concentrations showed an instant increase after stress as well. The mechanism of this quick response to stress is unclear at this stage but it is consistent with previous studies (27,51). After the initial response, however, the increase did not persist. One possibility is that the rising plasma corticosterone concentrations inhibit the synthesis and release of oestradiol because an inhibitory effect has been reported in several studies (24). However, we are aware that these are only correlatively data and further experiments are needed to confirm the inhibitory effect of corticosterone in our experimental conditions.

In humans, the responsivity of the HPA axis to a stressor differs across the menstrual cycle (52,53). In the present study, stress exposure increased plasma corticosterone concentrations during all oestrous cycles. However, different stages of the oestrous cycle showed significant differences in basal and stress-induced plasma corticosterone concentrations. The results of the present study are consistent with previous studies in rodents showing increased basal and stress-responsive corticosterone concentrations during pro-oestrus (54–57). The results also show that local production of oestradiol as well as aromatase and ERb mRNA expression in the brain was significantly changed only during pro-oestrus, and not during oestrus. In addition, the changes in plasma oestradiol concentrations differed between pro-oestrus and oestrus animals. These differences suggest that the response of the HPG axis to stress is higher when basal circulating plasma oestradiol concentrations are maximal.
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As for the apparently inconsistent findings with previous reports, we note that exposure to stress will change blood flow and blood–brain barrier permeability (58,59), which could affect the amount of circulating 17β-oestradiol in the extracellular fluid. However, this might explain our findings only if these changes are brain region specific because they are unable to explain localised changes in specific brain regions (60). Second, we investigated only mRNA expression in the PVN. Clearly, changes in mRNA expression do not necessarily reflect a change in protein level. Therefore, especially with regard to the reported changes in aromatase expression, further studies are needed to verify whether the increased mRNA expression translates into increased aromatase activity and a subsequent increase in oestradiol production.

In conclusion, the results obtained in the present study show that hypothalamic extracellular oestradiol concentrations, as well as ERb and aromatase mRNA expression, are increased in the PVN during acute restraint stress exposure in female animals. These changes appear to be modulated by the stage of the oestrous cycle, although the PVN increase in extracellular oestradiol persists in OVX animals. Our findings indicate that a positive feedback of oestradiol may be involved in the control of the HPG response to stress in female animals. These results have implications for the interpretation of changes in stress responsivity and mood fluctuations during the menstrual cycle in humans.

Acknowledgements

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Supplemental data figure 1

The relative mRNA expression level of the CRH, aromatase, ERα and ERβ genes before and after 30 min or 60 min of restrain stress in a hypothalamic tissue block.

A. The stress-induced increased expression of CRH mRNA was significant in both estrus and proestrus animals ($P=0.044$ in proestrus and $P=0.034$ in estrus according to one-way ANOVA).

B. A significant increase of hypothalamic aromatase mRNA expression after 1-h of restraint stress in the proestrus group ($P=0.029$; one-way ANOVA) was detected.

C. ERα expression in the hypothalamus did not change significantly during stress exposure ($P=0.348$; one-way ANOVA).

D. After 1-h of restraint stress, proestrus rats showed a significant increase in the ERβ mRNA level ($P=0.015$; one-way ANOVA).

The P-values in the figures indicate the result of post-hoc student t-tests (unpaired).
Supplemental data figure 2

The relative mRNA expression level of the GnRH gene before and after 30 min or 60 min of restrain stress in a hypothalamic tissue block.

GnRH mRNA expression was significantly elevated by 1hr stress in proestrus animals ($P=0.034$), but not in estrus animals ($P=0.058$).

The P-values in the figures indicate the result of post-hoc student t-tests (unpaired).
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Intrahypothalamic estradiol modulates hypothalamus-pituitary-adrenal axis activity in female rats
Intrahypothalamic estradiol modulates hypothalamus-pituitary-adrenal axis activity in female rats

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Key words: microdialysis, estradiol, HPA axis

Abstract

Estrogen plays an important role in the regulation of the hypothalamus-pituitary-adrenal (HPA) axis, but the neuroendocrine pathways and the role of ER subtypes involved in specific aspects of this interaction remain unknown. In a first set of experiments, we administered estradiol intravenously (i.v.), intracerebroventricularly (i.c.v.) and by intra-hypothalamic microdialysis to ovariectomized rats to measure plasma corticosterone (CORT) concentrations from carotid artery blood. Systemic infusion of estradiol did not increase plasma CORT, but i.c.v. estradiol induced a 3-fold CORT increase (p=0.012). Local estradiol infusions in the hypothalamic paraventricular nucleus (PVN) significantly increased plasma CORT (p<0.001). A similar CORT increase was seen after PVN infusion of the estrogen receptor α (ERα) agonist propylpyrazoletriol (PPT), whereas the estrogen receptor β (ERβ) agonist diarylpropionitrile (DPN) had no effect. In a second set of experiments we investigated whether estradiol modulates the HPA-axis response to acute stress by administering estradiol agonists or its antagonist ICI 182,780 (ICI) into the PVN during restraint stress exposure. After 30 minutes of stress exposure, plasma CORT had increased 5.0-fold (p<0.001). Estradiol and PPT administration in the PVN enhanced the stress-induced plasma CORT increase (8-fold versus baseline), whereas ICI and DPN reduced it, as compared to both estradiol and vehicle administration in the PVN. In conclusion, central estradiol modulates HPA-axis activity both in the basal state and during restraint stress. In the basal condition the stimulation is mediated by ERα sensitive neurons, whereas during stress it is mediated by both ERα and ERβ.

Key words: hypothalamus, stress, corticosterone, HPA-axis, estrogen, estrogen receptor
Introduction

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is considered to be a characteristic physiological response to stress. Interestingly, the activity of the HPA axis shows a pronounced sex difference. Compared to male rats, female rats have an increased basal corticosterone (CORT) secretion [1, 2]. In addition, female rats showed a higher stress response than male rats including increased ACTH and CORT responses [2-4]. Moreover, the female menstrual cycle is a modulator of HPA axis activity. In spite of similar ACTH and cortisol plasma levels in the basal condition during the early follicular and mid-luteal phase of the menstrual cycle, there is a pronounced difference in HPA axis responsivity across the menstrual cycle after stress exposure[5, 6]. Estrogen is thought to play a causal role in the gender or menstrual cycle dependent differential HPA axis responses, as ovariectomy (OVX) reduces basal CORT levels, while estrogen replacement restores basal plasma CORT concentrations [7, 8].

Estrogen receptors (ER) are expressed throughout the whole body including the brain. In the hypothalamus, ER expression has been reported in several nuclei, such as the paraventricular nucleus (PVN), the ventromedial hypothalamus (VMH), the lateral hypothalamus (LH) and the arcuate nucleus (ARC) [9, 10]. The PVN represents the central neuroendocrine setpoint of the HPA axis, balancing the secretion of two neuropeptides, i.e., corticotropin-releasing hormone (CRH) and vasopressin (AVP), during stress [11]. In the rat PVN, predominant ERβ expression was found with rather limited ERα expression [9, 12]. In line with these findings, we recently demonstrated an increased PVN expression of ERβ, but not of ERα, mRNA during acute stress [7]. There are many experimental data showing that ovarian steroids increase HPA axis activity [13, 14], although there are also reports indicating that ovarian hormones may decrease certain aspects of the HPA axis stress response[15, 16]. These differential results are perhaps explained by the two distinct receptor systems mediating the actions of estrogen. Initially, ERα was thought to be critical for its reproductive effects [17], while ERβ was thought to be more critical for synaptic plasticity and emotional behavior [18]. Recently, Lund et al reported that administration of an ERα agonist will increase anxiety-related behaviors, with opposite effects of an ERβ agonist [19]. Previously, they already had shown a similar differential effect on the stress-induced plasma CORT surge, by using E2-containing pellets [13].

Our own recent study showed a clear increase of plasma estradiol (E2) in female rats after exposure to acute stress [20]. Interestingly, local estradiol concentrations in the PVN were significantly elevated at the same time [20]. Consistent with this, PVN aromatase mRNA expression increased as well [20]. These data clearly indicated the PVN as a candidate site for estrogenic modulation of HPA axis activity.

In the present study, we investigated further the role of the different ER subtypes in the regulation of HPA axis activity, by using blood sampling and retro-dialysis techniques, under different conditions, i.e. basal and stress conditions.
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Material and methods

Animals
Adult female Wistar rats weighing 230-285g were housed in individual cages with food and water available ad libitum, with a room temperature of 23°C and a 12h:12h light/dark regimen (light on at 7:00 am). All experiments were performed between 11:00am and 13:45pm. Animals were allowed to adapt to the new environment for one week prior to the start of the experiments. Before surgery, rats were anesthetized using a mixture of ketamine and xylazine (100:10 mg/kg IP). Rats were allowed one-week of post-operative recovery before the start of the actual experiment. All experimental procedures were carried out with approval of the Animal Care Committee of the Academic Medical Center (AMC) in Amsterdam (The Netherlands) or the University of Science and Technology in Hefei (PR China).

Surgical procedures
All animals underwent a bilateral ovariectomy (OVX). Silicon catheters were inserted into the right jugular vein and left carotid artery, for intravenous infusions (i.v.) and blood sampling, respectively. With a standard Kopf stereotaxic apparatus, an intracerebroventricular (i.c.v.) guide cannula was placed into the lateral cerebral ventricle. Alternatively, we placed bilateral microdialysis probes next to the paraventricular nucleus of the hypothalamus (PVN) or inside the ventromedial nucleus of the hypothalamus (VMH). The coordinates were: a) i.c.v. - AP:1.0 mm, lateral:1.5 mm and 3.5 mm ventral from dura, b) PVN - AP:1.6 mm, lateral:1.8 mm (angled at 10°) and 9.1 mm ventral from the surface of the bone, and c) VMH - AP:2.5 mm, lateral:2.0 mm (angled at 10°) and 9.0 mm ventral from dura. We used cranioplastic cement to secure the i.c.v. cannula and the microdialysis probes and the jugular and carotid outlets to four stainless-steel screws inserted into the skull. All probe locations were checked in thionin-stained 35 μm cryostat sections after the animals had been sacrificed. Only the animals with a correct probe placement were used for data analysis.

During the experiments, animals were connected to blood-sampling and microdialysis lines, which were attached to a metal collar and kept out of reach from the rats by means of a counterbalanced beam. This allowed all manipulations to be performed outside the cages without handling the animals. The metal collars were attached at least 12h before the actual experiment. Animals were handled and sham blood was sampled (i.e., blood was withdrawn and immediately returned) regularly in the week before the first experiment began to familiarize them with the experimental procedures. As estradiol was introduced into the systemic circulation via the jugular vein catheter, all blood samples were drawn
via a catheter in the carotid catheter. Each withdrawal of a 0.2 ml blood sample was compensated for with the equivalent volume of saline.

β-estradiol (Sigma, St. Louis, USA) was dissolved in pure dimethyl sulfoxide (DMSO) and diluted 100 times with saline or Ringer solution. The ERα agonists propyl pyrazole triol (PPT) (displaying a 400-fold selectivity for ERα over ERβ) was bought from TOCRIS (Bristol, UK). The ERβ agonist diarylpropionitrile (DPN) (a highly potent estrogen ERβ receptor agonist with a 70-fold selectivity over ERα) was bought from TOCRIS (Bristol, UK). The agonists and the ER antagonist ICI 182,780 (TOCRIS, Bristol, UK) were dissolved in Ringer solution containing 1% DMSO. The compounds used in our studies were specifically chosen for their unique binding characteristics in previous studies [13, 21, 22]. In these earlier studies, 0.5 μM solutions of the compounds were used for pellet implantation and 10 mM to 20 mM for micro-infusions in the brain. We have not come across earlier studies using reverse microdialysis to administer estrogen agonists. With a measured estradiol recovery efficiency of 0.002% (in vitro), the drug concentrations used in the present study (i.e., 10 μg/ml) resulted in tissue concentrations of around 1.0 nM, which is close to the tissue concentration of estradiol as measured in our previous study [20]. The EC50 value of estradiol is around 0.15 nM for both ERα and ERβ. The EC50 of PPT is 0.2 nM and 80 nM for ERα and ERβ, respectively. The EC50 of DPN is 0.85 nM and 66 nM for ERβ and ERα, respectively. Therefore, the dose we chose for PPT will result in tissue concentrations that will only activate ERα, whereas those of DPN will only activate ERβ. The estradiol dose chosen will activate both ERα and ERβ. The higher dose of estradiol was chosen to investigate the dose dependency of the effect on plasma CORT.

Microdialysis

Dialysis probes were constructed according to procedures described previously [23]. Thin platinum wire (, 0.05 mm) was inserted into a piece of microdialysis tubing (molecular weight cutoff, 6000 kDa) and bent; each end was then inserted into one of two pieces of 25 gauge hypodermic tubing that had been soldered together. The dialysis tubing was then secured with epoxy. The U-shaped tip of the microdialysis probe was 1.5 mm long, 0.7 mm wide and 0.2 mm thick. The input port of the dialysis probe was connected to a remote syringe via a fluid swivel (375/22; Instech Laboratories, Plymouth Meeting, PA) interconnected with polyethylene tubing. The syringe contained Ringer’s. Perfusion of the microdialysis probes was performed by a syringe pump (Harvard Apparatus, Holliston, USA). Ringer’s perfusion (3 μl/min) was started 1 hr before the start of the drug infusion.

Experiment#1- i.v. estradiol infusion

From t=0 onwards estradiol (3.5 ng/min dissolved in saline containing 1% DMSO) or vehicle (saline containing 1% DMSO) were continuously infused via the jugular vein catheter in OVX animals. Blood samples (0.2 ml) were collected just before the start of the estradiol
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infusion at t=0, and t=15 min, 30 min, 45 min, 75 min, 105 min, 135 min and 165 min later via the carotid catheter. Blood samples from the vehicle animals were taken at the same time points.

Experiment#2 – i.c.v. estradiol infusion
A baseline blood sample (0.2 ml) was taken just before the start of the estradiol or vehicle (Ringer with 1% DMSO) infusion at t=0. Estradiol (200 pg/μl) was dissolved in Ringer containing 1% DMSO. The i.c.v. infusion started with a bolus (5.0 μl/5 min) and then continued with 5.0 μl/h (~16.7 pg/min, i.e., 0.5% of the i.v. dose) for 165 min. Blood samples were collected at t=15 min, 30 min, 45 min, 75 min, 105 min, 135 min and 165 min after the start of the infusion.

Experiment#3 – Administration of estradiol, ERα or ERβ agonist in the PVN via microdialysis
Ringer’s dialysis (3.0 μl/min) in the PVN via the microdialysis probes was started at t=-60 min. The perfusion solution was then changed to lower dose estradiol (10 μg/ml, 3.0 μl/min), higher dose estradiol (50 μg/ml, 3.0 μl/min), PPT (10 μg/ml, 3.0 μl/min), DPN (10 μg/ml, 3.0 μl/min) or Vehicle (1% DMSO in Ringer, 3.0 μl/min). A five min bolus infusion (15 μl/min) allowed the drugs to rapidly reach the tip of the microdialysis probes. Blood samples (0.2 ml) were collected just before the start of the estradiol, agonist or vehicle infusion (t=0) and 15 min, 30 min, 45 min, 75 min, 105 min, 135 min and 165 min after the start of the infusion via the microdialysis probes (i.e., after the change from Ringer’s to the estradiol (agonist)-containing solution or vehicle).

Experiment#4 - Administration of estradiol, ERα or ERβ agonist in the VMH via microdialysis
These experiments were identical to the protocol of Experiment#3, with the exception that the microdialysis probes were aimed at the VMH instead of the PVN.

Experiment#5 - Administration of estradiol, PPT, DPN or ICI 182,780 in the PVN via microdialysis during restraint stress
Ringer’s dialysis (vehicle, 3.0 μl/min) into the PVN via the microdialysis probes was started at t=-60 min. The solution was then changed to estradiol (50 μg/ml), PPT (10 μg/ml), DPN (10 μg/ml), ICI (10 μg/ml) or vehicle (1% DMSO in Ringer) at t=0 min. A five min bolus infusion (15 μl/min) allowed the drugs to reach the tip of the microdialysis probes rapidly. After the bolus infusion, the infusion rate was changed back to 3.0 μl/min and all animals were restrained in opaque plastic cylinders (18.0 cm length and 5.5 cm internal diameter) for 30 min [24], starting from t=0. Blood samples (0.2 ml) were collected at t=-5 min.
before the onset of the restraint stress and at t=30 min after the onset of the restraint stress.

Hormone measurement

Blood samples were immediately chilled on ice in tubes containing a 5 μl solution of heparin, and then centrifuged (at 4000 rpm, 15 min) at 4°C. Plasma was then stored at -80°C until additional analysis. Plasma estradiol concentrations were determined using an estradiol enzyme-linked immunosorbent kit (sensitivity range: 2–1000 pg/ml, inter-assay variation: 6.0%, intra-assay variation: 3.9%) (BioSource, Nivelles, Belgium). Plasma corticosterone concentrations were determined by a radioimmunoassay kit (sensitivity range: 10–9000 ng/ml, inter-assay variation: 6.5%, intra-assay variation: 3.4%) (MP Biomedicals, Eschwege, Germany).

Statistics

For statistical analyses we used SPSS V17.0 software. All results were initially analyzed by analysis of variance (ANOVA) with repeated measures or with a two-way ANOVA. When appropriate, further post-hoc tests were done with one-way ANOVA, and paired or unpaired student t-tests. Statistical significance was defined as p<0.05. Data are expressed as mean±S.E.M.

Results

Effects of i.v. and i.c.v. estrogen administration on plasma CORT

During the i.v. infusion of estradiol there was no significant difference in plasma CORT concentrations compared to the vehicle infused group (Time, F(7,63)=3.9, P=0.001; Group, F(1,9)=0.142, P=0.715; Time×Group, F(7,63)=0.642, P=0.719) (Figure 3.1A).

We measured plasma estradiol in the t=0 and t=165 min samples of the experimental animals. In the vehicle-infused group, plasma estradiol concentrations were similar before the start of the infusion and at t=165 min (P=0.38), while in the estradiol-infused group there was a significant increase (P<0.001) (Table 3.1). The plasma estradiol level in the estradiol infused group at t=165 min was comparable to that of intact animals (~100 pg/ml, data not show).

Unlike i.v. estradiol administration, i.c.v. infusion of estradiol in OVX animals markedly increased plasma CORT (Time F(7,70)=4.687, P<0.001; Group F(1,10)=9.244, P=0.012; Time×Group F(7,70)=6.485, P<0.001) (Figure 3.1B). I.c.v. administration of estradiol did not affect plasma estradiol concentrations.
Chapter 3

Effect of estrogen administration in the PVN and VMH on plasma CORT

The overall analysis of the microdialysis experiments in the PVN showed a clear effect of estradiol and estrogen receptor subtype-specific agonists on plasma CORT concentrations.

Table 3.1. Plasma estradiol concentrations during systemic and I.C.V. administration of estradiol

<table>
<thead>
<tr>
<th></th>
<th>Systemic infusion</th>
<th>I.C.V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Veh</td>
<td>Estradiol</td>
</tr>
<tr>
<td>Before infusion (pg/ml)</td>
<td>43,06±4,47</td>
<td>41,86±6,66</td>
</tr>
<tr>
<td>After infusion (pg/ml)</td>
<td>49,53±16,20</td>
<td>104,24±32,81</td>
</tr>
</tbody>
</table>

\( ^a P<0,001\), vs before infusion in the same group
The effects of Time $F(7,210)=7.65$, (P<0.001), and Group $F(4,30)=16.512$, (P<0.001) as well as Time×Group $F(28,210)=3.074$, (P<0.001) were very significant (Figure 3.2A). Post-hoc analysis showed that, compared to the vehicle group, all groups, except the DPN-infused animals, exhibited a significant Time×Group interaction (P<0.001). Only the high dose estradiol group (50 μg/ml) and the PPT group also showed a significant Group effect (P≤0.001). Plasma CORT increased already 15 min after the start of the infusion during both the high dose of estradiol (50 μg/ml) and PPT (P<0.001). However, the increase in plasma CORT during PPT was transient and had returned to baseline at 165 min, while plasma CORT increased further in the 50 μg/ml estradiol group. In the 10 μg/ml estradiol group, the plasma CORT increase was slower and did not reach significance until 135 min after the start of the estradiol infusion (P<0.001). In contrast to the PVN, administration

![Figure 3.2](image)

**Figure 3.2** Plasma corticosterone increased during administration of estradiol or PPT in the PVN (A), but not during administration in the VMH (B). In PVN group, Vehicle n=7; 10 ug E2 n=6; 50 ug E2 n=6; PPT n=9; DPN n=8. In VMH group, Vehicle n=7; 10 ug E2 n=6; 50 ug E2 n=8; PPT n=6; DPN n=5.
of estradiol, PPT or DPN in the VMH had no effect on plasma CORT (no significant effects of Time $F(7,189)=1.514$, $(P=0.165)$, Group $F(4,27)=1.159$, $(P=0.351)$ or Time×Group $F(28,189)=0.915$, $(P=0.593)$) (Figure 3.2B).

Effect of estradiol and estrogen (ant)agonist administration in the PVN on plasma CORT during restraint stress

As it was not possible technically to take blood samples via the jugular catheter when the animals were under restraint conditions, only pre-stress and post-stress samples were taken. Restraint stress significantly increased plasma CORT in vehicle, estradiol, E2-agonist and ICI-treated animals. ANOVA analysis showed an overall significant effect of Stress $F(1,26)=254.11$, $(P<0.001)$, but also the effects of Group $F(4,26)=11.13$, $(P<0.001)$ and Group×Stress $F(4,26)=11.33$, $(P<0.001)$ reached statistical significance (Figure 3.3), indicating that the increase in CORT significantly differed between treatment groups. Post-hoc analysis showed that restraint stress elevated plasma CORT concentrations significantly in all 5 groups ($p<0.001$). After 30 min of stress, estradiol and ER$\alpha$ agonist-infused animals had significantly higher plasma CORT concentrations than vehicle-infused animals ($P=0.008$ and $P=0.001$). Interestingly, the ER$\beta$-agonist and ICI-infused animals showed significantly lower plasma CORT concentrations after 30 min of restraint stress compared to the vehicle-infused animals ($P<0.001$ and $P=0.002$).

Figure 3.3 Administration of estradiol or PPT in the PVN increased plasma CORT in response to stress. Administration of DPN or ICI decreased plasma CORT in response to stress. a) Significantly higher than pre-stress levels ($P<0.001$); b) Significantly higher than vehicle stress response ($P<0.05$); c) Significantly lower than vehicle stress response ($P<0.05$).
Discussion

In the present study we observed a clear effect of E2 on HPA axis activity in OVX female rats after i.c.v. and PVN administration, but not after a systemic infusion or local administration in the VMH. Under basal conditions, the estrogenic effect was mainly mediated via ERα-sensitive neurons in the area of the PVN. During stress conditions, both ERα and ERβ in the area of the PVN were involved in the regulation of HPA axis activity, with ERα mediating a stimulatory effect and ERβ mediating an inhibitory effect.

The modulatory role of estrogen on HPA-axis activity has been clearly established [1, 25]. The first clear evidence for dichotomous effects of estrogen via opposing roles of the ERα and ERβ was provided by the ER-specific knockout mice [17, 18]. Initially it was thought that the distinct ER subtypes were involved in different functions, with ERα being mainly involved in reproductive behaviors and ERβ in non-reproductive behaviors [26, 27]. More recently, however, Lund et al. nicely demonstrated that opposing roles of the ER’s are also evident within one behavioral component. They showed that peripheral administration of an ERβ agonist decreases anxiety-related behavior, whereas administration of an ERα agonist produced opposite effects [19]. Similar effects were found for the anxiety-induced activation of the HPA-axis. Administration of the ERβ agonist decreased the stress-induced plasma CORT response and administration of the ERα agonist increased it. Later, the same group reported that the different ER effects on plasma CORT were mediated by the hypothalamus, since hypothalamic pellets containing the ERα agonist increased stress-induced c-fos expression in the PVN, whereas a similar administration of the ERβ agonist decreased it [21]. Our own previous study demonstrated an increased local production of estradiol in the hypothalamus during acute stress [20]. Together these pieces of evidence suggested an essential role for the PVN in estrogen effects on HPA axis activity. The use of reverse microdialysis and permanent vascular catheters allowed us to investigate rapid changes in plasma CORT during estradiol administration into the systemic circulation, the brain ventricular system, or locally within the hypothalamus, without additional stress for the animals. The results of the current study extend the previous results in that now it is clear that changes in hypothalamic E2 availability can acutely change HPA-axis activity, both during basal and stress-activated conditions. Moreover, the effect is specific for the PVN and not for the VMH.

Our first experiment did not yield any significant differences in plasma corticosterone levels after i.v. administration of estradiol or vehicle. However, the central administration of estradiol via an i.c.v. cannula did produce a clear difference. These data indicated that the estrogenic pathway probably is an important component of the central brain mechanisms controlling basal HPA-axis activity. Conversely, these results seemed counterintuitive as it is likely that the i.v.-infused estradiol will also reach these central targets. The most straightforward explanation seems to be that the i.v. concentration we used was too low to activate the HPA axis. However, the physiological concentrations reached with the
current infusion parameters argue against this explanation. An alternative explanation is that the i.v. infusion reaches additional targets, which inhibit activation of the HPA-axis, overruling its stimulatory effect via the ventricular compartment. Since the i.c.v. infusion was able to activate the HPA-axis, most likely via the PVN as shown by the microdialysis experiments, these inhibitory targets of the i.v. infusion should be either peripheral or quite distant from the ventricular compartment. For instance, the prefrontal cortex, which, unlike the hypothalamus, is quite a distance away from the ventricular compartment, and has a profound inhibitory effect on HPA axis activity [28]. At first sight, our results may also seem at variance with previous studies reporting that systemic administration of estradiol does regulate basal HPA-axis activity [29-31]. However, the experimental design of these studies included subcutaneous injections of estradiol in OVX animals for 4-6 weeks, while in the present study we administered estrogen i.v. for just 3 hours. We observed a rise in plasma CORT concentrations after E2 treatment at t=15 min. Importantly, a similar increase at the same time point was present, in the vehicle group. Therefore, we suspect this short surge of CORT release to be induced by DMSO or by the infusion itself, rather than by E2. Indeed, it has been shown previously that DMSO may affects plasma CORT concentrations [32]. We did not observe a similar phenomenon during the i.c.v. experiment, most likely because the total amount of DMSO infused during the i.c.v. experiment was much lower (0.05 μl/hr) than during the i.v. infusion (5.0 μl/hr).

After having established the central effect of estrogen, we focused on the hypothalamus. Effects of estrogen have been reported in various hypothalamic nuclei, including the PVN, VMH and arcuate nucleus [33, 34]. Within the hypothalamus, the PVN is the most important nucleus when it comes to determining HPA axis activity. While the PVN contains large numbers of CRH neurons [35], there are no reports showing CRH expression in the VMH. The present design involved infusion of E2 and agonists into the VMH as a negative control. As expected, VMH treatment did not affect HPA-axis activity at all as compared to vehicle infusion, while administration in the PVN mimicked the i.c.v. effects. Our findings highlight that, although both the PVN and VMH show abundant neuronal ER expression [12, 36, 37], the functional differences between the PVN and the VMH in terms of neurotransmitter content and/or connectivity are responsible for the differential effects of estradiol administration on HPA-axis activity in these nuclei.

When we administered the ERα agonist PPT into the PVN, it mimicked the E2 effects both in basal and stress conditions. ERα expression in the PVN seems limited [9, 12], but many ERα positive neurons are found in the immediate surroundings of the PVN [21]. The present effect we observed may be mediated by these neurons in the peri-PVN based on the following evidence: firstly, previous studies have shown that many GABAergic neurons surround the PVN [38, 39]. Among other things, this peri-PVN region contains GAD-IR neurons that project to the parvocellular PVN [40] and are inhibitory to the hypophysiotropic CRH neurons [41]. Secondly, the GABAergic neurons surrounding the
PVN express ERα [21]. Thirdly, E2 attenuates GABA-B responses in hypothalamic neurons [42] and suppresses the GABA_A-mediated inhibition [43].

In the PVN, ERβ-expressing neurons predominate compared to ERα positive neurons. Of the CRH neurons, 13% co-localize ERβ [9, 10, 44]. However, when we infused an ERβ agonist into the PVN under basal conditions, no effect was observed. Interestingly, the ERβ agonist did decrease stress-induced CORT release, suggesting that ERβ-sensitive neurons have an inhibitory function on the HPA axis only under stressed conditions. We previously showed that acute stress elevated ERβ mRNA expression in the PVN [20]. Consistent with this, Suzuki found that treatment with dexamethasone increased ERβ expression in the PVN [45]. This evidence partly explains why the ERβ agonist only affected the HPA axis under stressed conditions. Under basal conditions, the level of ERβ expression appears insufficient to regulate the HPA axis.

Our own previous work demonstrated an increased local production of estradiol in the PVN during acute stress in female rats, as well as an increased ERβ and aromatase mRNA expression [20]. Together with our present data these results indicate that local changes in estradiol bioavailability may play an important role in the fine-tuning of HPA-axis responsiveness during stress exposure. During stress, the E2- and PPT-treated animals showed higher plasma CORT responses, whereas administration of the ERβ agonist caused a decrease of the stress response. However, since E2 will activate both the ERα and the ERβ, a lower response in the E2- versus PPT-treated animals was to be expected. Therefore, we suppose that in the beginning of the stress response (low plasma CORT), only the ERα effect is available, but together with the increased aromatase mRNA expression and increased local availability of estradiol this causes a positive feedback. Once the HPA-axis is activated and ERβ mRNA expression is increased the inhibitory effect of ERβ activation will become apparent. Therefore, the final plasma CORT response is determined by the balance between ERα and ERβ activation. To test this time-dependent effect of ERα and ERβ activation, either more frequent sampling protocols or combined administrations of ERα and ERβ (ant)agonists, should be performed during stress exposure.

In conclusion, we have demonstrated an estrogenic stimulation of the HPA axis at the level of the PVN, mediated via the ERα. The stimulatory effect of local ERα activation is present both in the basal state and during restraint stress, whereas the inhibitory effect of ERβ receptor activation may only become apparent when the HPA-axis is activated.

Acknowledgements

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Hypothalamic estrogen regulates HPA


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Chapter 3


Chapter 4

Intrahypothalamic estradiol regulates glucose metabolism via the sympathetic nervous system in female rats
Intrahypothalamic estradiol regulates glucose metabolism via the sympathetic nervous system in female rats

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Abstract

Long-term reduced hypothalamic estrogen signalling leads to increased food intake, decreased locomotor activity and energy expenditure, which ultimately results in obesity and insulin resistance. In the current study we aimed to determine the acute obesity-independent effects of hypothalamic estrogen signaling on glucose metabolism. We studied endogenous glucose production (EGP) and insulin sensitivity during selective modulation of systemic or intrahypothalamic E2 signaling in rats one week after ovariectomy (OVX). OVX caused a 17% decrease in plasma glucose, which was completely restored by systemic E2. Likewise, the administration of E2 by microdialysis, either in the hypothalamic paraventricular nucleus (PVN) or in the ventromedial nucleus (VMH), restored plasma glucose. The infusion of an E2-antagonist via reverse microdialysis into the PVN or VMH attenuated the effect of systemic E2 on plasma glucose. Furthermore, E2 administration in the VMH, but not in the PVN, increased EGP and induced hepatic insulin resistance. E2 administration in both the PVN and the VMH resulted in peripheral insulin resistance. Finally, sympathetic, but not parasympathetic, hepatic denervation blunted the effect of E2 in the VMH on both EGP and hepatic insulin sensitivity. In conclusion, intrahypothalamic estrogen regulates peripheral and hepatic insulin sensitivity via sympathetic signalling to the liver.

Keywords: E2, hypothalamus, glucose, insulin sensitivity, sympathetic denervation
Estradiol (E2) plays a major role in the control of energy homeostasis (1, 2), as is exemplified by increased body weight after ovariectomy (OVX) in female rats, reversible with E2 replacement (3-6). E2’s effects on energy homeostasis are thought to be mediated primarily through the hypothalamus, as direct injections of E2 into the hypothalamic paraventricular nucleus (PVN), arcuate (Arc) or ventromedial nucleus (VMH) effectively reduce food intake and body weight after OVX in rodents (7-9). A link between hypothalamic E2 receptors and energy expenditure was elegantly shown by the obese phenotype induced by selective silencing of ERα in VMH (10, 11). Together with many more studies, these data have convincingly shown that reduced estrogen signaling in the hypothalamus increases body weight and is associated with impaired glucose tolerance and insulin resistance. At this stage it is less clear if estrogen affects glucose metabolism directly or whether it works indirectly - by inducing obesity. When ovariectomized rats are studied before the onset of obesity they exhibit higher glucose/insulin ratios (with decreased plasma insulin concentration) compared to intact rats, suggesting that ovariectomy increases insulin sensitivity (12). This surprising finding could represent a more direct obesity-independent effect of estrogen on glucose metabolism whose mechanisms have thus far been elucidated only partially.

The hypothalamus, has emerged as a key player in the regulation of glucose production (13). The suppressive effects of peripheral hyperinsulinemia on endogenous glucose production (EGP) can be blocked by the central administration of NPY (14) or insulin antibodies (13) (15). Moreover, the suppressive effect of central insulin on EGP can be largely abolished by selective hepatic vagal denervation (16, 17), whereas the intrahypothalamic administration of various neurotransmitters stimulates EGP via the sympathetic efferent nerves to the liver (18-20). These hypothalamic neurotransmitter systems probably act as targets for circulating hormones such as insulin (14), thyroid hormone (21) and glucocorticoids (22). E2 receptors (ER) are abundantly expressed in the hypothalamic PVN and VMH (23), nuclei that are key players in the hypothalamic regulation of glucose metabolism via autonomic outflow towards the liver (24). Considering that the hypothalamus plays a key role in both the regulation of body weight by estrogen and in controlling glucose metabolism, we hypothesized that the direct, obesity-independent, effects of estrogen on glucose metabolism are, at least in part, mediated via the hypothalamus and the autonomic nervous system.

To test our hypothesis we performed a series of experiments that involved the application of reverse microdialysis, selective hepatic autonomic denervations, euglycemic hyperinsulinemic clamps and stable isotope dilution. In order to prevent any effects of increased adiposity on glucose metabolism all experiments were performed 1 week after ovariectomy, i.e., before any increase in body weight or adiposity occurred.
Chapter 4

Research design and Methods

Animals

Female Wistar rats (Harlan, Horst), housed in a 12-h light–12-h dark schedule (lights on at 0700 h) were used for all experiments. Body weight was between 220 and 280 g. Food and drinking water were available ad libitum. All of the following experiments were conducted with the approval of the Animal Experimental Committee of the Academic Medical Center (AMC) in Amsterdam.

Surgery

Rats were anesthetized using a mixture of ketamine/xylazine (100:10 mg/kg IP). All animals underwent a bilateral ovariectomy (except for the control group in Experiment 1). Silicon catheters were inserted into the right jugular vein and left carotid artery for intravenous infusions (I.V.) and blood sampling, respectively. With a standard Kopf stereotaxic apparatus, bilateral microdialysis probes were placed adjacent to PVN or VMH. The coordinates for the PVN were AP: 1.6 mm, lateral: 1.8 mm (angled at 10°) and 9.1 mm ventral from the surface of the bone. The coordinates for the VMH were AP: 2.5 mm, lateral: 2.0 mm (angled at 10°) and 9.0 mm ventral from dura. We used dental cement to secure the microdialysis probes and the jugular and carotid outlets to 4 stainless steel screws inserted into the skull. Rats were allowed 1-week of post-operative recovery before the start of the actual experiment. The probe location was checked by thionin staining after sacrifice. Only the animals with correct probe placements were used for data analysis (supplemental data 1).

Hepatic sympathetic or parasympathetic branches were denervated according to our previously published methods (18). The effectiveness of the hepatic sympathetic denervation (SX) was checked by measuring the norepinephrine content in the liver (25). We have previously validated our method for selective hepatic parasympathectomy (PX) by using retrograde viral tracing (18).

During the experiments, animals were connected to blood-sampling and microdialysis lines, which were attached to a metal collar and kept out of reach from the rats by means of a counterbalanced beam. This allowed all manipulations to be performed outside the cages without handling the animals. The metal collars were attached at least 24 h before the actual experiment. Animals were handled and sham blood was sampled (i.e., blood was withdrawn and immediately returned) regularly in the week before the first experiment began to familiarize them with all the experimental procedures.

One mg β-E2 (Sigma, St. Louis, USA) was dissolved in 1 ml pure dimethyl sulfoxide (DMSO) and diluted 100 times with Ringer solution. The ER antagonist ICI 182,780 (TOCRIS, Bristol, UK) was dissolved at a final concentration of 10 μg/ml in Ringer solution containing 1% DMSO. With a measured estradiol recovery efficiency of 0.002% (in vitro), the drug
concentrations used in the present study (i.e., 10 μg/ml) are expected to result in tissue concentrations of around 1.0 nM, which is close to the tissue concentration of estradiol as measured in our previous study(26). The EC50 value of estradiol is around 0.15 nM for both ERα and ERβ. Thus, the doses used for infusion are expected to activate both ERα and ERβ. We cannot measure the recovery efficiency of the antagonist. The IC50 value of antagonist is 0.29nM. Thus if the recovery is similar to that of estradiol it will inhibit both ERα and ERβ.

Plasma measurements
Glucose enrichment was measured as described previously (27) (supplemental data2). Plasma insulin and corticosterone were measured by a commercially available ELISA. Plasma estradiol concentration was determined by ELISA kits (Biosource, Belgium).

Statistics
Data were analyzed by ANOVA with repeated measures, with Group (E2 or Veh) as the between-animal factor and Time as the within-animal factor. Post hoc tests (Tukey HSD) were performed if ANOVA revealed a significant effect. Significance was defined at P≤0.05.

Experiment 1 was designed to investigate the difference in plasma glucose concentrations between intact and ovariectomized (OVX) animals, and the effect of an intravenous infusion (I.V.) of E2 in OVX animals on plasma glucose concentrations. Blood samples from both intact and OVX animals were collected at 10:30 a.m. for the measurement of basal plasma glucose concentrations. In OVX animals, E2 (3.5 ng/min) or vehicle (saline containing 1% DMSO) was continuously infused via the jugular vein catheter for 165 min (start from t=15 min). Blood samples were collected from the carotid artery at t=0 (just before infusion) and t=30, 45, 60, 90, 120, 150 and 180 min after infusion. In the sham animals blood samples were collected at the same time point.

Experiment 2 was designed to investigate the changes in plasma glucose induced by the reverse microdialysis of the E2 receptor antagonist ICI 182,780 into the PVN and VMH combined with the I.V. administration of E2 in OVX animals. Ringer’s dialysis (3μl/min) in the PVN or VMH via the microdialysis probes was started at t= -60 min. E2 (3.5 ng/min) was infused I.V. starting at t=10 min, and E2 antagonist (10 μg/ml, 3μl /min) was infused via the microdialysis probes into the PVN or VMH starting at t=15 min. Blood samples were collected at t=0, 30, 45, 60, 90, 120, 150 and 180 min.

Experiment 3 was designed to investigate the effects of reverse microdialysis of E2 into the PVN and VMH of OVX animals on glucose kinetics. To study glucose kinetics, [6,6-2H2] glucose (as a primed [8.0 umol in 5 min]-continuous [16.6 umol/h] infusion) was used as tracer (>99% enriched; Cambridge Isotopes, Andover, MA). Blood samples were taken at t = -95 min for measuring background enrichment of [6,6-2H2]glucose, at t =0, 5 and 10
min for determining enrichment during the steady state, and at \( t = 30, 45, 60, 90, 120, 150 \) and 180 min for determining enrichment during the retrodialysis of E2 (non-steady state). Ringer’s dialysis (3μl/min) in the PVN or VMH via the microdialysis probes was started at \( t = -60 \) min. At \( t = 15 \) min E2 (10 μg/ml, 3 μl/min) or vehicle (Ringer with 1% DMSO, 3 μl/min) were infused by retrodialysis into PVN or VMH.

**Experiment 4** was designed to investigate the effects of reverse microdialysis of E2 into the PVN and VMH of OVX animals on insulin sensitivity. Background blood samples and isotope tracer infusion were the same as for Exp.3. At \( t = 15 \) min, insulin was administered in a primed I.V. infusion (3.6 mU/kg.min in 5 min for the “low” clamp#1, and 7.2 mU/kg.min in 5 min for the “high” clamp#2), followed by a continuous I.V. infusion (1.5 mU/kg.min and 3 mU/kg.min respectively). A variable infusion of a 25% glucose solution (containing 1% [6,6-2H2] glucose) was used to maintain euglycemia (5.5±0.5mmol/l) (supplemental data 3), as determined by carotid catheter blood sampling every 10 min. Thirty min after the start of the primary insulin infusion (\( t = 45 \)min), Ringer’s perfusion of the microdialysis probes was replaced by the E2 solution (10 μg/ml, 3 μl/min) or vehicle (Ringer’s containing 1%DMSO). At the end of the clamp, five blood samples were taken with a 5 min interval at \( t = 120, 125, 130, 135 \) and 140 min (supplemental data 4).

**Experiment 5** was designed to investigate the effect of selective hepatic autonomic nerve denervations on plasma glucose changes induced by the reverse microdialysis of E2 into the PVN and VMH. The experimental design is similar to that for Exp.3.

**Experiment 6** was designed to investigate the effects of a hepatic sympathetic nerve denervation combined with the reverse microdialysis of E2 into the VMH on hepatic insulin sensitivity. The experimental design was similar to that of Exp.4

**Results**

As expected, plasma E2 concentrations were lower in OVX than in intact animals (\( P = 0.004 \)) (Figure 4.1A). After 165 min of systemic E2 infusion, plasma E2 concentrations were higher in the E2 group (\( P = 0.002 \)), and comparable to those of the intact animals (\( P = 0.357 \)) (Figure 4.1A). OVX animals showed a 17% decrease of basal plasma glucose concentrations as compared to intact animals (\( P = 0.035 \)) (Figure 4.1B). During systemic infusion of E2, plasma glucose concentrations significantly increased as compared to the vehicle-infused group (\( Time \) effect: \( P < 0.001 \); \( Group \) effect: \( P = 0.028 \); \( Time \times Group \) effect: \( P = 0.001 \)) (Figure 4.1B). The effect of systemic E2 on plasma glucose was blunted by intrahypothalamic administration of the E2 antagonist ICI 182,780 (ICI). Plasma glucose levels were significantly lower during systemic E2 infusion and simultaneous retrodialysis of ICI in the PVN than during vehicle retrodialysis (\( Time, P < 0.001; Group, P = 0.02; Time \times Group, P = 0.064 \)) (Figure 4.1C). A similar effect was found after systemic E2 infusion and simultaneous retrodialysis of ICI in the VMH (\( Time, P < 0.001; Group, P = 0.009; Time \times Group, P < 0.001 \)) (Figure 4.1D).
Hypothalamic estrogen regulates glucose metabolism.

During retrodialysis of E2 in the PVN of OVX animals, plasma glucose concentrations increased compared to vehicle retrodialysis (Group, P=0.015; Time×Group, P<0.001) (Figure 4.2A). Similarly, E2 treatment in the VMH also resulted in higher plasma glucose levels (Group, P=0.006; Time×Group, P<0.001) (Figure 4.2D). During the retrodialysis of vehicle in the PVN and VMH EGP showed a slow decline, probably due to the prolonged fasting(20). Retrodialysis of E2 in the PVN did not affect EGP (Figure 4.2B), but the same treatment in the VMH increased EGP as compared to the vehicle treatment, i.e., EGP showed no steady decrease, showing significant effects of Group (P<0.001) and Time×Group (P<0.001) (Figure 4.2E). Importantly, plasma E2 concentrations were not affected by the intrahypothalamic infusions of E2 in either the PVN or VMH (Figure 4.2C & 4.2F).

**Figure 4.1** (A) Ovariectomy (OVX) reduced plasma E2 concentrations compared to intact animals. Intravenous E2 (E2 iv) administration restored plasma E2 concentrations (different letters indicate a significant difference, p<0.05). (B) After ovariectomy plasma glucose concentrations were lower compared to intact animals (p=0.035). Intravenous E2 administration acutely increased plasma glucose to concentrations observed in intact animals. The increase in plasma glucose concentrations during intravenous E2 administration was attenuated by simultaneous administration of the E2 receptor antagonist ICI 182,780 (ICI) in (C) the PVN (p=0.020 vs vehicle) and (D) VMH (p=0.009 vs vehicle). The hatched bars indicate the continuous infusion of vehicle, E2 and/or ICI.
We studied hepatic and peripheral insulin sensitivity using euglycemic hyperinsulinemic clamps at low and high insulin concentrations, respectively. Plasma insulin concentrations during the clamps were significantly higher than those during basal conditions in both the low- and high-dose clamp groups (time, P<0.001). No significant differences between the different infusion groups (i.e., Veh, PVN & VMH) were detected (group, P>0.27; time×group, P>0.48) (Figure 4.3C&F). The lower dose clamp experiment showed

Figure 4.2 (A) Administration of E2 (n=6) into the PVN increased plasma glucose concentrations (p=0.015), (B) but not endogenous glucose production (EGP) as compared to vehicle (n=7). (D) E2 (n=6) infusion into the VMH increased plasma glucose (p=0.006). (E) E2 infusion in the VMH increased EGP (overall Group effect P<0.001). (C+F) Plasma E2 concentrations were not different between vehicle and E2 infused animals, excluding leakage from the hypothalamus to the systemic circulation. The hatched bars indicate the continuous infusion of vehicle or E2.

We studied hepatic and peripheral insulin sensitivity using euglycemic hyperinsulinemic clamps at low and high insulin concentrations, respectively. Plasma insulin concentrations during the clamps were significantly higher than those during basal conditions in both the low- and high-dose clamp groups (time, P<0.001). No significant differences between the different infusion groups (i.e., Veh, PVN & VMH) were detected (group, P>0.27; time×group, P>0.48) (Figure 4.3C&F). The lower dose clamp experiment showed
similar basal EGP levels between the vehicle and E2 infusion groups (P=0.687), but the intrahypothalamic administration of E2 differentially affected the insulin-induced decrease of EGP (P=0.003) (Figure 4.3A). In the vehicle and PVN E2 infusion groups, EGP was suppressed by 30-40%, while in the VMH E2 infusion group the expected decrease in EGP induced by hyperinsulinemia was completely blunted (P=0.017 vs vehicle) (Figure 4.3B).

Figure 4.3 (A+B) Insulin decreased endogenous glucose production (EGP) by 30%-40% during vehicle (VEH; n=6) and E2 infusion in the PVN E2 (n=6). Insulin-mediated suppression of EGP was completely blunted by simultaneous E2 infusion in the VMH (n=6). (D+E) Insulin infusion also stimulated glucose uptake (Rd), which was partly prevented by infusion of E2 in either the VMH or PVN. (C+F) Plasma insulin concentrations in the different groups during the “low” (1.5mU/kg.min) (C) and “high” (3.0mU/kg.min) (F) clamps. Different letters indicate a significant difference (p<0.05).

During the higher dose hyperinsulinemic clamp, the insulin-induced increase in glucose uptake was reduced by E2 treatment both in the PVN and VMH (both P=0.012) (Figure 4.3D). In the vehicle group, as expected, glucose uptake was increased by 130%, whereas in the PVN and VMH treatment group, the increase in glucose uptake by insulin was attenuated (Figure 4.3E).
During E2 administration in the VMH, insulin-mediated EGP suppression was restored after sympathetic denervation (Sx) of the liver. (C) Insulin concentrations under basal conditions and during intravenous insulin administration. n=6 for all groups. Different letters indicate a significant difference (p<0.05). (insulin administration rate: 1.5 mU/kg.min).

During E2 administration in the VMH, insulin-mediated EGP suppression was restored after sympathetic denervation (Sx) of the liver. (C) Insulin concentrations under basal conditions and during intravenous insulin administration. n=6 for all groups. Different letters indicate a significant difference (p<0.05). (insulin administration rate: 1.5 mU/kg.min).
Lever noradrenalin levels were significantly lower (<10%) in all sympathetic (SX) groups as compared to both the sham-denervated and parasympathectomy (PX) groups (Supplemental data 5). During E2 infusion in the PVN, the increase in plasma glucose did not differ between sham, hepatic Sx and Px groups (Group, P=0.386; Time×Group, P=0.163) (Figure 4.4A). Likewise, there was no effect of hepatic denervations on EGP (Group, P=0.528; Time×Group, P=0.939) either during steady state or non-steady state conditions (Figure 4.4B). During E2 infusion in the VMH, however, plasma glucose concentrations were lower in the Sx group than in the Px and sham groups (post hoc Group effect: P=0.002 and P=0.001, Sx versus Px and sham, respectively) (Figure 4.4C). The stimulatory effect of E2 infusion in the VMH on EGP (Figure 4.2E) was abolished by Sx, but not by Px or sham denervation (Group, P=0.006 and Time×Group, P<0.001 for Sx versus sham, and Group, P=0.604 and Time×Group, P=0.487 for Px versus sham) (Figure 4.4D). During E2 infusion in the VMH, insulin suppressed EGP by 35% in the Sx group, but only by 10% in the group with intact sympathetic signaling to the liver (P=0.014) (Figure 4.5A,B), indicating the necessity of the sympathetic hepatic innervation for the modulation of hepatic insulin sensitivity by E2 in the VMH. Plasma insulin levels during the clamp were significantly increased as compared to basal conditions before the clamp, but no significant differences between the 2 groups (Sham and Sx) were found (p<0.001 and P=0.172, respectively)(Figure 4.5C).

**E2 effects on glucoregulatory hormones**

IV E2 infusions in Experiment#1 did not affect plasma insulin, corticosterone or glucagon levels when compared to the vehicle group (Table 1). Also E2 administration in the PVN or VMH did not affect plasma insulin or glucagon concentrations (Table 4.1). Both PVN and VMH administration of E2 resulted in increased plasma corticosterone values at t=180 min, but only the effect in the PVN reached significance (ANOVA basal values(t=0 min), p=0.637; t=180 min, p=0.009). The PVN-E2 corticosterone value differed significantly from

### Table 4.1 Plasma hormone measurements for experiment 1 and 3

<table>
<thead>
<tr>
<th>Groups</th>
<th>Insulin (ng/ml) Before infusion</th>
<th>Insulin (ng/ml) After infusion</th>
<th>Corticosterone (ng/ml) Before infusion</th>
<th>Corticosterone (ng/ml) After infusion</th>
<th>Glucagon Before infusion</th>
<th>Glucagon After infusion</th>
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</thead>
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<tr>
<td></td>
<td>(t=0)</td>
<td>(t=180)</td>
<td>(t=0)</td>
<td>(t=180)</td>
<td>(t=0)</td>
<td>(t=180)</td>
</tr>
<tr>
<td>IV veh</td>
<td>1.25 ± 0.28</td>
<td>1.51 ± 0.41</td>
<td>85.48 ± 10.46</td>
<td>152.20 ± 35.67</td>
<td>74.65 ± 24.77</td>
<td>64.88 ± 29.13</td>
</tr>
<tr>
<td>IV E2</td>
<td>1.59 ± 0.26</td>
<td>1.98 ± 0.43</td>
<td>75.83 ± 29.22</td>
<td>147.56 ± 52.76</td>
<td>76.69 ± 21.69</td>
<td>70.88 ± 14.01</td>
</tr>
<tr>
<td>PVN veh</td>
<td>0.90 ± 0.19</td>
<td>0.88 ± 0.13</td>
<td>77.75 ± 14.19</td>
<td>88.13 ± 16.66</td>
<td>80.14 ± 17.77</td>
<td>73.12 ± 17.76</td>
</tr>
<tr>
<td>PVN E2</td>
<td>0.97 ± 0.14</td>
<td>0.92 ± 0.25</td>
<td>81.00 ± 18.80</td>
<td>217.14 ± 34.64</td>
<td>73.02 ± 20.59</td>
<td>85.50 ± 16.31</td>
</tr>
<tr>
<td>VMH veh</td>
<td>1.26 ± 0.35</td>
<td>1.78 ± 0.28</td>
<td>88.33 ± 17.15</td>
<td>88.00 ± 27.12</td>
<td>68.47 ± 13.28</td>
<td>82.99 ± 13.36</td>
</tr>
<tr>
<td>VMH E2</td>
<td>1.56 ± 0.60</td>
<td>1.65 ± 0.35</td>
<td>55.40 ± 12.30</td>
<td>123.20 ± 31.08</td>
<td>72.41 ± 28.00</td>
<td>79.99 ± 12.96</td>
</tr>
</tbody>
</table>

a : p<0.05 as compared to t=0 min values.
both vehicle groups at $t=180$ min ($p<0.005$), whereas the VMH-E2 $t=180$ value differed neither from the 2 vehicle groups nor from the PVN-E2 group.

Discussion

The key finding of the present study is that local changes in hypothalamic E2 availability have site-specific effects on peripheral glucose metabolism and insulin sensitivity. More specifically, in ovariectomized rats local supplementation of E2 in the PVN and VMH decreased peripheral insulin sensitivity and reduced glucose uptake, whereas local E2 supplementation in the VMH also reduced endogenous glucose production and caused hepatic insulin resistance. Finally, we demonstrated that the effect of E2 in the VMH on hepatic insulin sensitivity is mediated by the sympathetic innervation to the liver.

The effects of ovariectomy in animal models include increased food intake and decreased running activity, all of which are reversed upon E2 replacement (28, 29). A series of experiments by the group of Deborah Clegg nicely showed that local effects of E2 in the brain play an important role in these restorative effects of E2. Recent reports showed that a major part of the profound effects of E2 on energy metabolism are mediated via the hypothalamus. For instance, ICV infusion of E2 was sufficient to restore the normal pattern of body fat distribution in OVX females (6) and a local knock-out of ERα in the VMH was sufficient for animals to become obese (10). Thus E2 withdrawal and substitution have profound effects on adiposity and lipid metabolism (3, 6, 30). Direct effects of E2 on glucose metabolism via the brain, however, are not evident yet. On the other hand, previous studies by us and others revealed that the hypothalamus also plays a crucial role in the regulation of glucose production (19, 21). To further examine the possible neural mechanisms behind the modulation of peripheral E2 on glucose metabolism, we used reverse microdialysis technology in order to be able to administer E2 locally into PVN or VMH. In our first set of experiments, strikingly lower plasma glucose levels in OVX animals were found, which could be reversed by a systemic E2 replacement. Interestingly, the increase in plasma glucose concentrations induced by systemic E2 was blocked by the hypothalamic administration of the E2 antagonist ICI, both in PVN and VMH. The data indicated that, like glucocorticoid and thyroid hormone, hypothalamic E2, too, may play an important role in the regulation of peripheral glucose metabolism.

Next we infused E2 directly into the PVN and VMH by reverse microdialysis. The increased plasma glucose levels were consistent with the results of the antagonist experiment. The E2-induced changes could not be explained by the changed corticosterone or insulin levels, as during the VMH infusions neither plasma corticosterone nor insulin concentrations were affected. Infusions in the PVN did not affect plasma insulin concentrations either. In accordance with the well-known effects of estrogens on the hypothalamo-pituitary-adrenal axis, corticosterone concentrations were changed by E2 administration in the
Hypothalamic estrogen regulates glucose

PVN. However, the E2-induced corticosterone changes occurred after $t=150$ min, which was much later than the E2-induced change in plasma glucose. Although both electrical stimulation of VMH neurons and direct insulin injections in the VMH have been reported to induce an increase of plasma glucagon concentrations (31-33), we did not find any change in plasma glucagon values after the different treatments in the present study. These data indicate that the increased EGP induced by VMH E2 is not mediated by an increased release of glucagon, and that different mechanisms maybe activated via the VMH. Although our denervation experiments suggest that the E2-induced increased EGP is caused by an increased sympathetic activity, at present we cannot exclude the involvement of other hormonal regulators such as the catecholamines.

Recently Yonezawa et al reported that during exposure to a high-fat diet both peripheral and central E2 receptors are involved in the regulation of glucose metabolism (34). However, central and peripheral E2 receptors seem to operate via different mechanisms. When treated with peripheral E2, fatty acid synthase was decreased in white adipose tissue. While treatment with central E2, changed both liver glucose production and peripheral tissue glucose uptake (34). Their results suggest that hypothalamic E2 may effect on EGP and glucose uptake by respectively increasing and decreasing insulin sensitivity. Estrogenic effects on peripheral organs were also addressed by several other studies. In the liver glucose homeostasis seems to be regulated mainly by estrogen acting via ER$\alpha$, which was associated with a pronounced hepatic insulin resistance (35). Immunohistochemical analysis revealed that ER$\alpha$ and ER$\beta$ are co-expressed in the nuclei of most muscle cells. These studies also showed that ER$\alpha$ is a positive regulator of GLUT4 expression, whereas ER$\beta$ has a suppressive role (36).

In the current studies, E2 administration in the VMH but not PVN increased EGP, indicating that the increasing plasma glucose concentrations observed after PVN infusion were mainly due to a decreased glucose uptake, whereas the increased plasma glucose concentrations after E2 administration in the VMH were mainly due to an increased EGP. The two classic E2 receptors, ER$\alpha$ and ER$\beta$, show a distinct hypothalamic distribution, with the VMH mainly containing ER$\alpha$ and the PVN mainly containing ER$\beta$ (23). At present it is not clear how this differential receptor distribution contributes to the different glucoregulatory effects of E2 in the PVN and VMH.

We used hyperinsulinemic-euglycemic clamps with 2 different insulin plasma levels. Consistent with the basal EGP results, PVN E2 treatment caused a peripheral insulin resistance. On the other hand, both peripheral and hepatic insulin resistance were found in the group treated with VMH E2. Previous studies in our group showed that the hypothalamus often increases hepatic glucose production by stimulating sympathetic efferent nerves (18, 19, 21). Also in the current experiments the stimulatory effect of E2 via the VMH on EGP (and plasma glucose concentrations) was abolished by a sympathetic, but not a parasympathetic, denervation of the liver. On the other hand, autonomic denervation of the liver (either sympathetic or parasympathetic) had no effect on the stimulatory effect
of E2 on plasma glucose concentrations via the PVN. There is no evidence for a direct neural connection between the VMH and autonomic nuclei in the brainstem or spinal cord, contrary to the PVN. On the other hand, the VMH has pronounced projections to the PVN, which functions as the hypothalamic integration center for autonomic and endocrine information and serves as the final neuroendocrine and autonomic output nucleus from the hypothalamus (18, 37-40). ERα is expressed in the majority of glutamatergic neurons in VMH (41) and the PVN is known to receive a strong glutamatergic input from the VMH (42). Therefore, we propose that ERα-containing glutamatergic neurons in the VMH that project to the PVN are activated by local administration of E2, thereby exciting sympathetic pre-autonomic neurons in the PVN, that in turn stimulate the hepatic sympathetic tone.

In line with our present findings, a number of previous experiments have provided evidence for hypothalamic effects on glucose uptake mediated via the autonomic nervous system (43, 44). From a physiological viewpoint the opposite effects of ERα stimulation in the VMH on hepatic glucose production and peripheral glucose uptake (stimulatory and inhibitory, respectively) are plausible, as in this way the two mechanisms will act in concert to increase plasma glucose concentration. In order to explain the opposite effect of the VMH on muscle-dedicated and liver-dedicated pre-autonomic neurons we propose that either the glutamatergic projection of the VMH to the muscle-dedicated pre-autonomic neurons involves a GABAergic interneuron in the subPVN or that the VMH contains GABAergic ERα-expressing neurons which contact and inhibit the muscle-dedicated pre-autonomic neurons directly. Indeed, E2 treatment has been shown to increase GABAergic activity in the VMH (45). Finally, the preferential effect of E2 in the PVN on peripheral glucose uptake indicates that in all likelihood the muscle-dedicated pre-autonomic neurons, but not the liver-dedicated pre-autonomic neurons, in the PVN express the ERβ. Although the expression of ERβ mRNA in pre-autonomic PVN neurons has been reported (46), the peripheral targets of these neurons are not known.

Together, our results show differential effects of intrahypothalamic E2 on hepatic and peripheral glucose metabolism that are, at least partly, mediated by the sympathetic branch of the autonomic nervous system. However, the current results seem contradictory with earlier studies that indicated increased plasma glucose levels in OVX animals. We think this apparent difference is induced by the different models used. Most studies thus far used a “chronic” model in which animals were studied at least one month after the OVX (47-49). Therefore, the increased levels of plasma glucose observed most likely are the results of hyperphagia and obesity. The current results indicate that the first effect of reduced plasma E2 concentrations is a lowering of plasma glucose levels. During the second stage, the OVX animals develop increased food intake and decreased energy expenditure and become obese. At the end of the second stage, the impaired energy homeostasis will overrule the glucose-lowering effect of E2 removal. How the short-term effects of intrahypothalamic E2 on glucose metabolism as observed in the present set
of experiments can be reconciled with the long-term effects of E2 deprivation on body weight and insulin sensitivity remains to be determined.

Acknowledgements

Ji Liu is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. J.Liu contributed to the experiment and wrote the manuscript. P.Bisschop researched the data and reviewed/edited the manuscript. L.Eggels contributed to the experiment. E.Foppen contributed to the experiment. M. Ackermans contributed to the EGP measurement. JN Zhou reviewed/edited the manuscript. E.Fliers researched the data and reviewed/edited the manuscript. A.Kalsbeek researched data, reviewed/edited the manuscript and contributed to discussion.

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Chapter 4

Reference


Supplemental data 1: Probe location
At the end of the experiments all the animals were sacrificed for histological verification of the probe location. One hundred and forty-one animals were used in this part of the study. The data from 19 animals had to be discarded because of incorrect probe placements (n=11) or an incomplete data set due to catheter problems (n=8).

Supplemental Figure. Representative sections and schematic drawings demonstrating probe locations in the PVN (A) and VMH (B).

Supplemental data 2: Protocol for measurement of [6,6-2H2]glucose enrichment
The protocol was described previously (1). Briefly, the samples (25μl plasma) were deproteinized by mixing with 1 mL methanol. After centrifugation, the supernatant was evaporated to dryness under a stream of N2. The aldonitrile penta-acetate derivative of glucose was prepared with 100 μL hydroxylamine in methanol (5 mg hydroxylamine and 12.5 mg sodium acetate in 1 mL methanol), and the mixture was heated for 60 min at 60 C. After drying the sample under a stream of N2, 100 μL acetic anhydride were added, and the sample was heated for another 60 min at 120 C. The reaction mixture was cooled and partitioned between water (750 μL) and methylenechloride (750 μL). The lower methylenechloride layer was dried and reconstituted in ethylacetate, which was injected into the gas chromatograph (model 6890 gas chromatograph coupled to a model
5973 mass selective detector, equipped with an electron impact ionization mode, Hewlett-Packard Co., Palo Alto, CA). The enrichment of [6,6-2H2]glucose was determined by dividing the peak area at M+2 by the total peak area at M, and correction for the natural abundance was performed by subtracting the natural abundance from the measured M+2 enrichment.

The calculation was performed as described before (2). Briefly, when endogenous glucose production (Ra) and glucose disposal (Rd) are calculated, the added source of labeled glucose entering the system and the exogenous glucose infusate should be taken into account. Thus, Ra and Rd were calculated with a modified form of the Steele equations as described by Finegood et al(3):

\[
R_a(t) = \frac{I}{Pct_p(t)} - \frac{pV G(t) [dPct_p(t)/dt]}{Pct_p(t)} + \left[ \frac{Pct_p(t) \times GInf(t)}{Pct_p(t)} \right] - pV \frac{dG(t)}{dt}
\]

and

\[
R_d(t) = \frac{I}{Pct_p(t)} - \frac{pV G(t) [dPct_p(t)/dt]}{Pct_p(t)} + \left[ \frac{Pct_p(t) \times GInf(t)}{Pct_p(t)} \right] - GInf(t)
\]

where I is the constant tracer infusion rate (mg · kg⁻¹·min⁻¹), t is time, Pctp(t) is the percentage enrichment in plasma glucose taken as the average of 2 consecutive samples, p is the pool fraction, V is the distribution volume of glucose, G(t) is the plasma glucose concentration taken as the average of 2 consecutive samples, dPctp(t)/dt is the rate of change in the percentage enrichment in plasma (min⁻¹), GInf(t) is the rate of infusion of exogenous glucose, Pctg is the percentage enrichment of the glucose infusate, and dG(t)/dt is the rate of change in the plasma glucose concentration; V (pV) was set at 40 mL/kg. The [6,6-2H2]glucose enrichment (tracer/tracee ratio) total assay CV was 1%, the intra-assay CV 1%, and the detection limit 0.04%.

Supplemental data 3: time schedule for clamping experiment (Experiment 4&6)

Background blood samples and isotope tracer infusion were the same as for Exp.3. At t=15 min, insulin was administered in a primed I.V. infusion (3.6 mU/kg.min in 5 min for the “low” clamp, and 7.2 mU/kg.min in 5 min for the “high” clamp), followed by a continuous I.V. infusion (1.5 mU/kg.min for the “low” clamp and 3 mU/kg.min for the “high” clamp). A variable infusion of a 25% glucose solution (containing 1% [6,6-2H2] glucose) was used to maintain euglycemia (5.5±0.5mmol/l), as determined by carotid catheter blood sampling every 10 min. Thirty min after the start of the primary insulin infusion (t=45min), Ringer’s perfusion of the microdialysis probes was replaced by the E2 solution (10 μg/ml, 3 μl/min) or vehicle (Ringer’s containing 1%DMSO). At the end of the clamp, five blood samples were taken with a 5 min interval at t=120, 125, 130, 135 and 140 min.
Supplemental data 4: Liver noradrenalin levels during denervation experiments. After homogenizing the tissue samples, the noradrenalin was measured by an in-house HPLC method. Essentially norepinephrine and epinephrine were selectively isolated by liquid-liquid extraction (4) and derivatized with the fluorescent 1,2-diphenylethylenediamine (5). The fluorescent derivatives were separated by reversed phase liquid chromatography and detected by scanning fluorescence detection.

**Supplemental Figure.** The liver noradrenaline concentration is reduced to <10% of control values in the sympathetic denervation groups, but not in the parasympathetic denervation groups. (A) Experiment 5 (P<0.001) and (B) Experiment 6 (P<0.001).
Supplemental data 5: Plasma glucose level and variable glucose infusion rate during clamping experiment (Experiment 4 & 6)

**Supplemental table.** The table presents the mean of the plasma glucose concentration in the basal state before (t= 0, 5, 10 min) and at the end of the different clamp experiments. Plasma glucose levels were around 5.5 mmol/l during both the basal state and the clamp. No significant group differences were detected.

<table>
<thead>
<tr>
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<th>basal</th>
<th>hyperinsulinemia</th>
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<tr>
<td>&quot;low&quot; clamp</td>
<td></td>
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<tr>
<td>vehicle</td>
<td>5.32±0.27</td>
<td>5.36±0.45</td>
</tr>
<tr>
<td>PVN E2</td>
<td>5.50±0.36</td>
<td>5.54±0.34</td>
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<td>VMH E2</td>
<td>5.56±0.33</td>
<td>6.02±0.38</td>
</tr>
<tr>
<td>&quot;high&quot; clamp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vehicle</td>
<td>5.23±0.34</td>
<td>5.49±0.56</td>
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<td>5.00±0.68</td>
<td>5.13±0.53</td>
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<tr>
<td>VMH E2</td>
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**Supplemental figure 5.** During the "low" dose insulin clamp, the variable glucose infusion rate was lower in both the PVN and VMH E2 treatment groups as compared to vehicle, but lowest in the VMH E2 group (Suppl. 5A). During the "high" clamp, the infusion rate was significantly lower in both the PVN and VMH E2 treatment groups as compared to vehicle (Suppl. 5B). After sympathetic denervation, the variable glucose infusion rate was highest in the SX group and comparable to the infusion rate in Vehicle treated animals of the "low" clamp group in suppl. 5A.
References


Chapter 5

Hypothalamic estradiol increases UCP gene expression in brown adipose tissue and lipolysis gene expression in abdominal white adipose tissue
Chapter 5

Hypothalamic estradiol increases UCP gene expression in brown adipose tissue and lipolysis gene expression in abdominal white adipose tissue

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Abstract

Reduced central estrogen (E2) signaling results in decreased energy expenditure, resulting in increased fat mass if energy intake remains the same. In addition, central E2 affects body fat distribution. In the present study, we explored the effects of central E2 on gene expression in brown and white adipose tissue (BAT and WAT) compartments. In experiment #1, we treated female rats after ovariectomy (OVX) with subcutaneous (sc) or intracerebroventricular (icv) E2 or vehicle for 4 weeks and studied its effect on WAT distribution using MRI. In experiment #2, we treated rats after OVX with intrahypothalamic E2 or vehicle for 3h via microdialysis in the hypothalamic paraventricular nucleus (PVN) or ventromedial nucleus (VMH), to study effects of hypothalamic E2 on gene expression in WAT and BAT using qPCR. In experiment #1, both sc and icv E2 reduced body weight compared with vehicle treatment, while perirenal (pWAT) and gonadal (gWAT), but not subcutaneous (sWAT) weight, was reduced. There was no difference in plasma E2 concentration between icv E2 treated and OVX animals. In experiment #2, E2 administration in the VMH, but not in the PVN, increased deiodinase type 2 (D2) and UCP-1 expression in BAT. Furthermore, E2 administration in the PVN and VMH increased HSL expression in pWAT and gWAT, but not in sWAT, suggesting that hypothalamic E2 selectively affects lipolysis in the various adipose compartments. Together, our results indicate that hypothalamic E2 affects body fat distribution independently of circulating E2. Hypothalamic effects of E2 on WAT are mediated by estrogen sensitive neurons in both the PVN and VMH, but hypothalamic effects on BAT are primarily mediated via the VMH. 

Key words: estradiol, UCP gene, fat distribution, adrenoceptor
Estrogen and fat distribution

Introduction

Estrogen plays a key role in energy homeostasis. Excessive body weight gain is observed after ovariectomy and can be prevented by 17β-estradiol (E2) replacement (1-3). A number of studies have indicated that increased body weight after ovariectomy is not only the result of increased food intake, but also of reduced energy expenditure (4, 5), as ovariectomized rats need less calories to maintain body weight compared to intact rats (6). One of the determinants of energy expenditure is adaptive thermogenesis in brown adipose tissue (BAT). Of interest, sympathetic denervation of brown adipose tissue (BAT) markedly impairs the estrogen-induced increase in oxygen consumption (7). This may be explained, at least in part, by effects of estrogen on the expression of uncoupling protein (UCP) in BAT, as UCP expression is down-regulated by ovariectomy and up-regulated by E2 supplementation (6). Selective inhibition of estrogen signaling in the hypothalamus also reduces total energy expenditure, suggesting an important role of the hypothalamus in the effects of estrogen on energy homeostasis (8). Whether hypothalamic E2 signaling is involved in the regulation of BAT activity is unknown at present.

E2 regulates not only energy expenditure but also body fat distribution (8, 9, 10). This has been documented in humans (9) and in rodent models (1), but the mechanism is unknown at present. Fat deposition in white adipose tissue (WAT) is mainly regulated by the rate-limiting enzymes involved in lipogenesis and lipolysis, i.e., fatty acid synthase (FASN) and lipoprotein lipase (LPL) at the one hand and hormone sensitive lipase (HSL) at the other hand. Previous studies have shown that systemic estradiol may regulate LPL and FASN gene expression in heart tissue and adipose tissue (10, 11). Whether hypothalamic E2 signaling can regulate gene expression in adipose tissue is unknown at present.

In the present studies, we aimed to further investigate the effects of hypothalamic E2, on body fat distribution, by studying the expression of key regulators of fat metabolism in BAT and in subcutaneous, perirenal and gonadal WAT after local hypothalamic administration of E2 to ovariectomized female rats.

Material and Methods

Animals

Female Wistar rats (Harlan, Horst), housed in a 12-h light–12-h dark schedule (lights on at 0700 h) were used for all experiments. Body weight was between 220 and 280 g at the time of experiments. Food and drinking water were available ad libitum. All of the following experiments were conducted with the approval of the Animal Experimental Committee of the Academic Medical Center (AMC) in Amsterdam.
Surgery

All animals underwent a bilateral OVX or sham operation under anesthesia by a mixture of ketamine/xylazine (100:10 mg/kg IP). With a standard Kopf stereotaxic apparatus, a single icv probe was placed into the lateral ventricle. Bilateral microdialysis probes were placed adjacent to the paraventricular nucleus of the hypothalamus (PVN) or the ventromedial nucleus of the hypothalamus (VMH). The coordinates for the icv probe were AP: 0.6mm, lateral: 1.5mm and ventral 3.5mm from the surface of the dura, for the PVN probes AP: 1.6 mm, lateral: 1.8 mm (angled at 10°) and 9.1 mm ventral from the surface of the bone, and for the VMH AP: 2.5mm, lateral: 2.0mm (angled at 10°) and 9.0 mm ventral from the dura. We used dental cement to secure the icv probe or the microdialysis probes with 4 stainless-steel screws inserted into the skull.

Experimental procedures

Experiment #1 was designed to investigate the body weight and fat distribution changes induced by 4 weeks of E2 treatment. After ovariectomy and placement of an icv probe, animals were divided into three groups: OVX plus vehicle, OVX plus subcutaneous (sc) 17β-estradiol and OVX plus icv 17β-estradiol treatment. Estradiol (Sigma, St. Louis, USA) was dissolved in sesame oil (Sigma, St. Louis, USA) to a concentration of 200 ng/ml and injected iv (100 μL) or icv (1 μL), every fourth day for one month. The same volume of sesame oil was injected in the vehicle animals, both sc and icv. After one month, the animals were sacrificed and scanned by a clinical 3.0 T Philips Intera MRI scanner (Philips Healthcare, Best, The Netherlands)(12). Image J software (National Institutes of Health, United States) was used to determine body fat distribution. To segment images, a manual threshold was set to separate the high intensity signal of adipose tissue from the lower intensity signal of non-fatty tissues and background. This was followed by manual separation of visceral and non-visceral fat in the images to determine relative visceral fat volume.

To exclude leakage of E2 from brain to the periphery, we measured the plasma estradiol concentration at 15min, 30min, 45min, 135min, 24hr, 48hr and 72hr after a bolus injection of icv E2 (200 ng/ml, 1 μl).

In experiment 2, rats were ovariectomized and microdialysis probes were implanted 1-week before the start of the experiment. The day before the experiment animals were connected to a metal collar which was kept out of reach from the rats by means of a counterbalanced beam. This allowed all manipulations to be performed outside the cages without handling the animals. 1 mg 17β-estradiol (Sigma, St. Louis, USA) was dissolved in 1 ml pure dimethyl sulfoxide (DMSO) and diluted 100 times with Ringer solution. Ringer’s dialysis (3μl/min) in the PVN or VMH via the microdialysis probes was started at t=-60 min. At t=0 min 17β-estradiol (10 μg/ml, 3 μl/min) or vehicle (Ringer with 1% DMSO, 3 μl/min) were infused by retrodialysis into PVN or VMH. After an infusion period of 180
min, animals were anesthetized and sacrificed in their own cage. BAT, subcutaneous, perirenal and gonadal white adipose tissue were dissected and frozen in liquid nitrogen immediately. Samples were stored in -80 until analysis.

RNA isolation and RT-PCR
Adipose tissue mRNA was isolated on the Magna Pure (Roche Molecular Biochemicals, Mannheim, Germany) using the Magna Pure LC mRNA tissue kit from approximately 30 mg of tissue. The protocol and buffers supplied with the kit were followed. cDNA synthesis was performed using the First Strand cDNA Synthesis kit for RT-PCR with oligo d(T) primers (Roche Molecular Biochemicals). Real-time PCR was performed using the Lightcycler 480 (Roche Molecular Biochemicals) and the Lightcycler 480 Sybr Green I Master kit (Roche Molecular Biochemicals). Primer pairs sequence for hypoxanthine phosphoribosyl transferase (HPRT; housekeeping-gene muscle) obtained from Sweet et al was used as house-keeping gene (13). The primers used in the present study are listed in Table 1. Primers were intron-spanning or alternatively, genomic DNA contamination was tested using a cDNA synthesis reaction without the addition of RT. Samples were corrected for their mRNA content using HPRT as a housekeeping gene. Samples were individually checked for their PCR-efficiency using the LinReg software (14). The median of the efficiency was calculated for each assay. Samples that differed more than 0.05 of the efficiency median value were not taken into account. Aberrant PCR-efficiencies occurred randomly and therefore did not bias the results.

Statistical analysis
Data are presented as mean ± SEM. All results were initially analyzed by analysis of variance (ANOVA). When appropriate, further post-hoc tests were done with Fisher LSD test.

Table 1 Primer sequence for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers Annealing Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>Forward: 5'-GCAGTACAGCCCCAAAAATGG-3', Reverse:5'-AACAAAAGTCTGGCCTGTATCCAA-3'</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Forward:5'-AACAGCTTGTCTTCCCTCA Reverse:5'-GCT TTGTGCTTGCATTCTGA</td>
</tr>
<tr>
<td>UCP-3</td>
<td>Forward:5'-GCACTGAGGCTTTCTGCTGA Reverse:5'-ATAAGTCAGATGTGTACCAGGA</td>
</tr>
<tr>
<td>HSL</td>
<td>Forward: CAC ACA GCA TGG ATT TAC GCA Reverse: ACC TGC AAA GAC GTT GGA CAG</td>
</tr>
<tr>
<td>LPL</td>
<td>Forward: CAA AAC AAC CAG GCC TTC GA Reverse: AGC AAT TCC CCG ATG TCC A</td>
</tr>
<tr>
<td>FASN</td>
<td>Forward: CTT GGG TGC CGA TTA CAA CC Reverse: GCC CTC CGG TAC ACT CAC TC</td>
</tr>
</tbody>
</table>
Chapter 5

Results

Effects of 4 weeks systemic or icv E2 on body weight and fat distribution

As expected, chronic subcutaneous estradiol administration reduced body weight gain in ovariectomized rats compared to vehicle. A similar reduction in body weight gain was observed after icv estradiol administration (figure 5.1A). Subcutaneous and icv estradiol administration reduced perirenal and gonadal fat accumulation to a similar extent (figure 5.1B/C). Plasma estradiol concentrations did not change after icv estradiol administration, (figure 5.1 D).

Figure 5.1 Intracerebroventricular and subcutaneous administration of estradiol reduces intra-abdominal fat deposition in ovariectomized rats. Effect of vehicle (OVX), 17β-estradiol iv (OVX+subE2) and 17β-estradiol icv (OVX+icvE2) on (A) body weight, (B) perirenal/subcutaneous fat ratio and (C) gonadal/subcutaneous fat ratio. (D) ICV bolus injections of E2 did not affect plasma E2 concentrations as compared to vehicle. ns = not significant; * p<0.05; ** p<0.01. OVX, n=6, OVX sub E2 n=5, OVX icv E2 n=10 for figure 5.1A. OVX, n=5, OVX sub E2 n=5, OVX icv E2 n=7 for figure 5.1 B, C. n=5 for each group in figure 5.1 D.
Effects of hypothalamic E2 within subcutaneous, perirenal and gonadal WAT.

Hormone sensitive lipase (HSL) catalyses the rate-limiting step in adipose tissue lipolysis. Ovariectomy decreased HSL expression in PWAT and GWAT, but not in SWAT (figure 5.2). E2 administration in the PVN and VMH increased HSL expression in PWAT and GWAT, but not in SWAT.

**Figure 5.2** Relative HSL mRNA expression in (A) perirenal white adipose tissue, (B) gonadal white adipose tissue and (C) subcutaneous white adipose tissue in intact and ovariectomized animals (PVN/VMH + VEH) and in response to ovariectomy plus E2 administration in the PVN (PVN + E2) or VMH (VMH + E2). Groups: intact, n=7; Veh, n=6; PVN E2, n=7; VMH E2 n=6. * p<0.05

Ovariectomy did not affect LPL expression in PWAT, while it decreased LPL expression in GWAT and increased LPL expression in SWAT (figure 5.3). E2 administration in the PVN only reversed the effect of OVX in GWAT.

**Figure 5.3** Relative LPL mRNA expression in (A) perirenal white adipose tissue, (B) gonadal white adipose tissue and (C) subcutaneous white adipose tissue in intact animals, in response to ovariectomy (PVN/VMH + VEH) and in response to ovariectomy plus E2 administration in the PVN (PVN + E2) or VMH (VMH + E2). * p<0.05. Groups: intact, n=7; Veh, n=6; PVN E2, n=7; VMH E2 n=6.
We did not observe an effect of ovariectomy or hypothalamic E2 administration on FASN expression in any of the adipose tissue compartments (figure 5.4).

**Effects of hypothalamic estradiol on BAT**

Ovariectomy reduced UCP-1 and UCP-3 expression in BAT. Administration of E2 in the VMH, but not in the PVN, increased both UCP-1 and UCP-3 expression in BAT compared to vehicle. Ovariectomy had no effect on DIO2 expression in BAT, but E2 administration in the VMH of ovariectomized rats significantly increased DIO2 expression in BAT compared to vehicle (Figure 5.5).

**Figure 5.4** Relative FASN mRNA expression in (A) perirenal white adipose tissue, (B) gonadal white adipose tissue and (C) subcutaneous white adipose tissue in intact animals, in response to ovariectomy (PVN/VMH + VEH) and in response to ovariectomy plus E2 administration in the PVN (PVN + E2) or VMH (VMH + E2). Groups: intact, n=7; Veh, n=6; PVN E2, n=7; VMH E2 n=6.

**Figure 5.5** Relative mRNA expression of (A) UCP1, (B) UCP3 and (C) DIO2 in brown adipose tissue in intact and ovariectomized animals (PVN/VMH + VEH) and in response to ovariectomy plus E2 administration in the PVN (PVN + E2) or VMH (VMH + E2). Intact n=7, PVN/VMH + VEH n=7, PVN + E2 n=6, VMH + E2 n=6. * p<0.05.
Discussion

The major finding of the present study is that hypothalamic E2 differentially modulates gene expression in white and brown adipose tissue. Earlier studies have shown that in rodents ovariectomy (OVX) increases body weight and adiposity, whereas systemic as well as central estrogen replacement can reverse these changes (15-17). In addition to fat mass, E2 also affects fat distribution. In the present study, we found decreased amounts of perirenal and gonadal, but unchanged amounts of subcutaneous fat after systemic and central E2 treatment, confirming a previous study (1). When on a restricted feeding schedule, OVX animals need less food in order to maintain a similar body weight as intact females (6, 18), which indicates that energy expenditure is reduced in these animals. Indeed, when given the same amount of food, OVX animals showed increased body weight (18). Adaptive thermogenesis, the dissipation of energy in the form of heat in response to external stimuli, is implicated in the regulation of energy balance. As E2 has profound modulatory effects on body temperature in rodents (19, 20) we decided to include BAT in the analysis of effects of central E2. UCP-1 is mainly present in brown adipocytes and its function is to create a fatty acid-activated uncoupling of respiration (21). UCP-2 and UCP-3 are not involved in cold-induced thermogenesis (21), and thought to be linked to resting metabolic rate (22). Previous studies have shown that UCP gene expression in both BAT and WAT is decreased after OVX and that this decrease is partly reversed by E2 replacement (6). These results indicate that E2 may affect the energy balance by modulating thermogenesis through activation of UCP gene expression. In the present study, we found both UCP-1 and UCP-3 gene expression to be decreased in BAT of OVX animals when compared to intact animals. The reduced UCP gene expression in OVX animals indicates lower energy expenditure. Interestingly, E2 treatment in VMH completely reversed the OVX induced down-regulation of both UCP-1 and UCP-3. In the last decade a large number of studies evidenced the primary role of the hypothalamus in the regulation of body weight and energy metabolism (18, 23). A loss of ERα in the VMH appeared to induce obesity and visceral fat accumulation (8). Recently, the VMH was also found to be implicated in the activation of UCP in adipose tissue (24). This suggests that VMH estrogen signaling plays an important role in the regulation of energy expenditure by activating BAT UCP gene expression. In the long run, these changes in UCP expression may affect fat accumulation and body weight gain.

LPL is the rate-limiting enzyme for hydrolysis of the triglyceride component of circulating lipoproteins and thereby is essential for lipogenesis. HSL functions to hydrolyze the first fatty acid from stored triacylglycerol molecules, and is considered the rate limiting step in lipolysis (25). In WAT, we found lower HSL and LPL expression after OVX. These observations are in line with other studies showing a lower HSL gene expression in the liver of OVX (26). Interestingly, higher LPL gene expression was found in SWAT after OVX as compared to intact animals. The changes in LPL expression therefore appear to reflect
the redistribution of body fat in favour of the abdominal compartment in OVX animals. E2 administration in either the PVN or VMH acutely reversed the lowered HSL expression in the visceral fat compartments of OVX animals. LPL expression, however, was regulated by hypothalamic estrogen signaling only in GWAT. We did not observe any significant effects of OVX or hypothalamic E2 signaling on FASN gene expression, suggesting that the hypothalamic E2 signal affects fat accumulation and distribution primarily by modulating lipolytic genes. We found no effect of hypothalamic E2 on SWAT, again pointing to the concept that the hypothalamic E2 regulation of fat distribution is primarily effectuated through the accumulation of visceral fat. Previously it was shown that intra-abdominal and subcutaneous fat depots are innervated by separate sets of autonomic neurons (Bartness) and, moreover, that these autonomic neurons in the spinal cord and brainstem are controlled by separate sets of pre-autonomic neurons in higher brain areas (27). These neural pathways may represent the anatomical substrate for the selectivity of the molecular effects of hypothalamic E2 as observed in the present study.

In the hypothalamus, multiple signals from the lateral hypothalamus and arcuate nucleus converge in the PVN to control energy metabolism (28, 29). Moreover, the PVN contains oxytocin, corticotrophin releasing hormone (CRH) and thyrotrophin releasing hormone (TRH) neurons that are likely engaged in the regulation of energy metabolism (30, 31). The hypothalamic VMH contains many glucose sensitive neurons and has direct connections with other hypothalamic nuclei that may be involved in energy homeostasis, i.e., the PVN and the dorsomedial hypothalamus (DMH) (28). Both PVN and VMH express estrogen receptors (ER). The predominant ER subtype distribution, however, is different between the two nuclei. Both ERβ and ERα are located in the PVN, whereas the VMH almost exclusively expresses ERα (32). The differential ER subtype distribution may represent an additional explanation for the differential effects of E2 administration in the PVN and VMH on WAT gene expression. Indeed, recently we demonstrated differential effects of E2 administration in the PVN and VMH on glucose metabolism (Liu et al., Diabetes 2013, in press).

In conclusion, PVN E2 increased LPL and HSL expression in gonadal white adipose tissue while VMH E2 increased UCP gene expression in brown adipose tissue. These physiological data support previous neuroanatomical data indicating separate neural control of different WAT compartments.
Estrogen and fat distribution

References


Central estrogen signaling regulates bone formation in the rat
Central estrogen signaling regulates bone formation in the rat

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Abstract

Background: Inhibition of sympathetic signaling to bone prevents loss of bone mass after ovariectomy in rodents. As sympathetic activity is controlled by the central nervous system we hypothesized that an interaction between estrogen and the sympathetic nervous system (SNS), relevant for the regulation of bone remodeling, takes place in the brain. Objective: To determine the effect of subcutaneous (SC) and intracerebroventricular (ICV) administration of 17β-estradiol (E2) on bone remodeling in ovariectomized rats. Methods: Adult female Wistar rats were ovariectomized and an ICV cannula was placed in the lateral cerebral ventricle. After surgery rats were divided in three groups: 1) VEH: OVX plus ICV vehicle injections (sesame oil) every 4th day (n=6), 2) OVX-SC: OVX plus SC E2 injections (2.0 ug) every 4th day (n=5), 3) OVX-ICV: OVX plus ICV E2 injections (0.02 ug) every 4th day (n=10). After 4 weeks bone mass and bone remodeling was determined by histomorphometric analysis of the tibia. Data were analyzed by ANOVA. Results: Bone volume was not different between the groups after 4 weeks (p=0.119), but bone formation rates were lower in OVX-SC and OVX-ICV as compared to VEH (p=0.019). Conclusion: Both subcutaneous and intracerebroventricular administration of estrogen reduces bone formation rate after ovariectomy, suggesting that part of the effect of estrogen on bone remodeling is mediated via the central nervous system.

Key words: estrogen, bone volume, bone resorption, bone mass
Introduction

Osteoporosis is characterized by a decrease in bone mass and impaired bone integrity. Bone mass is maintained by the balance between osteoblastic bone formation and osteoclastic bone resorption [1]. Postmenopausal status is an important risk factor for accelerated bone loss [2, 3] and osteoporotic fractures in women [4, 5]. Similarly, ovariectomy (OVX) in rats increases bone resorption, and – although to a lesser extent- bone formation, leading to an overall loss of mainly cancellous bone [6, 7].

A decrease in serum levels of sex steroids, especially estrogen, is thought to induce bone loss, because several studies indicated that OVX induces osteopenia in rats by increasing the bone turnover [6, 7], while estrogen replacement decreases bone turnover [8] [9]. Thus, the bone loss during estrogen deficiency is attributable to an increase in bone resorption, together with a smaller increase in bone formation which results in a net bone loss. Indices of formation as well as resorption are suppressed by replacement of estrogen which results in a net gain, preventing further bone loss [6, 7, 10, 11].

It is generally thought that the effect of estrogen on bone metabolism is a direct local effect within the bone compartment. However, in vitro studies on cultured osteoblasts/osteoclasts showed rather modest effects of estrogen on osteoblast proliferation, that were only observed when the cells were transfected with a high number of estrogen receptors [12]. These observations suggest additional pathways by which estrogen controls bone metabolism. Groundbreaking studies have revealed an important role for the brain in the regulation of bone metabolism via leptin, neuropeptide Y (NPY) and sympathetic signaling [13, 14].

Estrogen receptors (ER) are expressed throughout the brain. In the hypothalamus, ER expression has been reported in several nuclei, including the paraventricular nucleus (PVN), the ventromedial hypothalamus (VMH), the lateral hypothalamus (LH) and the arcuate nucleus (ARC) [15, 16]. In the brain estrogen interacts with leptin and NPY, both known to be involved in neural regulation of bone remodeling. However, whether estrogen signaling in the brain is also involved in the neural regulation of bone remodeling is not known. In the present study, we aimed to investigate the effect of centrally administered estrogen on bone metabolism.

Materials and Methods

Animals

Female Wistar rats (Harlan, Horst), housed in a 12-h light–12-h dark schedule (lights on at 0700 h) were used for all experiments. Body weight was between 220 and 280 g. Food and drinking water was available ad libitum. The experiments were conducted with the
approval of the Animal Experimental Committee of the Academic Medical Center (AMC) in Amsterdam.

All animals underwent a bilateral ovariectomy (OVX) or sham operation under anesthesia by a mixture of ketamine/xylazine (100:10 mg/kg IP). With a standard Kopf stereotaxic apparatus, a single intracerebroventricular (ICV) probe was placed into the lateral ventricle. The coordinates for the ICV probe were AP: 0.6mm, lateral: 1.5mm and ventral 3.5mm from the surface of the dura. The animals were divided into three groups: OVX plus ICV vehicle treatment (VEH) (n=5), OVX plus subcutaneous estradiol (OVX-SC) (n=5) and OVX plus ICV estradiol treatment (OVX-IVC) (n=7). Estradiol (20ug/ml; Sigma, St. Louis, USA) was dissolved in sesame oil (Sigma, St. Louis, USA) for injection. Estradiol was injected subcutaneously or ICV, 100 μl (i.e. 2 μg) and 1 μl (i.e. 0.02 μg) respectively, every fourth day for four weeks. The same volume of sesame oil was injected ICV in the vehicle animals. Nine and three days before sacrifice, a single dose of tetracycline was administered by i.p. injection (20 mg/kg, Merck Millipore, Amsterdam, The Netherlands).

Tissue preparation

The right tibia was dissected and freed of soft tissue, and one end of the tibia was cut off and flushed by cold PBS. The tissue was fixated in 4% formaldehyde for 24 hours at room temperature, washed twice with phosphate buffer solution for 30 minutes, dehydrated through graded ethanols and embedded in methylmethacrylate. Five μm thick undecalcified sections were cut with a Polycut 2500S microtome (Reichert Jung, Nussloch, Germany) and stained with Goldner's trichrome. Tetracycline labels were measured on unstained sections.

Histomorphometry

Histomorphometry was performed automatically using NIS-Elements AR2.10 (Nikon GmbH) at x100 magnification. All histomorphometric indices were defined and calculated according to the ASBMR nomenclature [17]. Trabecular bone volume and bone surface, single and double labeled surface, interlabel thickness and osteoclast number was measured. We calculated labeled surface, mineral apposition rate, bone formation rate and osteoclast surface and volume.

Statistical analysis

Data are presented as mean ± SEM. All results were initially analyzed by analysis of variance (ANOVA). When appropriate, further post-hoc tests were performed with Fisher LSD test.
Results

As expected, subcutaneous and ICV E2 treatment reduced body weight gain compared to vehicle, although the effect was less pronounced for the E2-ICV than for the E2-SC group (P=0.017, ICV vs VEH; P<0.001, SC vs VEH) (Figure 6.1).

Bone volume was not significantly different between the groups after 4 weeks of treatment (P=0.119) (Figure 6.2A). However, bone formation rate was lower after E2-SC and E2-ICV compared to OVX (E2-SC 0.2082±0.117 μm³/μm²/day, E2-ICV 0.2998±0.071 μm³/μm²/day, OVX 0.7358±0.168 μm³/μm²/day, p=0.019) (Figure 6.2B). Post-hoc analysis showed that both SC and ICV treatment with E2 significantly decreased bone formation rate when compared to the vehicle treatment group, and there was no significant difference between the two E2 treatment groups (OVX vs E2-SC p=0.014, OVX vs E2-ICV p=0.015, E2-SC vs E2-ICV p=0.618). Mineral apposition rate (MAR) was not significantly affected by treatment, either in the trabecular bone or in the cortical bone (P=0.141 and P=0.108, respectively) (Figure 6.2C and 6.2D). In addition, we did not find significant changes in osteoclast number, whether expressed per surface or per volume (P=0.198 and P=0.214, respectively) (Figure 6.2E and 6.2F).
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To exclude leakage of estradiol from the cerebroventricular compartment to the systemic circulation, we measured plasma estradiol concentration 15 min, 45 min, 75 min, 135 min, 1 day, 2 days and 3 days after an ICV bolus of estradiol. No significant differences were found between the estradiol and vehicle treated groups (time effect $p=0.001$, group effect $P=0.436$, time *group effect $P=0.72$) (Figure 6.3)

Figure 6.2 The effect of SC and ICV E2 treatment on several parameters of bone metabolism in ovariectomized rats. (A) bone volume; (B) bone formation rate (BFR); (C) and (D) mineral apposition rate in trabecular and cortical bone; (E) and (F) osteoclast number expressed per surface or per volume. N = 5 – 7 animals per group.

To exclude leakage of estradiol from the cerebroventricular compartment to the systemic circulation, we measured plasma estradiol concentration 15 min, 45 min, 75 min, 135 min, 1 day, 2 days and 3 days after an ICV bolus of estradiol. No significant differences were found between the estradiol and vehicle treated groups (time effect $p=0.001$, group effect $P=0.436$, time *group effect $P=0.72$) (Figure 6.3)
Central estrogen reduces bone formation

Discussion

In the present study, we found that not only systemic, but also central treatment of OVX animals with estrogen decreases bone formation rate. Although the dose used for the ICV administration was 100 times less than that of the SC injections the ICV effect on BFR was similar to that of the SC injections. This result clearly indicates that estrogentic modulation of bone formation is at least partly mediated by estrogen signaling in the central nervous system. Previous studies indicated that the reduction in bone formation was caused by a suppression of bone resorption which was not fully compensated for by an increased bone formation rate [18-21]. Thus, the estrogen treatment, either peripherally or centrally, most likely decreases OVX induced bone resorption.

Estrogens are important endocrine regulators of skeletal growth and maintenance, as demonstrated in both animal models and human studies [12, 22, 23]. A decline of endogenous estrogen, induced either by OVX in animal models or by menopause in women, reduces bone mass, whereas estrogen replacement reverses it [7] [24, 25]. The physiological effects of estrogen are mainly exerted via the two classic nuclear estrogen receptors (ERs), ERα and ERβ, which are ligand-activated transcription factors. Previous studies using transgenic mouse models showed that signaling via ERα protects against OVX-induced trabecular bone loss [24, 26, 27]. ERα is widely expressed in the hypothalamus, especially in the ventromedial-hypothalamus (VMH) and arcuate nucleus. Estrogen and leptin receptors are co-localized in neurons within these areas [28], while leptin signaling is well known to modulate bone metabolism [29]. These findings highlight the VMH and ARC as potential brain sites for estrogentic modulation on bone formation. Ducy et al [29] first provided evidence that the central nervous system regulates bone mass by showing that leptin-deficient mice have a higher bone mass, and that this
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phenotype could be reversed by ICV injections of leptin [29]. Furthermore, in mice lacking the β2-adrenergic receptor, the central bone-reducing effects of leptin were blunted, demonstrating that the central effects of leptin on bone mass may be mediated by the sympathetic nervous system [30]. Of interest, both efferent and afferent fibers have been shown to innervate bone tissue [31].

We did not detect any change in bone volume, mineral opposition rate or osteoclast indices, neither after systemic nor after ICV E2 treatment. The lack of SC effects seems inconsistent with previous studies. However, the dose, group size and treatment period in the present study were different from previous studies. For example, Wronska et al [1988] found that estradiol treatment (10ug, 25ug and 50ug/kg) for 35 days increased trabecular bone volume while it decreased bone formation rate [32]. Yeh et al [1997] showed bone density and total bone volume to be changed by treatment with 10ug/kg estradiol for 6 weeks [33]. In order to unmask more subtle effects of SC and/or ICV estrogen signaling on bone metabolism, probably longer periods of treatment and/or higher doses will be needed in future experiments.

To conclude, the estradiol-induced decrease in bone formation rate is at least partly mediated by the central nervous system.
Central estrogen reduces bone formation

Reference


Chapter 7

General discussion
General discussion

In the present thesis we examined the effects of estrogen, mediated via the central nervous system (CNS), on hypothalamo-pituitary-adrenal (HPA) axis activity, glucose homeostasis, and lipid and bone metabolism. We found that local estradiol synthesis in the paraventricular nucleus of the hypothalamus (PVN) is rapidly elevated by restraint stress, indicating that changes in central E2 concentrations may be involved in the modulation of the stress response. Additional studies in this thesis revealed that within the PVN, E2 modulates the activity of the HPA-axis both in basal (by ERα) and stress conditions (by ERα and ERβ). In addition, we demonstrated that hypothalamic estrogen signaling, independently of circulating estradiol, is crucial for several aspects of energy homeostasis. First, hypothalamic estradiol was found to regulate hepatic glucose production, as well as hepatic and peripheral insulin sensitivity. Second, we showed that hypothalamic estradiol regulates UCP gene expression in brown adipose tissue as well as the expression level of a number of lipolytic genes in white adipose tissue. Third, we provided the first evidence that central E2 affects bone formation rate. In part of these studies the hypothalamic effects of E2 could be characterized further with regard to the specific estrogen receptors or hypothalamic nuclei involved, using local administration of ER subtype specific (ant) agonists via microdialysis.

Part 1 Estrogen regulation of HPA axis activity

1.1. Crosstalk between the HPA and HPG axis

It is well known that sex hormones interact with the HPA axis at several levels. In Chapter 2, we demonstrated that acute stress increased hypothalamic GnRH mRNA expression, indicating activation of the HPG axis. Indeed, we found an increase of plasma estradiol concentrations during the stress condition. On the other hand, *in vitro* studies showed that corticosterone blocks the E2-induced LH release in primary cultures of rat pituitary cell(1) (2, 3).

In Chapter 2, we also found an increase of the local estradiol concentration in the hypothalamic PVN during the stress condition. These results were supported by the local increase in PVN aromatase gene expression during acute stress. The *in vivo* microdialysis studies in Chapter 3 extended the observations in Chapter 2 and revealed how local changes in PVN estradiol concentrations may modulate HPA-axis activity during basal and stress conditions (Figure 7.1).
1.2 The neuronal pathway and ER subtypes involved in the effects of estrogen on the HPA axis

Previous studies in our group revealed that the ER-mediated regulation of CRH promoter activity results from the two ERE half sites, in which the -316 ERE site contributes more to the constitutive CRH expression than to the -480 ERE site (4). Based on these in vitro studies, we investigated the effects of an infusion of ER subtype specific agonists directly in the PVN, where most CRH neurons are localized, on HPA-axis activity. The results indicated that both ERα and ERβ are involved. During basal conditions ERα activation increased HPA activity. During stress conditions ERα also increased HPA-axis activity, but ERβ activation decreased HPA-axis activity. We did not investigate whether estradiol is involved in the regulation of the negative feedback via the GR, but previous studies have shown that treatment with estradiol or a selective ERα agonist diminishes the suppressive effect of dexamethasone (DEX) on the stress-induced release of corticosterone, whereas treatment with an ERβ agonist enhances the suppressive effect of DEX on the stress-induced corticosterone release (5). Together, these data indicate that ERα inhibits, whereas ERβ enhances the negative feedback of glucocorticoids by elevating or suppressing CRH expression in the PVN.

The positive effect of the ERα agonist on the HPA-axis seems somewhat paradoxical since very few ERα positive neurons can be found within the PVN. On the other hand, the peri-PVN region contains many GABA-immunoreactive neurons that express ERα and project to the PVN (5, 6), exerting an inhibitory effect on the hypophysiotropic CRH neurons (7). Moreover, there is evidence showing that E2 attenuates GABA-B responses in hypothalamic neurons (8) and that E2 suppresses the GABA-A-mediated inhibition (9).
Thus we hypothesize that estradiol may increase CRH expression via the (peri)PVN by inhibiting, via ER\(\alpha\), the GABAergic neurons that surround the PVN (Figure 7.2). Most likely the inhibitory effect of E2 via the ER\(\beta\) only becomes apparent during stress conditions because at this time the CRH neurons are activated and thus an inhibitory effect can be observed more clearly.

**Figure 7.2** A schematic pathway for estrogen regulation of the HPA axis via the (peri)PVN. 1) E2 activates the CRH neurons by suppressing the inhibitory effect of GABAergic neurons on CRH release through ER\(\alpha\) in GABAergic neurons in the peri-PVN. 2) E2 can directly affect CRH neurons within the PVN via its effect on ER\(\beta\) in CRH neurons. E2, estradiol; ER, estrogen receptor; CRH, corticotrophin release hormone; GABA, GABAergic neurons; ACTH, Adrenocorticotropic hormone; GR, glucocorticoid receptor; CORT, corticosterone.

### Part 2 Regulation of glucose, lipid and bone metabolism by hypothalamic estrogen and the autonomic nervous system

Estrogen receptors are expressed in many areas of the hypothalamus and colocalize not only with insulin (10) and leptin receptors (11), but also with NPY (12) and POMC (13), i.e., neurotransmitters that are known to be involved in energy metabolism. As indicated by previous studies by our group and others (14, 15), pre-autonomic neurons in the hypothalamus are likely to play an important role in the regulation of lipid, glucose and bone metabolism.

#### 2.1.1 Hypothalamic estrogen and glucose metabolism

It has been well established that the autonomic innervation of the liver is involved in the control of hepatic glucose metabolism (16, 17). However, only recently it has become clear how the hypothalamus controls peripheral glucose metabolism through the autonomic nervous system. Surgical denervation of the sympathetic nerves innervating the liver severely affects the regulatory influences of many hypothalamic signals on
hepatic glucose production (14, 15, 18). In chapter 4 we showed that the stimulatory effect of E2 via the VMH on endogenous glucose production (EGP) and plasma glucose concentrations is abolished by a sympathetic, but not a parasympathetic, denervation of the liver. On the other hand, autonomic denervation of the liver (either sympathetic or parasympathetic) had no effect on the stimulatory effect of E2 on plasma glucose concentrations via the PVN. There is no evidence for a direct neural connection between the VMH and autonomic nuclei in the brainstem or spinal cord, contrary to the PVN. On the other hand, the VMH has pronounced projections to the PVN, which functions as the key hypothalamic integration center for autonomic and endocrine information, serving as the final neuro-endocrine and autonomic output nucleus from the hypothalamus (19, 20). ERα is expressed in the majority of glutamatergic neurons in the VMH (21) and the PVN is known to receive a strong glutamatergic input from the VMH (22). Therefore, we propose that the ERα-containing glutamatergic neurons in the VMH that project to the PVN are activated by local administration of E2. This may excite sympathetic pre-autonomic neurons in the PVN that in turn stimulate the hepatic sympathetic tone. In order to explain the opposite effect of the VMH on muscle-dedicated and liver-dedicated pre-autonomic neurons we propose that the glutamatergic projection of the VMH to the muscle-dedicated pre-autonomic neurons involves a GABAergic interneuron in the subPVN which contacts and inhibits the muscle-dedicated pre-autonomic neurons directly (Figure 7.3). Clearly this hypothesis needs to be investigated further in future studies.

**Figure 7.3** Hypothetical pathway to explain the hypothalamic effects of E2 on glucose production and glucose uptake. In this scheme 2 separate pre-autonomic PVN neurons for the control of muscle glucose uptake have been indicated, but of course, it is also possible that these are one and the same neuron, i.e., that the PVN neurons that are contacted via the VMH – subPVN projection also express the ERβ.
The autonomic nervous system not only affects hepatic glucose production, but also peripheral glucose uptake. Activation of adrenergic receptors was reported to inhibit insulin-stimulated glucose uptake by 2T3-L1 adipocytes (23) and to stimulate glucose uptake in brown adipocytes (24). It is not possible to perform similar surgical denervations of muscle as we did for the liver, but beta-adrenergic antagonists and knock-down of skeletal beta adrenergic receptors attenuated the modulatory effects of VMH signaling on glucose uptake (25). In Chapter 4, we observed that both PVN and VMH E2 signaling decreased insulin-dependent glucose uptake, taking into account the studies mentioned above, this indicates that E2 signaling in the PVN and VMH may also regulate glucose uptake by changing autonomic nervous activity. The effect of E2 in the PVN on glucose uptake indicates that the pre-autonomic neurons in the PVN that control the autonomic nervous input to the muscle probably contain ERs, in contrast to the pre-autonomic PVN neurons that control hepatic glucose production (Figure 7.3).

2.1.2 Hypothalamic estrogen and adipose tissue metabolism

In Chapter 5, we found both β-adrenergic receptor (AR) and uncoupling protein (UCP) gene expression in BAT to be increased after hypothalamic E2 administration, which indicates that hypothalamic E2 signaling may also play an important role in the modulation of energy metabolism via its effect on the autonomic input into brown adipose tissue. The autonomic nervous system is not only involved in the control of BAT activity, but also in that of the activity of white adipose tissue (WAT). PVN E2 treatment increased both LPL

![Figure 7.4 Hypothetical pathway to explain the hypothalamic effects of E2 on UCP gene expression in BAT and the expression of lipolysis genes in abdominal WAT.](image)
and HSL expression. This is in line with previous studies showing that activation of the sympathetic nervous system increases lipolysis in WAT (26, 27) (Figure 7.4).

2.1.3 Hypothalamic estrogen and bone metabolism
In Chapter 6, we showed that central estrogen signaling decreases bone formation rate, suggesting the presence of a functional brain-bone metabolism pathway. As we discussed before (see general introduction), hypothalamic NPY and POMC neurons are involved in the central regulation of bone metabolism (28). Whether the central effects of E2 on bone metabolism are also mediated via the NPY/POMC arcuate and the autonomic nervous system will have to be the subject of future studies.

Part 3 General perspective and future work

3.1 Perspective
Taken together, estrogen acts within the hypothalamus on different neural pathways via its two nuclear receptors, ERα and ERβ. The effects of estrogen are mediated via interactions with both neuro-endocrine and pre-autonomic neurons, as well as with interneurons. Within the hypothalamus, estrogen up- or down-regulates the activity of pre-autonomic neurons that are involved in the control of ANS projections to liver, muscle, BAT, WAT and possibly bone. In addition to directly affecting the activity of the pre-autonomic neurons, estrogen also modulates the activity of the pre-autonomic neurons via its action on other neurons, such as the GABA-containing neurons in the periPVN region (chapter 2), the glutamate containing neurons in the VMH (chapter 4), and the NPY-containing neurons in the arcuate nucleus (29). Varying parts of this neural network may also be involved in the effects of estrogen on appetite, body weight and insulin sensitivity (30). Future studies are needed to further unravel the neural pathways involved in the different effects of estrogen, as well its interaction with other hormones such as leptin and insulin that may use very much similar neural pathways to impose their effect.

Part 4 Clinical relevance
Menopause is associated with a shift towards a more masculine body fat distribution, metabolic syndrome and osteoporosis. Although estrogen hormone replacement therapy is one of the most efficient ways to relieve these symptoms, the clinical treatment is challenging since systemic estrogen replacement increases the risk of breast cancer and venous thrombosis (31, 32). Based on recent studies, including studies in the present thesis indicating central pathways for effects of estrogen that occur independently of estrogen concentrations in the circulation, it may be feasible to develop new and more specific
selective estrogen receptor modulators that primarily target estrogen signaling in the brain (33). In view of the selectivity of estrogen's central effects, this may be an alternative way to prevent or at least relieve the systemic side effects, i.e. the risk of breast cancer. Our data provide a strong argument to further explore potential estrogen drug targets in the hypothalamus.
Chapter 7

Reference


Summary
Summary

Sex differences have been found in many homeostatic domains, including the stress response, energy metabolism and fat distribution. In the present thesis, I studied the involvement of the female hormone estrogen in the occurrence of these sex-differences, more specifically I focused on the brain-mediated effects of estrogen on the regulation of a number of neuroendocrine responses.

In the first Chapter, I shortly reviewed the current knowledge on the synthesis of endogenous estrogen in the central nervous system, the estrogen signal transduction cascade and hypothalamo-pituitary-gonadal (HPG) axis regulation. In the hypothalamus, estrogen is locally produced from testosterone by the enzyme aromatase. Local estrogen levels in the brain may therefore fluctuate independently from circulating estrogen levels in plasma. In addition, I discussed the distribution of the different estrogen receptors within hypothalamus and the possible functional connection between brain estrogen on the one hand, and HPA-axis activity, energy expenditure, body fat distribution and bone metabolism on the other hand.

In Chapters 2 and 3, I presented experimental evidence that central estrogen influences HPA-axis activity during both basal and stress conditions. In Chapter 2 we show that exposure to stress affects local hypothalamic estrogen production and estrogen receptor expression. These findings drove us to unlock further the details of this interaction in Chapter 3. We found that under basal conditions stimulation of the estrogen receptor alpha exerts a positive effect on HPA-axis activity, while during stress conditions both estrogen receptor alpha and beta activation modulate HPA-axis activity. Therefore, the final output of the HPA-axis is determined by the opposite effects of estrogen receptor alpha and beta activation.

In Chapters 4 and 5 I studied the hypothalamic effects of estrogen on glucose metabolism and fat distribution. We revealed -for first time- the direct effects of hypothalamic estrogen on glucose metabolism. The stimulatory effect of estrogen in the ventromedial hypothalamus (VMH) on hepatic glucose production is mediated via the sympathetic nervous input to the liver. Most likely also the effects of estrogen in the PVN and VMH on glucose uptake are mediated via the autonomic nervous system, although we have not been able to prove this in our current series of experiments. The modulatory effects of estrogen on glucose metabolism may ultimately also affect energy metabolism, and result in altered fat deposition and body weight gain. These findings led me to investigate further the effects of hypothalamic estrogen on body fat distribution and gene expression in adipose tissue in Chapter 5. We showed that hypothalamic estrogen is sufficient to change body fat distribution, to activate brown adipose tissue, and to up-regulate lipolytic gene expression within white adipose tissue.

Bone loss is a well-known consequence of the decline of endogenous estrogen levels during the female menopause. In Chapter 6, we investigated possible regulatory effects of
Summary

hypothalamic estrogen on bone formation and found the first evidence that hypothalamic estrogen may be a determinant of bone formation. Finally, in chapter 7 I discussed the implications and limitations of the results presented in this thesis as well as their clinical relevance.
Samenvatting
Samenvatting

Veel fysiologische processen laten duidelijke sekseverschillen zien, zoals de activatie van de hypothalamus-hypofyse-bijnier as (HPA-as) tijdens stressreacties, het energiemetabolisme, de vetverdeling over het lichaam en de afname van de botmassa tijdens veroudering. In dit proefschrift heb ik mij gericht op de betrokkenheid van het vrouwelijke geslachthormoon oestrogeen bij deze processen.

In het eerste hoofdstuk beschrijf ik kort de huidige kennis over de synthese van oestrogeen in het centrale zenuwstelsel, de oestrogeen signaaltransductiecascade en de hypothalamus-hypofyse-gonade (HPG)-as. In de hypothalamus wordt oestrogeen lokaal geproduceerd door omzetting van testosteron in oestrogeen door het enzym aromataze. Lokale oestrogeen concentraties in de hersenen kunnen dus fluctueren onafhankelijk van circulerende oestrogeenconcentraties in het plasma. Daarnaast beschrijf ik kort de neuroanatomische verdeling van de verschillende oestrogeenreceptoren in de hypothalamus en de mogelijke koppeling tussen oestrogeenconcentraties in de hersenen en HPA-as activiteit, energieverbruik, lichaamsvetverdeling en botmetabolisme.

In hoofdstuk 2 en 3 presenteer ik experimenteel bewijs dat oestrogeen de HPA-as activiteit beïnvloedt tijdens zowel basale als stress condities. In hoofdstuk 2 laten we zien dat blootstelling aan stress de lokale hypothalam oestrogeenproductie en oestrogeenreceptor expressie beïnvloedt. Deze eerste bevindingen deden mij besluiten een poging te ondernemen de details van deze interactie verder te onderzoeken. In hoofdstuk 3 beschrijf ik de effecten van oestrogeen in de hypothalamus op HPA-as activiteit. Onder basale condities stimuleert oestrogeen via activatie van de oestrogeen receptor alfa de HPA-as, terwijl onder stressvolle omstandigheden dit effect tegengegaan wordt door activatie van de oestrogeenreceptor beta. De uiteindelijke activiteit van de HPA-as wordt dan ook mede bepaald door de tegengestelde effecten van oestrogeen op respectievelijk de oestrogeenreceptoren alpha en beta.

In hoofdstuk 4 en 5 heb ik de hypothalame effecten van oestrogeen op de glucosestofwisseling en de lichaamsvetverdeling bestudeerd. Hoofdstuk 4 beschrijft de experimenten waarin we voor de eerste keer de directe effecten van hypothalam oestrogeen op het glucosemetabolisme aantonen. Onder andere laten we zien dat de stimulerende effecten van oestrogeen in de ventromediale hypothalamus (VMH) op hepatische glucose productie worden gemedieerd via het sympathische zenuwstelsel. Waarschijnlijk worden ook de effecten van oestrogeen in de nucleus paraventricularis (PVN) en VMH op glucoseopname gemedieerd via het autonome zenuwstelsel. We zijn er echter niet in geslaagd om dit laatste te bewijzen in onze huidige reeks van experimenten. In hoofdstuk 5 beschrijf ik de effecten van oestrogeen in de hypothalamus op de lichaamsvetverdeling en genexpressie in vetweefsel. We hebben aangetoond dat de aanwezigheid van oestrogeen alleen in de hypothalamus voldoende is om de lichaamsvetverdeling te veranderen. We hebben ook aangetoond dat oestrogeen via de
Samenvatting

Hypothalamus een activering van bruin vetweefsel veroorzaakt en een verhoogde expressie van lipolytische genen in het witte vetweefsel induceert.

Botverlies is een bekend gevolg van de daling van de oestrogeenconcentraties die optreedt bij de menopauze. In hoofdstuk 6 vond ik het eerste bewijs voor een regulerend effect van oestrogeen via de hypothalamus op de botvorming.

Ten slotte bespreek ik in hoofdstuk 7 de implicaties en de beperkingen van de resultaten die beschreven zijn in dit proefschrift, alsmede de klinische relevantie.
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Publications
**Publications**


