Fertility, aging and the brain

Neuroendocrinological studies in female rats

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Annelieke N. Franke

Proefschrift

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Voor Maykel

Voorwoord

Hier is het dan eindelijk: mijn proefschrift, de afronding van een fantastisch leuke en leerzame tijd bij FMD. En wat is er de afgelopen 5 jaar veel gebeurd! Natuurlijk heb ik dit ook te danken aan een heleboel mensen en die wil ik hier graag bedanken.

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Annelieke

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It is well known that fertility decreases in female mammals with advancing age. In women this decrease already starts around the age of 30 and shows a large variation between individuals. The aim of this thesis was to elucidate changes in the reproductive system, especially in the brain, that may underlie the early decline in fertility with age. To this end, neuroendocrinological studies were performed in young and middle-aged females of two rat strains known to differ in the onset of infertility: the Wistar (WU) and (UxRP)F1 strain.

The results of the present thesis confirm the idea that the attenuation of the luteinizing hormone (LH) surge is one of the first indications of reproductive aging in rats. The LH surge is responsible for ovulation. It is induced by feedback mechanisms of ovarian steroid hormones estradiol and progesterone on the brain (i.e. on the secretion of gonadotropin-releasing hormone) and pituitary gland (i.e. on the secretion of LH and follicle-stimulating hormone (FSH)) that become operative when the ovarian follicles are matured, and involves estrogen and progesterone receptors in the brain.

Our results indicate that the attenuation of the LH surge in middle-aged rats likely results from an altered response of the brain to estradiol and possibly also progesterone feedback, since we found a dramatic decrease in the number of estradiol and progesterone-containing neurons in several brain areas known to be crucially involved in neuroendocrine regulation of the reproductive axis. In contrast, estradiol and progesterone levels were increased ((UxRP)F1) or even unchanged (Wistar) and the pituitary LH response to GnRH as well as the follicular progesterone production during the LH surge appeared to be comparable between young and middle-aged rats. This suggests that pituitary and ovary functions were still intact. Therefore, changes at the level of the brain may be at the start of the decline in fertility with age in rats.

Interestingly, we found strain differences in the regulation of the reproductive axis. There was, for instance, a difference between F1 and Wistar rats in the magnitude of the LH surge (F1>Wistar) and the magnitude of the pituitary LH response to GnRH (F1<Wistar). Also, middle-aged F1 rats appeared to be reproductively aged to a further extend compared to Wistar rats, as judged by the number of changes in the reproductive system.

Although in women ovarian aging appears to be the dominant reason for fertility decline, there is evidence for considerable variation between individuals in the mechanisms underlying reproductive aging. Based on our present findings and literature, we hypothesize that hypothalamic aging may also contribute to the decline in fertility in some women.

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Chapter 1

General Introduction

1.1 Female fertility

The ability to produce offspring in mammals depends mainly on the female, since it is the female that is fertile for only a short period during a reproductive cycle. In addition, unlike male mammals that produce new spermatozoa during their entire adult life, female mammals are born with a fixed number of primordial follicles (i.e. oocytes surrounded by flat granulosa epithelial cells). During fertile life cohorts of these follicles are recruited into the growing pool of follicles. Most of these follicles never reach the preovulatory stage, but degenerate somewhere along this developmental pathway. Depending on the species, only one or a few follicles will finally ovulate and can be fertilized each 'ovarian cycle'. The growth of follicles as well as the events resulting in ovulation are tightly orchestrated.

Since the total amount of follicles is limited, the 'follicular pool' will decline during life. A decline with age is also seen in female fertility, which eventually results in infertility. The beginning of the infertile period in women is characterized by 'menopause', i.e. the last menstrual cycle, although fertility has ended long before menopause. Thus, the time that females are fertile is limited and, what may be even more alarming, the decline in female fertility starts relatively early during life. There are indications that in women the decline in fertility starts already around the age of 30 years (248) (Figure 1).

Because women in western countries often postpone their wish to have children and have a career first, it is generally expected that more and more couples will face fertility problems. Hence, the need to know what changes in the female reproductive system underlie the decline in fertility with age is growing. By gaining insight we might be able to help young couples that face fertility problems to increase the chance of a successful conception, pregnancy and of course: a healthy baby!

As mentioned above, one of the most obvious changes in the female reproductive system is the decline in the 'ovarian follicular pool' with age. The classical idea with respect to reproductive aging is that this depletion of oocytes, leaving too few or only 'bad ones', may explain the age-related decline in fertility in

female mammals. A more detailed study of this decline in ovarian reserve revealed a clear acceleration in follicular loss in women around the age of 37 years, and already before this acceleration occurs fertility is decreasing (Figure 1).

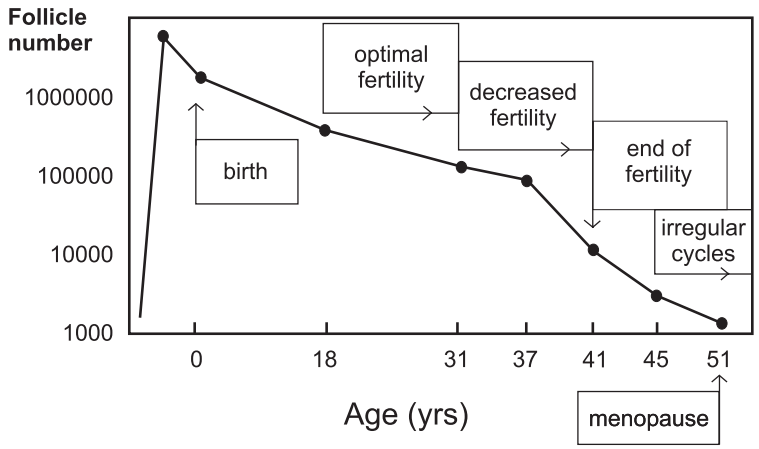

Figure 1. Schematic representation of the decline in follicle number in women with age, including the associated stages of fertility (adapted from 248).

In many aspects, the regulation of the reproductive system appears comparable between humans and rodents, but in rodents the ovarian follicular pool is not depleted when fertility ceases (270). This suggests that other factors, for instance at the level of the hypothalamus and pituitary gland, may be involved in the reproductive decline with age as well. Based on the presence of a significant number of oocytes when fertility ceases in rats, but not in humans, the contribution of these factors to reproductive aging appears to be larger in rodents compared to humans. The aim of this thesis is to gain insight in the contribution of the hypothalamus to the process of reproductive aging and therefore the rat was chosen as an animal model.

1.2 The reproductive system

The female reproductive system consists of the preoptic area and hypothalamus in the brain, the (anterior) pituitary gland and the ovaries and uterus (Figure 2).

The preoptic area and hypothalamus

The preoptic area and hypothalamus play a key role in the regulation of estrous cyclicity in female rats. Gonadotropin-releasing hormone (GnRH)-producing cells are situated in these areas and feedback mechanisms of ovarian steroid hormones, like estradiol (E_2) and progesterone (P), act on neurons in these brain areas to control both the basal pulsatile and the preovulatory surge release of GnRH.

The GnRH system

The GnRH system in rats consists of approximately 1600 cells that secrete the GnRH decapeptide (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly.NH_2), also known as luteinizing hormone-releasing hormone (LHRH) (11), from which multiple forms are present within and among species (183,293).

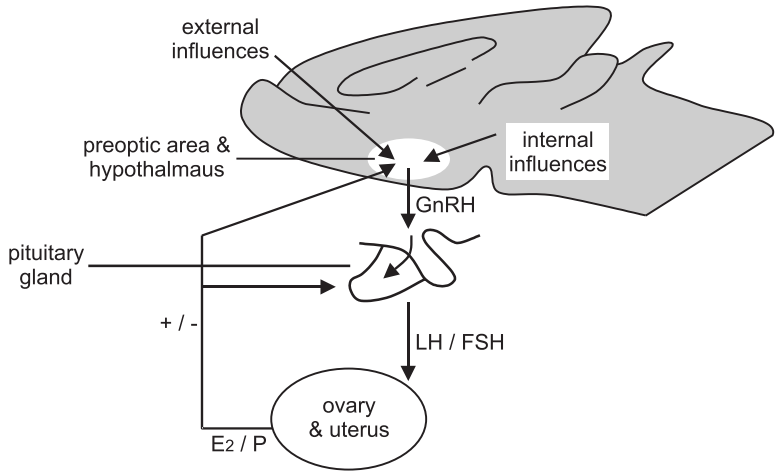

Figure 2. Simplified representation of the reproductive axis. For abbreviations see text. (Modified after 258).

These GnRH cells originate from the olfactory placode and are not of neuronal origin. During brain development they migrate along the vomeronasal/nervus terminalis into the rostral forebrain and towards the infundibulum and median eminence (221). This migration pattern explains the dispersed distribution of GnRH cells throughout the ventral forebrain. In rats, GnRH cell bodies are located in the septum, the vertical limb of the diagonal band of Broca, the organum vasculosum of the lamina terminalis (OVLT), the preoptic area (POA), and they extend laterocaudally along the medial forebrain bundle up to the retrochiasmatic area and mediobasal hypothalamus (MBH). Although this distribution pattern is similar in females and males, some small differences can be observed among species. In general, the longer the period of brain development of a species, the more GnRH cells are located in the caudal regions.

There are indications that GnRH-containing neurons can be divided into distinct functional groups: a rostral group located at the level of the OVLT and anterior POA is essential for the generation of the preovulatory GnRH surge and hence of the luteinizing hormone (LH) surge (120), while a caudal group (MBH) appears to act as the driving force to induce pulsatile GnRH and LH secretion (119). Although GnRH neurons project to various (extra-) hypothalamic areas, suggesting a role for GnRH as a neurotransmitter (11), almost all GnRH neurons (>90%) (11,292) project to the median eminence (ME). Here, GnRH is stored in small granules within axon terminals and varicosities of the GnRH neurons. The GnRH-containing terminals lie within close proximity of capillaries of the highly vascularized ME. The decapeptide is released in a pulsatile pattern in the portal vasculature of the ME and thus stimulates the release of gonadotropic hormones (e.g. LH) by the anterior pituitary. By proper ovarian steroid hormone feedback and hypothalamic signaling, the GnRH pulse frequency will increase, resulting in a GnRH surge. It is this GnRH surge that stimulates the events that induce ovulation of the mature oocyte(s).

GnRH neurons are in synaptic contact with each other, glia cells, endothelial cells, and other neuronal cells (202). In this way, GnRH release can be affected by for instance GnRH itself via an ultrashort feedback loop, pituitary hormones, gonadal hormones, and by neurotransmitters such as amino acids or neuropeptides that innervate GnRH cell bodies or GnRH terminals (111,293).

Ovarian steroid hormone feedback

The effect of ovarian steroid hormones on neurons in the hypothalamus, POA,

and pituitary gland, but also on many other tissues throughout the body, are mediated by nuclear steroid hormone receptors. The classical model for steroid hormone action is initiated with the intracellular binding of the ligand (e.g. E_2 and P) to its receptor (e.g. ER, PR). This binding causes an allosteric change, thereby allowing the hormone-receptor complex to dimerize. Subsequently, the complex enters the nucleus and binds to a DNA response element (e.g. ERE, PRE) in the promoter region of target genes, where it is able to modulate the transcription activity of these genes. In addition, several coactivators and corepressors may affect the activity of the hormone-receptor-complex (178).

Two types of estrogen receptor forms have been identified, ERα and ERβ, which have different expression patterns and functions (168,230). The expression patterns and studies in receptor knockout mice suggest that ERα plays a more important role in the regulation of the female reproductive system than ERβ (168). One of the important functions of activation of ERα is the induction of PR mRNA transcription (65,151) by binding to an estrogen-response element (ERE) in the PR gene promoter (132). E_2-induced PR expression shows a clear regional sex difference (23,94,222), which suggests a specific role in the reproductive system. Indeed, PRs play a crucial role in the generation of the GnRH surge in females (151). Two types of PRs have been characterized in mammals, PR-A and PR-B, which have different transcription activation properties and elicit distinct responses to P exposure (40,41). Studies in PR knockout mice demonstrate that expression of PR-A is essential for female fertility, while PR-B is required for the effect of P on the mammary gland (41).

The preoptic area (POA)

Since GnRH neurons appear to express only low concentrations of ERβ (100) and do not contain ERα or PRs, the proposed feedback action of E_2 and P on the GnRH system is thought to be indirect and involves other neurons. Neurons in the POA are likely candidates for this ovarian hormone feedback. The POA can be divided into two areas, the anteroventral periventricular nucleus (AvPv) and the medial preoptic area (MPO). Both areas contain high concentrations of ERs (237) and PRs (25) (see also Figure 3), and both ER and PR gene expression changes dramatically during the estrous cycle (231). Moreover, lesions of these areas block the preovulatory LH surge (88,116,120). Implantations of estrogen (E) or E+P directly into the MPO of ovariectomized rats result in an LH surge, while implants of

anti-estrogen into this region block the LH surge in these rats (83,84). Furthermore, the release of several neurotransmitters changes prior to and during the LH surge in the POA (108). Therefore, both AvPv and MPO are thought to play a role in the generation of the preovulatory LH surge. In addition, the MPO is involved in maternal (187,240) and sexually motivated behavior (113).

The mediobasal hypothalamus (MBH)

The MBH consists of the arcuate nucleus (ARC) and the ventromedial hypothalamic nucleus (VMH). Both areas play an important role in the regulation of the reproductive system and are controlled by steroid hormone feedback mechanisms (25,141,232,273) (see also Figure 3). ARC neurons project to the POA and to GnRH nerve terminals at the ME. Neurons in the ARC synthesize several neuropeptides (neuropeptide Y, β-endorphin, galanin, neurotensin) that can modulate GnRH release into the portal system (111). In addition, E-induced synaptic plasticity (107,176) in the ARC-ME area and in GnRH terminals (115) is thought to influence the synaptic contacts between GnRH terminals and the portal vasculature during the estrous cycle, thus enabling the release of large quantities of GnRH during the GnRH surge.

The VMH plays a role in the expression of sexual behavior by the female. In this area, E_2 induces the expression of PRs that are activated by dopamine (DA) via a P-independent mechanism. This activation of PRs in the VMH results in the expression of lordosis (i.e. sexual behavior) from late proestrus until early estrus (151), which is necessary for copulation and thus for a successful fertilization.

The biological clock

The suprachiasmatic nucleus (SCN) or so-called mammalian ‘biological clock’ plays a central role in the regulation of neuroendocrine and behavioral rhythms. Lesions of this area result in the loss of diurnal rhythms in circulating plasma hormone levels (175,177) and in the condition of constant estrus in female rats (17). The observation that administration of pentobarbital (a hypnotic) during a ‘critical period’ on the afternoon of proestrus can postpone ovulation in rats by exactly one day, led to the hypothesis that a daily neuronal event during this critical period is necessary for the induction of an LH surge and subsequent ovulation (72). In addition, shifting the light-dark cycle by a couple of hours also shifts the critical period by a corresponding number of hours. Only on proestrus a distinct

GnRH and LH surge are generated, while on other days of the estrous cycle pituitary LH only shows a small elevation at the midpoint of the light period. Furthermore, SCN neurons project directly to GnRH neurons (261) and to neurons in the AvPv (275) and in the ARC (80). These data suggest that a neuronal signal, which is necessary for the induction of the LH surge, depends on the light-dark cycle and is likely to be under control of the biological clock.

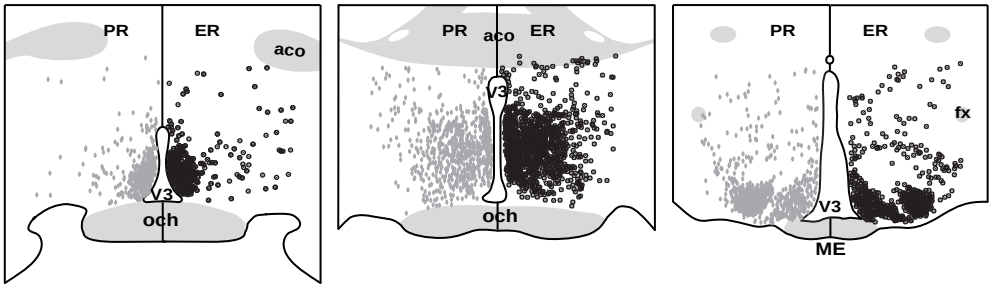

Figure 3. Representative image of the AvPv (A), MPO (B), VMH (C), and ARC (C), and the distribution of cells containing ER- (only left side of the brain shown) and PR- (only right side of the brain shown) immunoreactivity. 3V, third ventricle; aco, anterior commissure; fx, fornix; ME, median eminence; och, optic chiasm.

Thus, there appear to be three systems in the hypothalamus important for the regulation of the estrous cycle and ovulation. (I) GnRH-containing neurons are necessary to stimulate the release of gonadotropins by the anterior pituitary gland, (II) steroid hormone sensitive neurons in the AvPv, MPO, VMH and ARC are necessary for feedback actions of gonadal steroids on hypothalamic GnRH secretion, and (III) the SCN, which is proposed to play a role in the entrainment of the reproductive cycle to the environmental light-dark cycle, i.e. the activity cycle.

The anterior pituitary gland

The anterior pituitary gland, or adenohypophysis, synthesizes two gonadotropic hormones: follicle stimulating hormone (FSH) and LH, which both are glycoproteins consisting of two chains of glycosylated polypeptides. The α-chain of both hormones is identical, while the β-chain is different for LH and FSH.

FSH stimulates the development of ovarian follicles, while LH stimulates ovarian androgen production, ovulation and corpus luteum (CL) formation. The synthesis

and release of both hormones is primarily stimulated by the pulsatile release of GnRH from the hypothalamus, resulting in a basal pulsatile 'circhoral' rhythm in LH and FSH plasma concentrations (i.e. frequency approximately 1 h) (29,61,128). LH release is maximally stimulated when 20% of the GnRH receptors is occupied (241). There are two GnRH receptor forms in mammals (166) and the binding of GnRH to its transmembrane receptor activates second messenger systems. The number of GnRH receptors on gonadotropic cells determines which second messenger system is activated (241). In addition, the pituitary shows a GnRH 'priming' mechanism, which results in a higher LH and FSH release after the second and third GnRH stimulus compared to LH and FSH release after the first GnRH stimulus. This mechanism is thought to play an important role in the generation of the preovulatory LH and FSH surges (61,244).

The synthesis and release of both hormones is also regulated by the feedback actions of the gonadal hormones E_2 and P at the level of the hypothalamus and pituitary gland. E_2 is able to stimulate LH release during the preovulatory phase: E_2 increases the number of GnRH receptors on gonadotropic cells and inhibits LH and FSH release while still stimulating the synthesis of LH and FSH.

Besides LH and FSH, two other hormones that play a role in the regulation of the reproductive cycle are produced by the pituitary gland: activin and follistatin. Activin is a glycoprotein that consists of two β-subunits (239). Three forms exist: activin A (βA-βA), activin B (βB-βB) and activin AB (βA-βB). The present data suggest that activin selectively stimulates FSH synthesis and release (252) and stimulates GnRH binding by the pituitary (38), while follistatin is thought to indirectly inhibit FSH release by binding both activin (101) and inhibin (239).

The ovaries

The ovarian follicles secrete several sex steroids. The theca cells of the follicles synthesize testosterone (T), while aromatization of T in the granulosa cells of the follicles results in the formation of E_2. Also, P is formed by granulosa cells as well as by the luteinized cells of the CL and is, like T, synthesized from cholesterol. In addition, P can be produced in the adrenal gland as well as the brain (74), and P from adrenal origin may also play a role in the generation of the LH surge (158).

Apart from T, E_2, and P, the ovary also produces several other substances: inhibin, activin and follistatin. Inhibin and activin are members of the transforming growth factor (TGF)-β superfamily of growth factors (101) and are both produced

by granulosa cells. Inhibin is a heterodimeric glycoprotein that consists of a common α-subunit and a β-subunit: α-β_A in inhibin A and α-β_B in inhibin B. Granulosa cells of preantral and dominant follicles are the main source of inhibin B and A, respectively (87). Both forms suppress FSH synthesis, GnRH-stimulated FSH release, and possibly also GnRH secretion (252), but have little or no effect on LH secretion. Activin predominates in immature follicles (101) and appears to play a role in local processes, just like follistatin from ovarian origin.

Although estrogens have reported to play a role in sexual differentiation in fetal, neonatal, and pubertal life and in sexual function in adult life, also ischemic injury (282), Alzheimer's disease (90), Parkinson's disease (90), and osteoporosis (90) appear to be affected by this hormone. Nevertheless, the most important and well-known role for estrogen is its role in the regulation of the female reproductive axis (158).

E_2 and P are of crucial importance for the induction of the preovulatory LH surge. Depending on its concentration, E_2 can (a) have inhibitory or stimulatory effects on gonadotropin secretion, (b) regulate the synthesis of GnRH in hypothalamus, (c) work in concert with GnRH to stimulate the synthesis of pituitary GnRH receptors, (d) sensitize the pituitary to GnRH, (f) induce the synthesis of hypothalamic and pituitary PRs, and (e) lower the opioid tone in the hypothalamus, thus reducing an important inhibitory factor of GnRH release (158).

In addition, P has been demonstrated to play a role in the timing and magnitude of the LH surge. Injections of P on the day prior to proestrus can advance LH release and ovulation by 24 h in rats with a 5-day estrous cycle (217), while administration on the early morning of proestrus advances the LH surge by several hours (73). P released at proestrus prevents the daily expression of LH surges that are normally seen in ovariectomized E-treated rats (72). Furthermore, P is able to enhance the ongoing LH surge (73) by increasing the number of activated GnRH neurons (146), by stimulating the GnRH priming mechanism via pituitary PRs (274), and by reducing the hypothalamic and plasma GnRH degradation activity (158). In conclusion, E_2 and P are able to modulate GnRH output and the response of the pituitary to GnRH.

1.3. The reproductive cycle

Although the length of the reproductive cycle varies between mammalian species, the regulation of the cycle in for instance humans, rats, mice, hamsters, and monkeys shows many similarities (270) (Figure 4). In all mammals, the hormones originating from the hypothalamus, pituitary and ovaries (see above) mainly regulate reproductive cyclicity and ovulation. In general, the ovarian cycle is characterized by a follicular phase, a (pre)ovulatory phase, and a luteal phase.

During the follicular phase, the follicles grow and produce increasing amounts of E_2. The rising E_2 levels will initially inhibit (negative feedback) and eventually, when above threshold level for a minimal time, stimulate (positive feedback) several events culminating in a massive secretion of GnRH by the hypothalamus. The GnRH surge is followed by the secretion of LH and FSH by the pituitary gland, i.e. the preovulatory LH and FSH surge. The LH surge leads to the luteinization of granulosa and theca cells of mature follicles and, depending on the species, the rupture of the mature follicle(s) ('ovulation') about 14 (rats) to 24 h (human) later. The luteinized cells will form a CL, which mainly secretes P. P inhibits the occurrence of a GnRH and LH surge on subsequent days of the cycle, i.e. this is the mechanism that inactivates the system after the positive feedback (706). Ovulation entails the release of the ovum by the ruptured follicle, whereafter the ovum is transported via the oviduct to the uterus. When no fertilization occurs, P secretion will decrease due to the regression of the CL. In case of a successful fertilization and implantation, the CL is 'rescued' from regression. The persistent high levels of P secreted by this CL are able to maintain pregnancy (73,128).

Cycle length

Women have a so-called 'menstrual cycle' as reproductive cycles include a menstrual period characterized by the loss of the upper layers of the endometrium together with some blood (i.e. 'menses'). Physiologically, the menstrual cycle is divided into three sequential phases. The follicular phase begins with the onset of menstrual bleeding and averages 14 days. The ovulatory phase lasts for 1 to 2 days and culminates in ovulation, and the luteal phase is normally about 14 days in length. The overall duration of a normal menstrual cycle averages 28 days, although there is a striking individual and age-related variation in cycle length (128) (Figure 4).

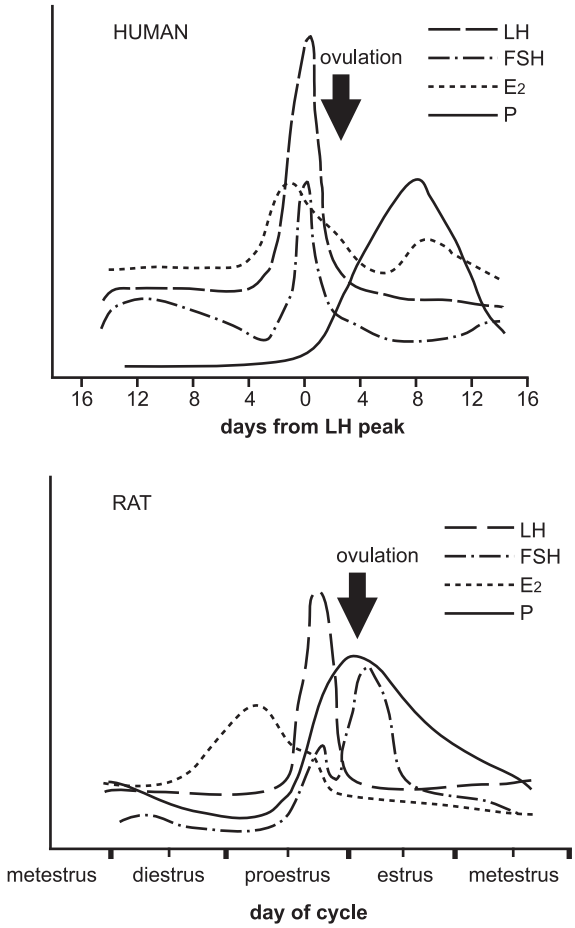

Figure 4. Schematic representation of hormone concentrations during the human menstrual cycle (above) and the rat estrous cycle (below) (adapted from 62).

In general, the reproductive cycle of the rat resembles that of humans, although the estrous cycle of rats lacks a distinct luteal phase and has no menstrual period. The estrous cycle in rats lasts for 4 or 5 days and is divided into proestrus, estrus, metestrus, and diestrus. These different stages of the estrous cycle can be determined microscopically by rating the cell types present in vaginal smears or lavages (changes are the result of E_2 and P exposure!). Proestrus is characterized by the predominance of small round nucleated epithelial cells and the absence of leukocytes. On the day of estrus, large clusters of cornified squamous epithelial cells can be found. These cells are without visible nuclei, contain a granular

cytoplasm, and are irregularly shaped. The day of metestrus is characterized by the presence of many leukocytes between the clusters of cornified epithelium. During diestrus predominantly small leukocytes can be found in the vaginal smear along with smaller numbers of other types of epithelial cells. The lengthening of the estrous cycle by one day in 5-day cyclic rats may appear as an additional day of cornification (estrus) or leukocyte infiltration (diestrus).

In rats, the light-dark cycle plays an important role in the control and duration of the estrous cycle. In addition, estrous cycles are characterized by 'estrous' behavior, the receptive behavior of a female rat towards a male rat from late proestrus until early estrus. This typical behavior is stimulated by the odor and/or presence of a male rat, and consists of ear wiggling, hopping and darting of the female.

The follicular phase

In women, plasma FSH and LH levels are low at the end of the luteal phase. One to two days before menses FSH levels begin to rise, which is followed by a rise in plasma LH levels. Since FSH stimulates the growth of follicles that produce E_2, also E_2 levels increase gradually during the first half of the follicular phase. This process is enhanced by the positive feedback of E_2 on FSH and ER expression. Subsequently, FSH increases the responsiveness of granulosa and theca cells to LH by indirectly increasing the number of LH receptors. During the second half of the follicular phase, the growth of the dominant follicle leads to an increase in inhibin A levels, resulting in a decline in FSH levels whereas LH levels continue to rise slowly. At the same time, LH stimulates the release of E_2 by the dominant follicle by increasing T production in the theca cells, which is converted into E_2 by aromatase in granulosa cells. Only just before ovulation, granulosa cells express LH receptors, so that E_2 levels increase rapidly, reaching peak values before ovulation. This rise in E_2 stimulates hypothalamic GnRH and consequently also pituitary LH secretion. Furthermore, LH stimulates the production of P by the granulosa cells of the dominant follicle (128).

As in humans, also in rats the follicular development from antral to mature Graafian follicle takes about 14 days. Since the length of the estrous cycle in rats is only 4 to 5 days, this implies that it takes several estrous cycles before a follicle, which is recruited from the follicular pool, is ready to ovulate. Although no distinct follicular phase is present in rats, the last growth spurt of the dominant follicles one

to two days before ovulation is associated with a clear rise in E_2 levels.

The (pre)ovulatory phase

During the ovulatory phase gonadotropin levels show a peak ('surge'). The LH surge is much higher than the FSH surge. This surge is evoked by the positive feedback of still rising E_2 levels on the pituitary gland and hypothalamus. In women the preovulatory gonadotropin surge starts between midnight and 8:00 AM (28). After ovulation E_2 levels decrease, while a small but significant increase in P levels is observed.

In rats, the preovulatory phase starts around mid-proestrus, when E_2 reaches peak levels and the preovulatory GnRH surge is generated. This results in a rapid increase in LH levels and a subsequent LH surge in the late afternoon. The proestrous LH surge induces follicular rupture and ovulation on the early morning of estrus. In addition, P displays a peak during the late afternoon of proestrus due to the fact that the granulosa cells of the preovulatory follicles have already started to luteinize. E_2 levels fall rapidly at the end of the light period of proestrus. Pituitary FSH levels show the same pattern as LH: basal levels during the metestrus and diestrus and a rise on proestrus.

The luteal phase

In women the luteal phase is characterized by a ten-fold increase in P originating from the CL and a decline in FSH and LH levels with the nadir just before menses. E_2 and inhibin are also secreted by the CL and this results in a broad second peak for both hormones. If there is no pregnancy the cycle will end with a dramatic decrease in gonadal steroids (especially P) and inhibin, a small consequent rise in plasma FSH levels, and the start of menses approximately 3 days after P concentrations have fallen below a certain level (128).

In contrast, rats display no distinct luteal phase. There is no development of a functional CL, unless the rat becomes (pseudo)pregnant. Therefore, the initial P production of each newly formed CL is very small. It starts at the afternoon of metestrus and only lasts for about one to two days depending on cycle length, unless a pregnancy occurs. As in humans, each inactive CL is present for about 13 days before regression occurs. In addition, on estrus morning a rise in FSH but not LH levels can be seen, which is under control of inhibin and/or activin.

1.4. Reproductive Aging

During aging numerous changes occur in the regulation of the reproductive system. For instance changes can be seen in cycle length, hormone concentrations, the number of successful pregnancies, and sexual behavior. Although these changes may vary among species, there are also many similarities (270).

The reproductive system during aging in humans

In humans 'normal' fertility or fecundity has not been properly defined. Women are said to have a decreased fertility if they do not achieve a pregnancy within approximately one year, i.e. within 12 unprotected cycles. This decreased fertility can be the result of the genetic background of a person causing the sub- or infertility, but it can also be the result of reproductive aging. In women a decline in fertility caused by reproductive aging can be found as early as 20 years before menopause (Figure 1); menopause being defined as the moment of the last menstrual cycle. Since the age at which natural menopause occurs varies considerably among women, i.e. between 45-55 years, a large variation in the onset of reproductive aging is to be expected (248). Besides a large genetic component (85%) (42), the age of natural menopause is also affected by genito-urinary tract infections, immunological or autoimmune disorders, malnutrition, smoking, parity, the use of oral contraception, socio-economic status, and possibly the body mass index of a person (43,114,266,267).

Fertility starts to decline between the age of 25 and 35 (57,248) and the incidence of spontaneous abortions, dizygotic twinning, fetal trisomy (i.e. 'Down syndrome') and other chromosomal abnormalities increases with maternal age. In addition, the percentage of anovulatory cycles increases with age (3-7% between the age of 26-40 and 12-15% between the age of 41-50) (26). While the length of the menstrual cycle alters from about 28 days at the age of 20 years to 26 days at 40, the variance in cycle length in an individual reaches lowest values around 7 years prior to menopause. Thereafter, the cycle length becomes increasingly variable and therefore this period is also called the period of irregular menstruation before menopause. Cycles are shorter due to a reduced length of the follicular phase, or are longer due to the failure to ovulate. Either short or long cycles are associated with reduced fertility. In women, the end of fertility precedes menopause

by several years (248) (Figure 1).

The first period of reproductive senescence (age 30-40) is characterized by a decrease in inhibin B and E_2 levels and a concurrent increase in FSH levels (122,123,239). It is thought that both the reduced E_2 and inhibin B levels result from the decline in the ovarian follicular reserve, i.e. less follicles in the preantral stage. Since inhibin B inhibits FSH synthesis and release, the decrease in inhibin B is thought to result in an elevation of plasma FSH levels, in particular during the early follicular phase of the reproductive cycle (27,224,239,248). Elevated FSH levels during early reproductive aging are accompanied by a sharp decrease in the probability of pregnancy with regard to in vitro fertilization (IVF) (248). In addition, the rise in FSH levels appears to be associated with an accelerated follicular maturation as well as an increase in follicular atresia, resulting in an accelerated follicular loss (Figure 1), and subsequently to a shorter follicular phase and cycle length. Furthermore, there are indications that in case of IVF, the age of the oocyte donor affects the outcome of the treatment: (pre)menopausal women have a higher chance to become pregnant when the embryos have developed from oocytes of young compared to aged donors (121).

Although the above mentioned data suggest an exclusive role for the ovary in reproductive senescence, there are indications that changes at the level of the hypothalamus-pituitary system may be involved as well. Firstly, the frequency of LH pulses (a faithful mirror of pulsatile GnRH release) decreases during the follicular phase (288). The observed rise in FSH with age could well be the result of this decreased GnRH pulsatility, since a small (110) but not a large (110,281) decrease in GnRH pulse frequency has been shown to stimulate FSH release. Secondly, there are indications that the pituitary LH responsiveness to GnRH is diminished (75) and that the preovulatory LH surge attenuates with age. The attenuation of the LH surge is associated with an impaired fertility (269) and therefore a reduced probability of conception (9). In summary, the GnRH pulse generator appears to slow down with age and the sensitivity of the pituitary to GnRH reduces with age resulting in an attenuation of the LH surge and a decreased fertility.

Considering the fact that the output of GnRH neurons during the reproductive cycle depends on other (extra-)hypothalamic areas, like the SCN, POA, and ARC, changes in these areas during aging could equally well contribute to reproductive senescence (288). These data suggest that there may be a hypothalamic-pituitary contribution to reproductive aging that affects oocyte maturation and embryo

implantation at a relatively early age.

After menopause, the ovarian production of E_2 and P ceases (205). This rapid decrease in sex steroid levels at menopause influences sexual drive and can lead to hot flushes, atrophy of the genito-urinary tract and of the mammary epithelial glands and ducts, and to osteoporosis. The severity of the latter changes differs greatly between individuals and can be prevented by estrogen replacement. Apart from these changes in E_2 and P levels, LH levels increase, while decreased GnRH levels are found in the hypothalamus. This decrease in GnRH content of the hypothalamus was also seen in premenopausal women from which the ovaries were removed, and in ovariectomized rodents from which it is known that GnRH secretion is increased. Therefore, these data suggest that postmenopausal women have an increased secretion of GnRH, resulting in decreased GnRH levels in the hypothalamus and elevated LH and FSH levels. Besides changes in the reproductive axis, aging humans also show age-related changes in other systems regulated by the hypothalamus (103).

The reproductive system during aging in rats

Many studies have been conducted in rats to elucidate the mechanisms underlying reproductive aging. Like in humans, the age-related reduction in fertility is illustrated by several changes, e.g. an increase in genetic and morphological abnormalities of oocytes, fetal resorption during midpregnancy, stillbirths, nonlethal developmental abnormalities, length and variability of reproductive cycles, and a decrease in litter size (138,163,270).

Transplantation experiments in rats and mice (184) suggest that the contribution of hypothalamic aging to the decline in fertility with age is larger in rats and mice than is expected based on our knowledge of reproductive aging in humans. For example, aged female mice with irregular cycles and with ovarian transplants of young regular cycling mice are less fertile (less pregnancies on day 10 after mating) than young female mice with regular 4-5 day estrous cycles and with ovarian transplants of aged, irregular cycling mice (195). This experimental evidence strongly supports a vital contribution of the hypothalamus, apart from the ovary, to the age-related decline in fertility.

In rats, the process of reproductive aging can be divided into different successive periods, which are characterized by changes in cycle length. The process of reproductive aging starts between 7 and 15 months of age (which may

depend on genetic background) with a lengthening, instead of a shortening as found in humans, of the estrous cycle and the transition from regular to irregular cycles. This period between 7 and 15 months is often called 'middle-age' and is followed by persistent vaginal cornification or persistent estrous (PE). During this first period of acyclicity, the ovaries are anovulatory and secrete E_2, but little P, and appear to be less responsive to these hormones (172). This period can be followed by a period with repetitive pseudopregnancy (RPP), in which ovulatory cycles display prolonged luteal phases (about 12 to 14 days), although this is not seen in all animals. Finally, female rats become infertile and anovulatory (around 18 months of age), a phase which is called persistent anestrus. During these subsequent periods several components of the reproductive system change, for instance hormone concentrations, responses to hormonal feedback of the hypothalamus, and sexual behavior.

Even before the lengthening of the estrous cycle occurs, an attenuation of the preovulatory LH surge can be detected (45,161,284). Previous studies indicate this may be due to age-related changes at the level of the hypothalamus (290) and pituitary gland (134,286). Although a decrease in the number of GnRH-containing neurons is only apparent at the age of 26 months (76), a decrease in the number of activated GnRH neurons at proestrus can already be demonstrated in regularly cyclic middle-aged females (153,213). The percentage of activated GnRH neurons on proestrus correlates with the amplitude of the LH surge (66). This decrease in activated GnRH neurons may be the result of an age-related decrease in excitatory input to GnRH cells and/or a decrease in sensitivity to this input, which may subsequently lead to a diminished GnRH synthesis, transport, and secretion (210,284). This induces an impairment of the response of pituitary gonadotrophs to GnRH (22,134) and consequently an attenuation of the LH surge with age. Apart from change in the magnitude of the LH surge, there is also a delay in the timing of the surge in middle-aged female rats (285) and alterations can be seen in the pulsatile release of LH (219). In addition, higher FSH levels were reported on proestrus and estrus in middle-aged rats, while the preovulatory FSH surge was delayed (45).

The presence of diurnal rhythms in the regulation of the reproductive system, especially in the timing of the LH surge, and the loss of these rhythms during the process of aging strongly indicate that the SCN is involved and that age-related changes in the SCN could very well influence reproductive senescence. Indeed,

aging has been shown to influence the SCN and its target areas. This notion is supported by the fact that: (a) local cerebral glucose utilization, an index of overall neural activity, exhibits a circadian rhythm in the SCN of young rats that is altered when animals are middle-aged (290), (b) the diurnal rhythms of neurotransmitters and their receptors in the hypothalamus are altered with age (290), (c) the amplitudes and/or periods of the circadian rhythm of behavioral parameters, including running, drinking, and sleeping, are dampened or altered in aged animals, and (d) the ability of the animal to entrain circadian behavior to changes in photoperiod decreases with age (280). Thus, the ability of the SCN to impel its circadian rhythm on different (endocrine) systems is impaired during aging. Two SCN-neurotransmitters are known to innervate the MPO: vasopressin (VP) and vasoactive intestinal peptide (VIP). Both are involved in the generation of the LH surge at proestrus. The circadian rhythm in VIP mRNA, but not that of VP mRNA, is demonstrated to alter with age, i.e. the peak of VIP mRNA during the day period decreases (131). It is thought that the VIP-input to the MPO, in particular to the GnRH neurons themselves, is important for the stimulation of GnRH release (104,130,238). Thus, the decreased activity of SCN-VIP neurons may directly be responsible for the decreased amplitude of the LH surge in middle-aged female rats.

Furthermore, proestrous E_2 as well as P levels decrease with age. The decrease in plasma P concentrations is associated with a decrease in P levels during gestation, suggesting an altered activity of the CL with age that may contribute to the decrease in fertility in middle-aged female rats (98). Finally, during persistent anestrus E_2 concentrations remain elevated due to the E_2 production by the remaining follicles, which differs from women after menopause.

An important factor that influences the rate of reproductive aging in rats is the life-time steroid hormone exposure. High levels of E_2 accelerate aging of the reproductive system and infertility (97), while repeated (pseudo)pregnancies or treatment of young female rats with P delays the reproductive decline (46,139,156). Since exposure to P can partly counteract the effects of E_2 exposure (140), a role for the E_2/P ratio in reproductive aging is suggested. In this view, elevated E_2 levels and decreased P levels (i.e. an increased E_2/P ratio) as seen during aging can lead to changes which may cause infertility in rats as well as humans. In addition, individual and strain differences can be the result of differences in life-time steroid hormone exposure.

In summary, the activity and sensitivity of the hypothalamus-pituitary-gonadal axis appear to decrease with advancing age. At the level of the hypothalamus-pituitary system, the activity of GnRH neurons on proestrus declines, possibly as a result of decreased sensitivity of GnRH neurons to excitatory input, and the response of hypothalamus-pituitary system to E_2 and P and of the pituitary to GnRH appear to decline with age.

1.5. Scope of the thesis

Based on the findings that (a) the release of many neurotransmitters, which are often regulated by steroid hormone feedback, changes during the early phase of reproductive aging and (b) life-time ovarian steroid exposure influences the rate of reproductive aging, it is hypothesized that an age-related change in the feedback of ovarian steroid hormones to the brain underlies the decrease in fertility with age in female mammals. In addition, it is hypothesized that the rate of reproductive aging differs between individuals and rat strains because of differences in life-time ovarian steroid exposure. To test these hypotheses, two rat (sub)strains were used: a Wistar strain (Harlan) that predominantly displays 4-day estrous cycles versus an F1 crossbred of two Wistar strains (UxRP) that predominantly displays 5-day estrous cycles. Apart from this difference in cycle length, fertility and fecundity appear to decline at an earlier age in the F1 strain. Already at the age of 7-9 months, smaller litters and an increase in fetal resorptions are observed in F1 females (163), and the onset of acyclicity occurs earlier in F1 rats compared to Wistar and other rat strains (270). Using this approach, insight in the common mechanisms that underlie reproductive aging may be gained.

Since one of the first indications of reproductive aging in rodents is an attenuation and delay of the LH surge, it was first investigated if the LH surge had already changed in regularly cyclic females of 8.5 months of age compared to young adult females (4 months) of both rat strains (**Chapter 2**). In addition, the possible underlying causes of changes in the LH surge profile were investigated. To this end, blood samples were taken to characterize proestrous plasma P surges (onset time, peak time, peak height, total amount released) in both strains since P release on proestrus is known to stimulate the ongoing LH surge. Also, the maximal amount of acutely releasable LH by the pituitary was tested on proestrus

using the potent GnRH analog Ovalyse® to test whether the capacity of the pituitary to secrete LH was changed with age.

Secondly, the hypothesis that a change in the magnitude of the LH surge with age is the consequence of a change in positive feedback actions of ovarian steroids to the brain was tested. The preoptic area (including the AvPv and MPO) is involved in this positive feedback and appears to be crucial for the initiation of the preovulatory GnRH surge. Therefore, the ovarian hormone feedback on preoptic area steroid receptors during the estrous cycle was studied in young (4.5 months) and middle-aged (9 months) females of both rat strains. To measure steroid hormone concentrations (E_2 and P), blood samples were taken twice a day during one complete estrous cycle. On specific days of the estrous cycle, rats were killed, whereafter brain sections were stained for PR (**Chapter 3**) or ER (**Chapter 4**). With a computer analysis program, the number of cells in the POA that contained high concentrations of the PR or ER was counted. Furthermore, the feedback of E_2 on brain ER and PR with age was studied in more detail by using ovariectomized females that were given a single subcutaneous injection with a physiological dose of estradiol benzoate. Hence, steroid levels were not directly influenced by strain or age. The number of cells in the POA that contained PR and ER was counted at specific time points after estradiol benzoate administration. In this way, it was possible to investigate PR induction (**Chapter 3**) and ER downregulation (**Chapter 4**) with age and between strains in a controlled setup.

Thirdly, reproductive aging in rats is characterized by an increase in cycle length. The decline in female fertility with advancing age may be the result of an age-related change in the feedback of ovarian steroid hormones to the brain. In **Chapter 3 and 4** already the positive feedback of steroid to the brain was investigated, showing a decreased activation of the GnRH system on proestrus. In addition, the negative feedback of steroid hormones to the brain could be affected by age. An age-related increase in negative feedback could lead to an increase in cycle length. Since the ARC is thought to play a role in the negative feedback of steroid hormones to the brain, steroid hormone receptor numbers were investigated in the ARC in cyclic young and middle-aged females of both rat strains (**Chapter 5**).

Fourthly, early reproductive aging in women is characterized by an increase in basal FSH concentrations, a decrease in the magnitude of the preovulatory FSH surge, and an increase in the postovulatory FSH surge. An increase in FSH levels

is thought to be an indicator for the sharp decrease in magnitude of the ovarian follicular reserve pool with age. Since most rat studies have focused on the LH surge and not FSH like in humans, it was investigated if FSH levels were already altered in the 8.5-month-old females in which also preovulatory LH and P profiles were determined. To this end, plasma FSH levels were measured in the same females of both rat strains (**Chapter 6**). In addition, it was tested whether FSH levels in rats also correlated with the magnitude of the ovarian follicular pool, like in women, by counting the number of primordial follicles in the ovaries of these female rats (**Chapter 6**). To gain more insight in possible strain differences in (rate of) reproductive aging, the magnitude of the follicular pool was also compared between strains.

Finally, in the summary and general discussion (**Chapter 7**) the main conclusions of this thesis are presented, and a mechanism is proposed by which (I) the normal reproductive cycle is regulated at the hypothalamic level, and (II) age-related changes in the female reproductive axis can influence fertility and fecundity.

Chapter 2

The age-related attenuation of the luteinizing hormone (LH) surge is not associated with changes in GnRH-induced LH release or progesterone (P) levels

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(to be submitted)

Abstract

The aim of the present study was to investigate changes in the female reproductive axis, which may underlie the attenuation of the LH surge with age. On proestrus, ten hourly blood samples were taken to characterize LH and P surges in young (4 months) and middle-aged (8.5 months) rats of a Wistar (4-day cyclic) and an F1 (UxRP; 5-day cyclic) strain. In the latter, fertility appears to decline earlier. Also, the amount of acutely releasable LH by the pituitary was tested on proestrus using the potent GnRH analog Ovalyse®. F1 females displayed a significantly higher LH surge compared to Wistar females, and both strains showed an attenuation of the LH surge with age. The P surge was slightly lower in F1 compared to Wistar rats, but showed no significant changes with age. Administration of Ovalyse® resulted in a rapid increase in LH plasma levels; total LH release was lower in F1 compared to Wistar rats, but was not affected by age.

Our results clearly indicate that the age-related attenuation of the LH surge is seen across rat strains and precedes other possible changes relevant for reproductive aging. Based on our data, we hypothesize that the attenuation of the LH surge is the result of a decrease in GnRH release and/or pituitary GnRH priming.

Introduction

Fertility and fecundity decline in female mammals long before reproductive senescence occurs. A general indication for reproductive aging is the length of the reproductive cycle. During adult life, the cycle of female rats, which is normally 4 or 5 days, becomes prolonged and irregular. This is often followed by a period of pseudopregnancy (PP) or persistent estrus (PE) and eventually leads to a state of persistent diestrus (PD) or anestrus (270). A change in cyclicity can also be seen in humans (248), but menstrual cycles that consist of approximately 28 days tend to become shorter with age before cyclicity ends at menopause. Although the sequence of events during aging is highly predictable in both species, the age at which the decline in fertility becomes evident varies considerably between individuals (248,270).

In the past decades, research has focused on the changes that lead to

reproductive aging. Changes during aging that may influence fertility and fecundity have been demonstrated at the level of the hypothalamus (39,77,85,172,215,251, 291), the pituitary gland (134,205,286) and the ovary and uterus (182,205,224, 248). An attenuation of the preovulatory LH surge, which is responsible for ovulation, appears to be one of the first indications of reproductive aging in rats (134,181,215,264,285,291), but the specific contribution of the hypothalamus, pituitary and ovary to the decreased preovulatory LH release is unclear.

In humans, the attenuation of the preovulatory LH surge is associated with a reduced probability of conception in healthy women (9) and an impaired fertilization of oocytes in vitro (269). In middle-aged female rats, an attenuated LH surge can be demonstrated even before estrous cycles become irregular (Sprague-Dawley: 215,277,285; Long-Evans: 134,181), and is strongly associated with the age at which rats become acyclic (181).

The preovulatory LH surge is induced by a tightly orchestrated series of events in the hypothalamus-pituitary-ovarian axis. During the follicular phase, growing ovarian follicles secrete increasing amounts of estradiol (E2). The increase in E2 (a) inhibits LH secretion by the pituitary gland, (b) stimulates storage of pituitary LH and hypothalamic GnRH (99), (c) stimulates the expression of pituitary GnRH receptors (158), (d) induces progesterone receptor (PR) expression in the hypothalamus and pituitary gland (6,32,99,158,246), and (e) results in a down-regulation of its own receptor (ER) (299). The ER- and PR-containing neurons in the preoptic area (POA) are hypothalamic components necessary for the induction of the LH surge. These neurons project directly to GnRH neurons (235) and play a crucial role in their activation (35). The activated GnRH neurons release GnRH at the median eminence (the so-called ‘GnRH surge’), which leads to an increase in synthesis and release of pituitary LH (the ‘LH surge’) (29,61). GnRH is secreted in bursts, which induces GnRH priming at the level of the pituitary gland (61,244). Apart from GnRH priming, the ongoing LH surge can be further enhanced by progesterone (P) release secreted by the luteinizing follicles (61,146,158,256).

The aim of the present study was to investigate changes in the reproductive axis that may underlie the attenuation of the LH surge. To this end, two different rat strains were used, a Wistar strain (Harlan) with regular 4-day estrous cycles and an F1 crossbred of two Wistar strains (UxRP) with regular 5-day estrous cycles. Apart from a difference in cycle length between these rat strains, fertility and fecundity appear to decline earlier in the F1 strain (270). Already at the age of 7-9 months

smaller litters and an increase in resorptions are observed in (UxRP)F1 females (163). In addition, the onset of acyclicity occurs relatively early in (UxRP)F1 rats compared to Wistar and other rat strains (270).

In regular cyclic females of both rat strains, proestrous LH and P profiles were characterized at the age of 4 and 8.5 months. Furthermore, the amount of acutely releasable LH was tested during the following proestrus using a potent GnRH analog.

Materials and Methods

Animals

Virgin female (UxRP)F1 rats (N = 60), a locally bred hybrid of two Wistar sub strains (RP-inbred albino females and U-inbred hooded males, abbreviated as F1), were obtained from the university animal care facility at 9-10 weeks old (163,265). Virgin female (N = 60) and male (N = 16) Wistar rats (HsdCpb:WU, Wistar Unilever; abbreviated as WU) were obtained from Harlan (Horst; The Netherlands) at respectively 9 or 12 weeks old. Rats were housed four or five per cage under regular light-dark cycles (L/D 12:12, lights on at 3:00 h defined as 'zeitgebertime' 0 or ZT 0) with free access to standard pelleted food (Hope Farms B.V., Woerden, The Netherlands) and water.

Estrous cycles were monitored by daily vaginal lavage for a period of approximately 7 weeks, i.e. from one week after arrival until the end of the experiment (young females; 17 weeks old at the end of the experiment; n=30 for each rat strain) or from the age of 29 weeks until the end of the experiment (middle-aged females; 36 weeks old at the end of the experiment; n=30 for each rat strain). In addition, receptive behavior was monitored daily. To this end, a naive male WU rat was introduced briefly in the female home cage approximately one hour before lights off. Display of hopping and darting, ear wiggling, and lordosis posture by the female was checked. Each female was housed individually one week before cannulation until the end of the experiment. Body weight of the female rats had reached a plateau at the age of 31 weeks, and amounted to 286 ± 5 g for WU and 229 ± 2 g for F1 females. The experiment was approved by the animal experimental committee of the Wageningen University.

Experimental design

To investigate differences in proestrous LH surge characteristics between young and middle-aged females, regular 4-day cyclic WU and 5-day cyclic F1 females were used for blood sampling and hormone analyses. An additional group of 5-day cyclic young (n=6) and middle-aged (n=6) WU rats was included in this study to account for differences in hormone profiles caused by cycle length. Hourly blood samples were taken on proestrus to measure plasma LH and P levels. To investigate pituitary LH responsiveness a potent GnRH analog (Ovalyse®; des-Gly10-GnRH-ethylamide, Upjohn, Ede, The Netherlands) was used on the following proestrus. Ovalyse® (100 ng in 0.25 ml physiological saline (0.9% NaCl w/v) containing 1% bovine serum albumin (BSA)) was administered via the jugular vein cannula immediately after the first blood sample was drawn. Previous studies have shown that this dose of Ovalyse® induced a 3-5 times higher LH surge than the spontaneous surge (165) and has no effect on ovulation rate (163).

Cannulation

To obtain stress-free blood samples, the right jugular vein of females was cannulated according to the method of Steffens (242). Females we anaesthetized by an intraperitoneal (ip.) injection with a Ketamine/Rompunâ mixture, i.e. 60 mg Ketamine (Kombivet, Etten-Leur, The Netherlands) and 3.3 mg Rompunâ (Bayer, Leverkusen, Germany) per kg bodyweight, followed by a subcutaneous (sc.) injection with an antibiotic (Duplocillineâ, Mycofarm, De Bilt, The Netherlands) after 5 minutes. The cannula was filled with polyvinyl pyrrolidon (PVP-25, Merck, Darmstadt, Germany) solution (50% w/w PVP, 500 IU/ml heparin in saline). In order to prevent clotting, each cannula was flushed with heparinized saline solution twice a week, whereafter it was filled with fresh PVP solution. Young and middle-aged female rats were cannulated and allowed to recover from surgery for at least five days prior to blood sampling.

Blood samples

From each female, ten hourly blood samples of 170 ml were taken on the day of proestrus from ZT 5.5 to ZT 14.5. Blood samples were collected in heparinized, air-dried vials (25 IU heparin, Leo Pharma BV, Breda, The Netherlands) and kept on ice until centrifugation at 13,000 rpm for 5 minutes. For LH measurements, 75 ml plasma was 1:4 diluted in phosphate buffered saline (1M PBS, pH 7.5) containing

0.1% BSA (Sigma Chemical, St. Louis, MO, USA) in polystyrene vials. For P measurements, 30 ml of the 1:4 dilution was added to 120 ml PBS containing 0.1% BSA (0.5 M PBS, pH 7.0; final dilution 1:20). All diluted plasma was stored frozen at –20 °C until RIA.

Radio Immuno Assays

LH and P plasma levels were determined by validated RIA's, that are routinely used in our department.

LH plasma levels were analyzed by a double-antibody RIA. The first antibody used was NIDK-anti-rLH-S-11 (dilution 1:500,000) and the tracer (NIDK-rat LH-I-9) was iodinated with 125I by the chloramine-T method. The second antibody was a donkey-anti-rabbit antibody (AA-SAC1 Donkey-anti Rabbit) supplied by Wellcome Reagents (Beckenham, UK) and was diluted 1:2 with PBS containing 0.1% BSA (pH 7.5). Plasma LH levels were expressed in terms of the reference standard NIDK-rLH-RP-2 (AFP-5666C). The assay sensitivity was 0.2 ng/ml for LH at 90% of the maximal binding. LH concentrations were determined in four separate assays. The inter- and intra-assay coefficients of variation were determined using pooled rat serum, and amounted to respectively 12.1% and 10.8% (259).

P plasma levels were determined using a single-antibody RIA as previously described (262). A specific rabbit antiserum against 4-pregnene-6b-ol-3,20-dione-hemisuccunate-BSA was used in a dilution of 1:15,000 with PBS containing 0.1% BSA. Progesterone (P-9776, Sigma Chemical, St. Louis, MO, USA) was used as a reference standard and [1,2,6,7-3H]Progesterone (TRK 413, specific activity 87.0 Ci/mmol, Amersham, UK) was used as the tracer. P levels were determined in 14 separate assays. The assay sensitivity was 2 ng/ml for P at 90% of the maximal binding. The inter- and intra-assay coefficients of variation were determined using pooled rat serum, and amounted to 15.8% and 6.2%, respectively.

Data processing and statistics

Data were expressed as mean ± SEM and analyzed using SPSS (version 10.1). To determine the effects of age and rat strain on the LH surge several characteristics were defined: i.e. basal levels, onset time, peak time, peak height and the total amount of LH released during the LH surge. Basal LH levels were defined as the average LH concentration per animal of the first three blood samples taken (ZT 5.5, ZT 6.5, and ZT 7.5). In case of an extremely early rise in

LH levels, i.e. an increase at ZT 7.5 (n=3), the first two blood samples were taken to calculate basal LH levels. Onset time was defined as the sample hour (ZT; mean ± SEM expressed as h:min ± min) at which LH levels exceeded basal LH levels plus 3 x the standard deviation of these basal LH levels, while LH levels continued to rise in the next blood sample(s). Peak time of the LH surge was defined as the sample hour (ZT; mean ± SEM expressed as h:min ± min) at which the highest LH concentration was measured. The highest amount of LH measured at that time was defined as the peak height. The total amount of LH released during the LH surge was determined in each animal by the cumulative value of LH levels during the complete sampling period.

LH surge characteristics were also measured after the administration of the GnRH analog. In this case, the LH surge was divided in two parts: the LH surge directly after Ovalyse® administration (ZT 5.5 - ZT 8.5; 'induced' LH surge) and the LH surge occurring after ZT 8.5 until the end of the sampling period (ZT 14.5; 'spontaneous' LH surge). For the 'induced' LH surge, peak time and peak height were determined as described above. Since the second LH surge was strongly influenced by the previous exposure to high levels of GnRH (i.e. Ovalyse® administration), no statistics were performed on these LH levels.

Finally, the total amount of P released was defined as the cumulative value of measured P levels during the complete sampling period. Since the proestrous P surge showed no clear basal levels, onset time and peak time within the sampling period, we used the LH surge data to define basal P levels and preovulatory P surge levels. Thus, the first three blood samples were defined as basal P levels (average) and the following samples as the P surge (cumulative value).

Data were analyzed by two-way analyses of variance (age vs. strain) in case of total P levels, total LH levels and peak height during the LH surge, including the first 'induced' LH surge after Ovalyse® administration. Basal LH and onset time of the LH surge, peak time of the normal and first 'induced' LH surge and total P levels after Ovalyse® administration were analyzed per group (young and middle-aged 4-day cyclic WU and young and middle-aged 5-day cyclic F1 rats) by the nonparametric Kruskal-Wallis test. A GLM-multivariate test was performed to test whether strain and/or age differences existed in basal and surge P levels between the groups. In addition, a Pearson correlation test was used to test for correlations between LH levels and P levels at specific time points. Furthermore, data of 5-day cyclic WU rats were compared to data of 4-day cyclic WU and 5-day cyclic F1 rats

by a GLM-multivariate test (age, strain, and cycle length; total LH levels and peak height during the LH surge, total P levels and surge P levels; or per age in case of basal P levels) or nonparametric Kruskal-Wallis test (basal LH, onset time and peak time; total LH levels and peak height during the LH surge after Ovalyse® administration). A $p<0.05$ was considered significant.

Results

The proestrus LH surge

All animals with regular cycles that were selected for hormone analysis showed an increase in LH release during the sampling period. This included 15 young and 9 middle-aged WU females with regular 4-day cycles (at least 2 consecutive 4-day cycles) and 10 young and 14 middle-aged F1 females showing regular 5-day cycles (at least 2 consecutive 5-day cycles). The profile of the LH surge in time was comparable between groups. In all experimental groups, basal plasma LH levels averaged 0.3 to 0.4 ng/ml and the onset of the LH surge occurred around ZT 9.5, whereafter LH levels increased rapidly and reached peak levels around ZT 12 when the lights went off. Subsequently, LH levels gradually declined (Figure 1 and Table 1). A clear strain difference ($p<0.001$) was found in the magnitude of the LH surge. F1 rats displayed significantly higher peak LH levels and total amount of LH released during the surge compared to WU females (Table 1).

Table 1. LH surge characteristics of 4-day cyclic WU and 5-day cyclic F1 rats: basal levels, onset time, peak time, peak levels, and total LH levels. Group means ± SEM for onset time and peak time are expressed as ZT time in h:min. a, significant age difference ($p< 0.05$); b, significant strain difference ($p<0.05$).

strain	cycle	age (months)	basal (ng/ml)	onset time (ZT)	peak time (ZT)	peak LH[a b] (ng/ml)	total[a b] (ng/ml)
WU	4-day	4	0.3±*0.04*	9:50±*25*	12:10±*16*	12.1±*1.2*	38.2±*3.8*
		8.5	0.4±*0.07*	9:37±*23*	11:37±*19*	8.3±*1.4*	25.2±*4.6*
F1	5-day	4	0.3±*0.04*	9:24±*14*	11:48±*18*	21.2±*2.6*	69.0±*6.9*
		8.5	0.4±*0.06*	9:47±*18*	12:22±*6*	14.8±*1.9*	46.0±*4.2*

In addition, a clear effect of age was found. In 8.5-month-old females the LH surge was significantly attenuated compared to 4-month-old females in both rat strains (Figure 1 and Table 1). Both the LH peak concentration and total LH levels were significantly lower in 8.5-months-old females compared to those measured in 4-months-old rats (Table 1; p=0.003 and p=0.001, respectively). No differences were found in basal plasma LH levels or in timing of the LH surge (onset time and peak time) with age or between strains.

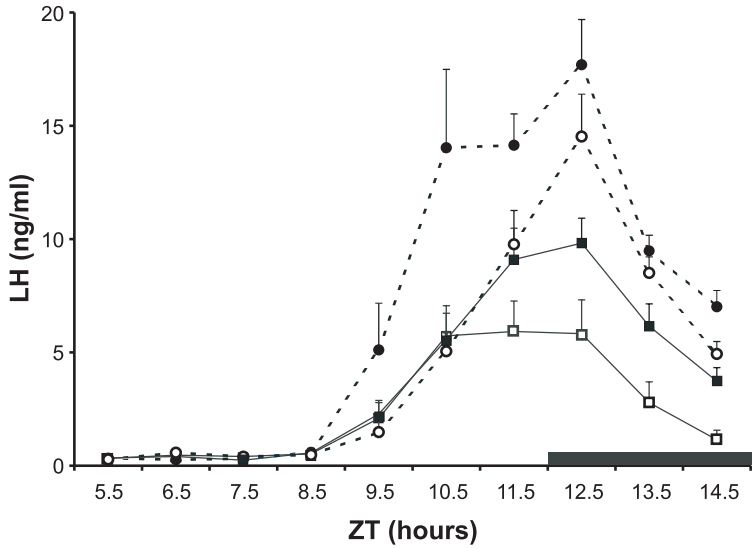

Figure 1. The proestrous LH surge in young (closed squares; n=15) and middle-aged (open squares; n=9) 4-day cyclic WU rats and in young (closed circles; n=10) and middle-aged (open circles; n=14) 5-day cyclic F1 rats. The black bar on the X-axis indicates the dark period of the light-dark cycle.

The profile of the LH surge in the additional group of 5-day cyclic WU females resembled that of the 5-day cyclic F1 females, which was confirmed by the absence of significant differences between LH surges of 5-day cyclic F1 (Table 1) and 5-day cyclic WU (Table 2) rats. When LH data of all groups (young and middle-aged 4- and 5-day cyclic WU and 5-day cyclic F1 rats) were compared, we found that total LH levels of 5-day cyclic rats were significantly (p=0.010) higher than those of 4-day cyclic rats. In addition, total and peak LH levels were significantly reduced with age in all groups (p=0.007 and p=0.007, respectively), but no differences in basal LH levels or timing of the LH surge (onset time and peak time)

were found with cycle length, between age or strain.

Pituitary responsiveness

Administration of Ovalyse® at ZT 5.5 resulted in a rapid and consistent increase in LH plasma levels in all animals. Highest plasma LH levels were measured at 1 or 2 h after Ovalyse® administration and decreased thereafter. After ZT 8.5, LH levels increased again resulting in the spontaneous LH surge.

Four-day cyclic WU rats showed a significantly higher LH peak height ($p=0.015$) as well as higher total LH levels ($p<0.001$) compared to 5-day cyclic F1 rats during the induced LH surge (Figure 2). In addition, the profile of the induced LH peak differed between strains. In WU females, LH levels in the two blood samples after Ovalyse® injection were of comparable height, while in F1 females LH levels were highest in the first blood sample and already decreased in the second blood sample after Ovalyse® injection. This is reflected in a significant difference in peak time of the induced LH peak between groups ($p=0.004$; young WU at ZT 7:07 ± 8 and middle-aged WU at ZT 7:12 ± 9, young F1 at ZT 6:30 ± 0 and middle-aged F1 at ZT 6:39 ± 6). When data of 5-day cyclic WU were included in the analysis, we found that total LH release ($p=0.006$) and peak LH levels ($p=0.030$) after Ovalyse® administration were significantly higher in WU (4-day as well as 5-day cyclic) compared to F1 females (5-day cyclic; Table 2). No significant differences between young and middle-aged WU or F1 females were found.

Table 2. LH peak levels and total LH levels of the natural LH surge in young (n = 6) and middle-aged (n = 6) 5-day cyclic WU rats and of the LH surge after Ovalyse® administration (until ZT 8.5) in young (n = 3) and middle-aged (n = 4) 5-day cyclic WU rats. Group means ± SEM for onset time and peak time are expressed as ZT time in h:min. a, significant difference compared to 5-day cyclic F1 rats ($p<0.05$; see Table 1).

			LH surge		Ovalyse®	
strain	**cycle**	**age** (months)	**peak LH** (ng/ml)	**total** (ng/ml)	**peak LH**[a] (ng/ml)	**total**[a] (ng/ml)
WU	5-day	4.5	15.9 ± *2.5*	58.1 ± *12.6*	33.0 ± *2.3*	64.1 ± *2.7*
		9	15.4 ± *5.3*	47.8 ± *17.9*	34.8 ± *2.2*	69.5 ± *5.3*

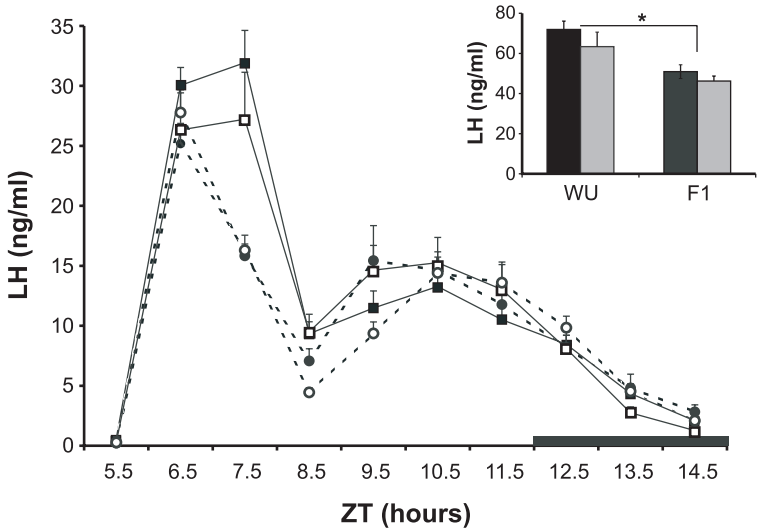

Figure 2. The proestrous LH surge after Ovalyse® administration in young (closed squares; n=14) and middle-aged (open squares; n=10) 4-day cyclic WU rats and in young (closed circles; n=8) and middle-aged (open circles; n=13) 5-day cyclic F1 rats. The inserted graph shows total plasma LH of the induced LH surge (until ZT 8.5; i.e. the acutely releasable LH) in young (black bars) and middle-aged (gray bars) 4-day cyclic WU rats (left) and 5-day cyclic F1 rats (right). The black bar on the X-axis indicates the dark period of the light-dark cycle. * significant difference between rat strains ($p<0.001$).

The proestrus P surge

Plasma P levels increased gradually during proestrus, but we observed no distinct P peak during the time window evaluated (Figure 3). Total P levels during proestrus, i.e. the cumulative value of measured P levels during the sampling period, as well as basal P levels showed no significant differences with age or between strains. Surge P levels, defined as the cumulative value of ZT 4 to ZT 10, showed a significant difference between rat strains ($p=0.033$), but not with age when 4-day cyclic WU and 5-day cyclic F1 were compared.

When data of 5-day cyclic WU rats were included in the analysis, the strain difference in surge P levels was lost. This was mainly due to the high variance and intermediate levels in P levels of 5-day cyclic WU, i.e. in young 5-day cyclic WU rats, P levels approach those from F1, while in middle-aged rats, levels approached those from WU. When data of all groups were included, however, a significant difference in the average basal P levels was found only in young

females between rat strains (p=0.006) and cycle length (p=0.002). P levels after Ovalyse® administration increased gradually but were not significantly different between groups (data not shown).

Finally, we found that preovulatory LH and P levels were positively correlated at ZT 9.5 (r=0.433 with p=0.003) and at ZT 10.5 (r=0.407 with p=0.006). In addition, LH levels at ZT 8.5 were positively correlated with P levels at ZT 9.5 (r=0.588 with p<0.001).

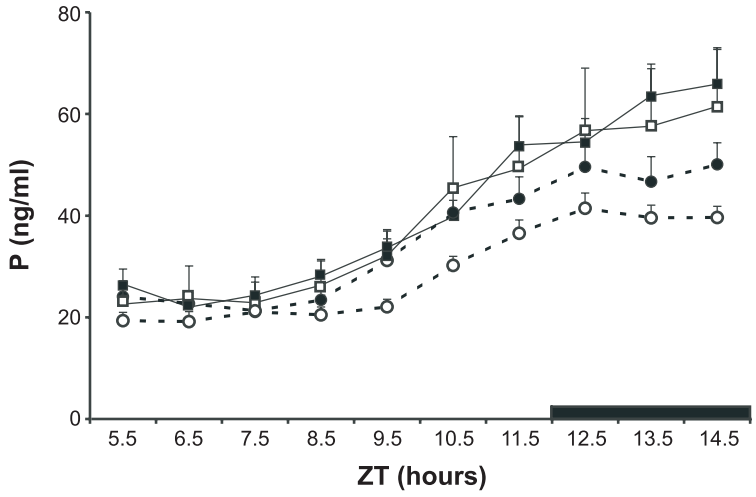

Figure 3. The proestrous P surge in young (closed squares; n=12) and middle-aged (open squares; n=9) 4-day cyclic WU rats and in young (closed circles; n=10) and middle-aged (open circles; n=14) 5-day cyclic F1 rats. The black bar on the X-axis indicates the dark period of the light-dark cycle.

Discussion

To our knowledge, this is the first study which clearly demonstrates that the initial change in LH surge profile with age, i.e. the attenuation of the LH surge, is not accompanied by a decrease in acutely releasable pool of LH or a decrease in proestrous P levels. Since these results were found in two rat strains, they strengthen the idea that the attenuation of the LH surge is indeed an initial, universal change in the sequence of events during reproductive aging. This

universal change in LH release occurs despite the fact that the magnitude of the preovulatory LH surge, the amount of acutely releasable LH by the pituitary gland, and the magnitude of the preovulatory P surge show considerable strain differences.

Our data that show that the LH surge is attenuated in 8.5- compared to 4-month-old rats of the WU and F1 strain are in accordance with previously reports by other groups for young and middle-aged rats of different rat strains (134,181,215,285). In several studies it was suggested that the decrease in proestrous LH levels with age is caused by changes in LH storage and/or release (152,286). Our results show that total LH release after administration of the potent GnRH analog Ovalyse® was comparable between 4- and 8.5-month-old females of both strains. In addition, LH levels after Ovalyse® in F1 rats were comparable to those found in previous experiments in our lab (165). Because Ovalyse® was administrated before the natural LH surge occurred, the first LH surge measured (until ZT 8.5) likely reflects the acutely releasable pool of LH. This implies that our 8.5-month-old WU and F1 females showed no reduction in the acutely releasable pool of LH. Yet, the acutely releasable pool of LH was reduced at the age of 9-12 months in 4-6 day cyclic Sprague-Dawley rats (286), suggesting that reproductive aging may eventually result in a decrease in the releasable pool of LH.

Our data clearly show that proestrous P levels are comparable between young and middle-aged female rats and therefore can not be the underlying cause of the attenuated LH surge at 8.5 months old. In addition, preovulatory LH and P levels were positively correlated at ZT 9.5 and ZT 10.5, just when the LH surge is initiated, which strongly suggest that the initiation of the LH surge is related to the initiation of proestrous P release. Moreover, our results that LH levels at ZT 8.5 are correlated with P levels at ZT 9.5 substantiate the idea that the P surge results from the increase in LH levels. Indeed, 12-month-old cyclic Long-Evans rats display an attenuated preovulatory LH as well as P surge (170) and it has been suggested (37) that the attenuated P levels result from a decrease in proestrous LH levels. Furthermore, the responsiveness of the ovary to hCG stimulation was increased in regular cyclic middle-aged compared to young Long-Evans rats (37). Altogether, these results suggest that the attenuation of the LH surge with age precedes a possible decrease in proestrous P levels and therefore we assume that the attenuation of the LH surge is caused by alterations in the hypothalamus-pituitary system, and not at the level of the ovary.

Potassium-evoked GnRH release was comparable in isolated hypothalami of young and middle-aged female Sprague-Dawley rats, while LH release was reduced with age (209). This suggests that the secretory capacity of the GnRH system is still intact in middle-aged rats, but that the pituitary responsiveness to GnRH and/or the activity of the GnRH system itself is reduced. Yet, 20% binding of GnRH to its pituitary receptor results in 80% of maximum LH release (180). Thus, if the decrease in preovulatory LH release with age is caused by a reduced number of GnRH receptors, the number of GnRH receptors should be decreased dramatically. There are, however, no indications that the number of GnRH receptors is affected with age in female mice (12). Yet, during the LH surge the LH secretion per GnRH burst appears to decrease with age (161). This is thought to be the result of a deficient GnRH drive, a reduced responsiveness to GnRH signaling, and/or a reduction in cellular LH with age (152,161). Indeed, pituitary responsiveness to GnRH is decreased *in vitro* in 10-12 month-old Long-Evans rats that show attenuated LH surges (22) and in 9- compared to 4-month-old WU rats that were tested *ex vivo* in a superfusion system in our lab (117). Since the reduction in LH release after GnRH stimulation with age was more evident during the second and third GnRH stimulation (22), this suggests that the GnRH priming mechanism is affected with age. Furthermore, the activity of GnRH neurons is altered at a relatively early age (8-11 months old) (76), but the number of immunoreactive GnRH neurons decreases only at a progressed age (26 months old) (212). Several studies indicate that in middle-aged female rats the number of activated GnRH neurons (76,291) and GnRH release (211) are reduced on proestrus. GnRH neurons are regulated by many different neural signals (238) and several of the systems involved in the regulation of the GnRH surge are also affected with age (68,77,173,215,291). Taken together, this suggests that the input of GnRH neurons changes with age, resulting in less activated GnRH neurons and subsequently a reduced GnRH release that, together with a reduction in GnRH priming, would lead to a decrease in LH secretion on the day of proestrus.

Previous studies also suggested that changes in the SCN with age may underlie changes in LH release (291), since the SCN is crucial for the induction and timing of the preovulatory LH surge (88). In the present experiment, however, we found no changes in timing of the LH surge (i.e. LH surge onset and/or peak) in middle-aged female rats. Nevertheless, the peak time of LH surges in young 5-day cyclic F1 females showed large individual differences. While LH surges of these

females could be divided in 'early surges' with high total LH levels (peak time < ZT 12; n=7) and 'late surges' with lower total LH levels (peak time > ZT 12; n=3), the old 5-day cyclic F1 rats displayed only 'late LH surges' (n=14). Likewise, early and late surges were observed in the small group of 5-day cyclic WU rats, but the shift with age was not as robust as in F1 rats. The increase in animals that display late LH surges with age suggests that an age-related delay in the LH surge exists in 5-day cyclic females, although it is very modest. Since a clear delay in timing of the LH surge at the age of 7-10 months is only demonstrated in Sprague-Dawley rats (215,285) and not in Long-Evans, WU, or F1 rats, this suggests that the age at which changes occur in the SCN drive of the LH surge may be strain specific.

Although in the present study the changes in LH release between young and middle-aged females were comparable between rat strains, the magnitude of the LH and P surge and the releasable pool of LH differed between rat strains. The releasable pool of LH and the proestrous P surge were smaller in 5-day cyclic F1 rats compared to 4-day cyclic WU rats, but the preovulatory LH surge was higher in these females. At first sight, this appears to be paradoxical. Lower P levels on proestrus (256) are thought to result in a lower, and not a higher, LH surge. In addition, although the effect of the magnitude of the releasable pool to the magnitude of the LH surge is still unclear, one can hypothesize that a smaller releasable pool of LH could result in a lower LH surge as well. Based on these results, we hypothesize that the endogenous GnRH release is higher and/or the GnRH priming mechanism is more effective in F1 compared to WU females. Moreover, these data suggest that, in addition to strain differences in rate of reproductive aging, considerable strain differences in the normal regulation of the reproductive axis exist.

Apart from strain differences, cycle length could contribute to differences in hormone concentrations between WU and F1 rats. Indeed, hormone concentrations of 4-day and 5-day cyclic females differ (96,265). For example, E_2 levels are elevated for one day (4-day cyclic) or two days (5-day cyclic) during the estrous cycle (Sprague-Dawley: (186), Long-Evans: (185), F1: (69,70)). Indeed, the LH surge profile in 5-day cyclic WU rats resembled that of 5-day cyclic F1 rats, suggesting that E_2 exposure might play a role. Yet, 5-day cyclic WU rats, are not exposed for two, but for only one day to elevated E_2 concentrations during an estrous cycle (unpublished observation). In addition, the releasable pool of LH was

still significantly higher in 5-day cyclic WU compared to F1 females. This suggests that strain differences may be present in estrogen sensitivity, but also in GnRH input, GnRH release and/or GnRH priming.

Conclusion

This is the first study, which clearly demonstrates the existence of strain differences in regulation of the reproductive system. The magnitude of the preovulatory LH and P surge, as well as the amount of releasable pool of LH differed between 4-day cyclic WU and 5-day cyclic F1 females. These differences could not simply be explained by a difference in cycle length or estrogen exposure.

Based on the data of our two rat strains, we conclude that one of the first, universal changes during reproductive aging is an attenuation of the LH surge, which precedes possible changes in the releasable pool of LH, preovulatory P levels, and timing of the surge. The lack of concurrent changes in the other parameters measured led us to hypothesize that the attenuation of the LH surge is the result of a decrease in hypothalamic GnRH release and/or pituitary GnRH priming.

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Chapter 3

The estradiol-induced increase in preoptic area progesterone receptors (PR) is reduced with age

Annelieke N. Franke, Victor M. Wiegant, Hans J.M. Swarts, Jan Kastelijn, Eline M. van der Beek

(to be submitted)

Abstract

To investigate the contribution of the hypothalamus to the decline in fertility with age, we studied steroid feedback to the brain. Using 4.5- and 9-month old female Wistar (4-day cyclic) and (UxRP)F1 (5-day cyclic) rats we assessed the dynamics over the estrous cycle of circulating estradiol (E_2), progesterone (P) and progesterone receptor-immunoreactive (PR-IR) cell numbers in the anteroventral periventricular nucleus (AvPv) and medial preoptic area (MPO). We also tested the capacity of E_2 to induce PRs using ovariectomized estradiol benzoate-treated (OVX+EB) rats.

Both strains demonstrated changes in E_2, P, and PR-IR cell numbers over the estrous cycle. Although E_2 and P concentrations were unchanged (Wistar) or increased ((UxRP)F1) with age, both strains showed a clear decrease in PR-IR cells in the AvPv and MPO with age. In OVX+EB females PR-IR cell numbers were increased 26 and 32 h after EB administration, but only (UxRP)F1 females showed an age-related decline in PR-IR cells.

Since the number of hypothalamic PR containing cells decreased with advanced age in cyclic rats, this decrease, possibly as a consequence of impaired E_2-mediated induction of PRs, may be a general mechanism during reproductive aging.

Introduction

The age at which the decline in fertility becomes evident in female rats varies considerably between strains and individuals (270). One of the first changes in the reproductive system associated with a decline in fertility is the attenuation of the preovulatory LH surge (22,45,67). Indeed, regular cycling middle-aged rats with an attenuated LH surge become acyclic at an earlier age than rats of the same age that display a normal LH surge (181). Since we were interested in the contribution of the hypothalamus to the decline in fertility with age, changes between young and middle-aged cyclic females in one of the crucial mechanisms that regulate the LH surge, i.e. steroid feedback to the brain, were studied in more detail in the present study.

In general, estrous cycles are regulated by positive and negative feedback

effects on (neuroendocrine) mechanisms that involve several hypothalamic areas and neurotransmitters (111,238). Previous studies showed that lesions of the preoptic area (POA), in particular of the anteroventral preoptic area (AvPv) or the suprachiasmatic nucleus (SCN), eliminated spontaneous as well as steroid induced LH surges (88,116,144). Besides the AvPv and SCN, the medial preoptic area (MPO) is thought to be involved (111,199,238). The MPO plays an additional role in maternal (240) and sexually motivated behavior (113). Both the AvPv and MPO contain high concentrations of the progesterone receptor (PR) and the estrogen receptor (ER) (25), and PR and ER gene expression changes dramatically over the estrous cycle (231). In the regulation of estrous cycles, the rise in estradiol (E_2) levels during follicular growth plays an instrumental role. E_2, bound to its own receptor, can induce the expression of PRs by binding to an estrogen-responsive element (ERE) in the PR gene promoter (132), thereby stimulating the transcription of PR mRNA (65). Progesterone (P) is thought to downregulate its own receptor, but the underlying mechanism remains unclear (7,220,255).

At present, it is thought that exposure to rising levels of E_2 during the follicular phase of the cycle stimulate the expression of PRs (32,73) in estrogen-receptive neurons of the AvPv. These neurons are innervated by the SCN (275) and this nucleus is responsible for a daily neuronal signal that activates the PRs. In contrast to ovariectomized (OVX) E_2-treated female rats that display daily LH surges, the daily neuronal signal in cyclic rats results in an LH surge only once every 4 or 5 days, because of the additional feedback of P. It has been shown that neurons in the AvPv and MPO project to the vicinity of GnRH neurons (92,143,235) and that GnRH neurons in these areas are the first to be activated on proestrus (147). Therefore, the activation of preoptic area PRs may result in activation of GnRH neurons, thereby initiating the GnRH surge resulting in the preovulatory LH surge and ovulation (34,35).

Previous research showed that the attenuated LH surge in aging female rats was accompanied by a reduced activation of GnRH neurons (153,212). Nevertheless, the capacity of the hypothalamus to secrete GnRH was unaltered with age (209). These data suggest that the activation of GnRH neurons on proestrus declines with age as a result of changes in input to the GnRH neuron. Indeed, an attenuated LH surge is associated with a decline in both Fos-expressing GnRH neurons as well as Fos-expressing cells in the AvPv (144). Thus, the AvPv plays a key role in the stimulatory feedback effects of gonadal steroids on the

GnRH surge.

Based on the role of PR in the AvPv and MPO in the regulation of the LH surge, we hypothesize that specific changes in the feedback of E_2 and P on the number of PRs in these areas may be instrumental for the age-related decline in fertility leading to the attenuation of the LH surge. Therefore, the aim of this study was to investigate changes in profiles of the ovarian hormones E_2 and P over the estrous cycle in relation to the number of PR-containing cells in the preoptic area in young (4.5 months) and middle-aged (9 months) female rats. In addition, the hypothesis that the capacity of E_2 to induce PRs in the AvPv and MPO is altered with age was tested in ovariectomized female rats at the age of 4.5 and 9 months.

There is evidence that exposure to high E_2 levels during life advances the decline in fertility with age (97,155,207). In contrast, exposure to (high) P delays the decline in fertility (156). Thus, individual and strain differences in exposure to E_2 and P during the estrous cycle could theoretically lead to differences in the rate of reproductive aging. In the present study, we used two rat strains. Firstly, the (UxRP)F1 strain, a strain that predominantly displays 5-day estrous cycles and shows impaired fertility and fecundity at a relatively early age (163). Secondly, a Wistar strain (HsdCpb:WU), that displays predominantly 4-day estrous cycles and in which cyclicity is thought to end at a much later age compared to (UxRP)F1 rats (270). We anticipated to gain more insight in the mechanism that underlies reproductive aging by comparing the effects of steroid feedback on PR numbers between these two rat strains.

Materials and Methods

Animals

Virgin female F1 rats, a locally bred hybrid of two Wistar sub strains (RP-inbred females (albino) and U-inbred males (brown hooded); abbreviated as F1), were obtained from the university animal care facility at 9 weeks old. Virgin female and male Wistar rats (HsdCpb:WU, Wistar Unilever; abbreviated as WU) were obtained from Harlan (Holst, The Netherlands) at 9 weeks old (females) or at 12 weeks old (males). Rats were housed five per cage under regular light-dark conditions (L/D 12:12, experiment 1: lights on at 5:00 h (‘zeitgeber time 0’ or ZT 0), and experiment 2: lights on at 3:00 h (ZT 0)) and had free access to standard pelleted food (Hope Farms B.V., Woerden, The Netherlands) and tap water.

Estrous cyclicity was monitored by daily vaginal lavages from one week after arrival until the end of the experiment or ovariectomy (young females; 4.5 months old at the end of the experiment; n=70 for the F1 strain and n=62 for the WU strain) or from the age of 7.5 months until the end of the experiment or ovariectomy (middle-aged females; 9 months old at the end of the experiment; n=80 for each rat strain). In addition to vaginal lavages, receptive behavior was monitored daily. To this end, a naive male WU rat was introduced to the female rat home cage one hour before lights off, where after display of hopping and darting, ear wiggling, and lordosis posture by the female was checked. One week before cannulation, each female was housed individually until the end of the experiment. The experiment was approved by the animal experimental committee of the Wageningen University.

Experimental design

Experiment 1: cyclic female rats

To study age-related changes in the feedback of gonadal hormones on brain progesterone receptors (PR), we measured steroid hormone levels (E_2 and P) over the estrous cycle in young and middle-aged rats. In addition, the number of PR-expressing cells was measured in the preoptic area at specific time points during the estrous cycle. To this end, young WU (n=20) and F1 (n=40), and middle-aged WU (n=40) and F1 (n=40) rats were used. Rats were cannulated at 11 weeks (young), or at 36 weeks (middle-aged) old. After a recovery period of at least 5 days, a blood sample of 300 µL was taken twice a day at 10:00 h (ZT 5) and 16:00 h (ZT 11) during one complete estrous cycle when the females were at the age of 4.5 months (young) or 9 months (middle-aged). Based on the vaginal lavages and receptive behavior prior to and during blood sampling, only females with a distinct and regular 4-day (WU; n=14) or 5-day (F1; n=32) estrous cycle were included in the experiment. The percentage of WU rats that displayed only 4-day cycles during the last three estrous cycles decreased with age from 40% (young) to 20% (middle-aged), while in F1 rats (5-day cyclic) it decreased from 53% (young) to 34% (middle-aged). Note that the number of regular cyclic females was higher in young and middle-aged F1 compared to WU rats. Immediately after the last blood sample was taken, each animal was perfused. Perfusions were performed at specific time points of the estrous cycle: i.e. on metestrus (10:00 h = ZT 5), diestrus II (10:00 h = ZT 5), or proestrus (16:00 h = ZT 11). Each group contained 3-6 animals. Brain

sections of the preoptic area were immunocytochemically stained for PR in three staining runs.

Experiment 2: ovariectomized EB-treated female rats

To study age-related changes in the sensitivity of hypothalamic PR to E_2 feedback in more detail, we used 4.5- and 9-month-old ovariectomized WU and F1 rats (n = 30 for each group) subcutaneous (sc.) injected with a physiological dose estradiol benzoate (EB). In addition, we tested whether P influenced the number of PR-expressing cells in the preoptic area in the time course normally found to enhance the LH surge (30). All young and middle-aged female WU and F1 rats were randomly divided over five experimental groups: four groups which only received EB and were perfused 2, 8, 26 or 32 h (ZT 5 and ZT 11) after EB administration, and one group 32 h exposed to EB which received an additional P injection 4.5 h before perfusion. Because the experiment was split into two parts, i.e. young and middle-aged females, a group of 12 young WU females was included in the second part of the experiment. These females were perfused 2 h (n=6) and 32 h (n=6) after EB administration. One blood sample was taken via cardiac puncture to measure E_2 and P concentrations just before perfusion. Brain sections of all females were processed for PR immunocytochemistry.

Cannulation

To obtain stress-free blood samples, the right jugular vein of all females was cannulated according to the method of Steffens (242). Females were anaesthetized by an intraperitoneal (ip.) injection with a Ketamine/Rompun® mixture, i.e. 60 mg Ketamine (Kombivet, Etten-Leur, The Netherlands) and 3.3 mg Rompun® (Bayer, Leverkusen, Germany) per kg bodyweight, followed by a sc. injection with an antibiotic (Duplocilline®, Mycofarm, De Bilt, The Netherlands) after 5 minutes. The cannula was filled with polyvinyl pyrrolidon solution (PVP-25, Merck, Darmstadt, Germany; 505 w/w, 500 IU/ml heparin in saline). In order to prevent cannulae from clotting, each cannula was flushed with heparinized saline solution twice a week, where after it was filled again with PVP solution (242).

Blood samples

Blood samples were collected in heparinized, air-dried vials (25 IU heparin, Leo Pharma BV, Breda, The Netherlands) and kept on ice until centrifugation at 13,000

rpm for 5 minutes. For E_2 measurements, 100 µl pure plasma was collected into glass vials and stored frozen at -20°C until further processing. For P measurements, 20 µl plasma was 1:20 diluted in phosphate buffered saline (PBS; pH 7.0) containing 0.1% bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO, USA) in polystyrene vials, where after the diluted plasma was stored frozen at -20°C until further processing.

Ovariectomy and steroid injections

All females were bilaterally ovariectomized under Ketamine/Rompun® anesthesia (60 mg Ketamine/kg and 3.3 mg Rompun®/kg bodyweight; ip. injected). Visagel (Eurovet, Bladel, The Netherlands) was applied to the rat eyes to prevent them from dehydration. Animals were ovariectomized via one incision in the abdominal cavity. On day 13 after ovariectomy, each female was s.c. injected with 6.25 µg EB in 0.2 ml cottonseed oil at ZT 3. On day 14 after OVX, six females per group received a sc. P injection (0.5 mg P in 0.2 ml cottonseed oil) at ZT 6.5. Plasma E_2 and P levels were determined as described below.

Radio immuno assays (RIA's)

E_2 and P levels were determined by validated RIA's routinely used in our department. E_2 plasma levels were determined by extraction using dichloromethane and were expressed in terms of pg/ml estradiol (E-1132; Sigma Chemical, St. Louis, MO, USA). The assay sensitivity was 5 pg/ml for E_2 at 90% of the maximal binding. The intra- and inter-assay variation coefficient were determined using pooled rat serum and amounted to 10.3% for the intra-assay and to 12.5% for the inter-assay variation in the E_2 assays at 50% of the maximal binding (263).

P plasma levels were determined using a single-antibody RIA as previously described (262). A specific rabbit antiserum against 4-pregnene-6β-ol-3,20-dione-hemisuccunate-BSA was used in a dilution of 1:15,000 with PBS containing 0.1% BSA. Progesterone (P-9776, Sigma Chemical, St. Louis, MO, USA) was used as a reference standard and [1,2,6,7-^{3}H]Progesterone (TRK 413, specific activity 87.0 Ci/mmol, Amersham, UK) was used as the tracer. P levels were determined in 14 separate assays. The assay sensitivity was 2 ng/ml for P at 90% of the maximal binding. The intra- and inter-assay coefficients of variation were determined using pooled rat serum and amounted to 15.2% and 12.0% respectively at 50% of the

maximal binding.

Perfusion and sectioning of the brain material

Experiment 1. Each animal was deeply anesthetized by an intravenous (iv.) or s.c. (in case the cannula was blocked) injection with an overdose of Nembutal® (CEVA sante animale B.V., Maassluis, The Netherlands; 60 mg/ml; 1.5 to 3 times bodyweight (kg) in ml). A blood sample was taken via cardiac puncture to measure E_2 and P concentrations (last blood sample of hormone profile). Thereafter, each animal was transcardially perfused with 250-350 ml 0.9% NaCl solution followed by 200-300 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, brains were removed from the scull and postfixed for 25 h in the same fixative. Brains were stored at 4°C in Tris-buffered saline (TBS; pH 7.4) containing 0.1% sodiumazide until further processing. Each brain was embedded in 20% gelatin in TBS and postfixed for 4-6 h (91). Subsequently, 40 μm thick brain sections were cut on a vibratome (1000 classic, Vibratome Company, St. Louis, MO, USA) from the coronal level containing the medial septum and the diagonal band of Broca to the posterior hypothalamus. Sections were stored in TBS containing 0.1% sodiumazide at 4°C until immunocytochemistry.

Experiment 2. Protocols for perfusion of the animals and sectioning of the brain material are as described for experiment 1 (see above), except that in the second experiment the hypothalamic sections were stored at 4°C in cryoprotectant buffer (500 ml 0.1 M PBS, 300 g sucrose, 5 g polyvinyl-pyrrolidon (MW 40,000; PVP-40), 300 ml ethylene glycol per liter) (276). One month before staining, the sections were placed in TBS containing 0.1% sodiumazide.

Immunocytochemistry

Every third section was used for PR immunocytochemistry. Briefly, free-floating sections were incubated for 20 minutes in TBS containing 0.1% sodiumborohydrate, washed in TBS several times and incubated in TBS containing 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity (243). Thereafter, sections were washed three times in TBS and incubated in the first antibody, a rabbit polyclonal antibody directed against the DNA binding domain of the human PR (dilution 1:500-600; A0098; DAKO Inc., Glostrup, Denmark), which detects both the A and B isoforms of PR (253). Sections were incubated for 4-6 h at room temperature, followed by four nights at 4°C. Then, sections were

washed and incubated in biotinylated Goat-anti-Rabbit (1:400; Vector Laboratories, Inc., Burlingame, CA, USA) for 90 minutes at room temperature. Sections were washed thoroughly with TBS again and incubated in Vector Elite Avidin-Biotin-peroxidase complex (1:1200; Vector Laboratories, Inc., Burlingame, CA, USA) for 2 h at room temperature. After washing in TBS, immunoreactivity was visualized by 0.05% 3,3'-diaminobenzidine (DAB) in a TBS solution containing 0.02% nickelammoniumsulfate and H_2O_2 (30%; 3 µl in 10 ml) for 20 minutes. Primary and secondary antibodies were diluted in TBS solution containing 0.3% Triton X-100 and 0.25% gelatin. Sections were mounted on glycerin albumin-coated slides, air dried, dehydrated, and coverslipped using Depex mounting medium.

Image analysis

Images of the right side of the brain containing the AvPv (standardized to 3 images per rat brain) and MPO (standardized to 5 images per rat brain) were captured using a microscope (10 × 10 magnification) with a digital black-and-white CCD camera (Sony, XC-77CE) coupled to a PC. The eight consecutive sections (80 µm apart) containing the AvPv and MPO were selected using several criteria, including the shape of the third ventricle and the optic chiasm and the staining pattern. The second section of the AvPv and the third section of the MPO corresponded to levels of the rat brain atlas of Swanson (level 18 and 20 respectively) (245). The size of the captured images was 530 x 795 µm (horizontal x vertical). By capturing images immediately adjacent to the third ventricle, just above the optic chiasm or optic nerves, we captured the entire AvPv. In case of the MPO, a selection of the total staining area containing the medial preoptic nucleus (MPN) completely, but not the more lateral located nuclei, was captured.

Thereafter, images were analyzed using an image analysis program (Scion Image Beta, version 4.02; Scion Corporation, Maryland, USA) using gray level threshold discrimination as previously described (14). To be able to compare PR data of multiple stainings, each section was captured and analyzed with standardized procedures. This method was checked for reproducibility by comparing the four staining runs of experiment 2. These resulted in a comparable number of PR-IR positive nuclei in each experimental group. A background correction was made by capturing each image of the POA at the same gray level (i.e. the average gray level of the entire image). After capturing all the images, the mean background was calculated for each staining procedure by measuring the

gray level in an area that did not contain PR-positive nuclei in a representative selection of the images (10-40%). Using these background measurements the gray level threshold was determined: the mean maximal gray level in background + 3 x mean S.D. of the background was used as the threshold, which theoretically results in a $p<0.001$ to include false positive nuclei in the counting procedure. Using this threshold, the image was converted in a binary image in which gray levels above threshold were colored red (i.e. the positive staining), and particles smaller than a minimal number of pixels (n = 3) were automatically excluded. Artifacts that were colored red were also excluded by hand. Finally, individual PR-positive nuclei were counted automatically. Using this method, only dark stained PR cells were counted, which corresponds to cells with a high concentration/density of PR (18). Cells with a lower concentration of nuclear PR from which the staining intensity did not reach threshold levels were not included in our counting result. Only one third of the brain sections was stained for PR and only the right side of the brain was analyzed. Since we found no significant differences in total number of PR-positive cells between the left and right side of the brain (van der Beek and Franke, unpublished data), we calculated the total number of PR-positive cells per brain area by multiplying the counted nuclei by six.

Statistics

Data were analyzed using SPSS 10.1 and expressed as mean ± SEM. In experiment 1, the effect of age on hormone profiles within a rat strain was determined by LN transformation of the E_2 data and using a general linear model (GLM) for repeated measurements. For P data an ANOVA was used per experimental group (age and strain), because an interaction between age and estrous cycle day was present. Post Hoc tests were performed (Bonferroni, Dunnett T3) for E_2 and P data to test for significant differences between estrous cycle days. An independent-samples t-test was used to determine the time points during the estrous cycle with a significant difference in E_2 and P concentrations with age (F1). In addition, the average hormone concentration per estrous cycle was calculated, LN transformed, and tested for age and strain differences using a GLM – univariate test. A one-way ANOVA per rat strain was used to test if the age-related difference was significant for F1 and/or WU rats. To determine the effect of estrous cycle day and age on the number of PR-positive cells in the AvPv and MPO for each rat strain, data were LN transformed. In case of the WU strain, data

were analyzed per age, since an interaction between age and estrous cycle day was present. Age differences were further analyzed by comparing PR data between groups on each estrous cycle day with ANOVA. To test PR-IR cell numbers in the AvPv and MPO of F1 rats, and to test for strain differences on metestrus-morning and proestrus-afternoon, GLM – multivariate tests were performed.

In experiment 2, a total of nine females were excluded from data analysis, seven were outliers ('extremes' as revealed by boxplot analyses), and two were excluded because extreme high PR numbers in addition to low ER numbers (ER) at 2 or 8 h after EB administration suggested that these females were incompletely ovariectomized. We checked if E_2 concentrations at different perfusion times were comparable between groups using a GLM-univariate test, after square root transformation. The effect of EB-time and age on PR data of OVX-EB treated females (AvPv and MPO; analyzed per rat strain) as well as the existence of a strain difference in PR-IR numbers were investigated using GLM – multivariate tests. Post-hoc tests were performed for EB-time (Dunnett T3). The effect of a P injection on the number of PR-positive cells in the AvPv and MPO was tested by comparing PR data of females perfused 32 h after EB injection with or without P treatment (GLM – multivariate test). For all analyses, $p<0.05$ was considered to be significant.

Results

Experiment 1: cyclic female rats

Plasma E_2 levels changed significantly over the estrous cycle: E_2 concentrations were low during metestrus, gradually increased during diestrus, reached peak levels on proestrus, and were low again on estrus (Figure 1). E_2 levels appeared to be higher at diestrus I and proestrus in 9- compared to 4.5-month-old females of both rat strains. This difference was significant in 5-day cyclic F1 rats ($p=0.033$; t-test revealed a trend on the morning of diestrus-I ($p=0.060$) and proestrus ($p=0.087$)), but not in 4-day cyclic WU rats. The average E_2 concentrations during the estrous cycle amounted to 15.4 ± 0.8 pg/ml (young) and 20.2 ± 2.6 pg/ml (middle-aged) in 5-day cyclic F1 rats and to 15.1 ± 1.1 pg/ml (young) and 16.7 ± 0.9 pg/ml (middle-aged) in 4-day cyclic WU females. No significant differences between rat strains were found, but the average E_2 concentration was significantly higher in middle-aged compared to young F1 females ($p=0.039$; Figure 1, insets).

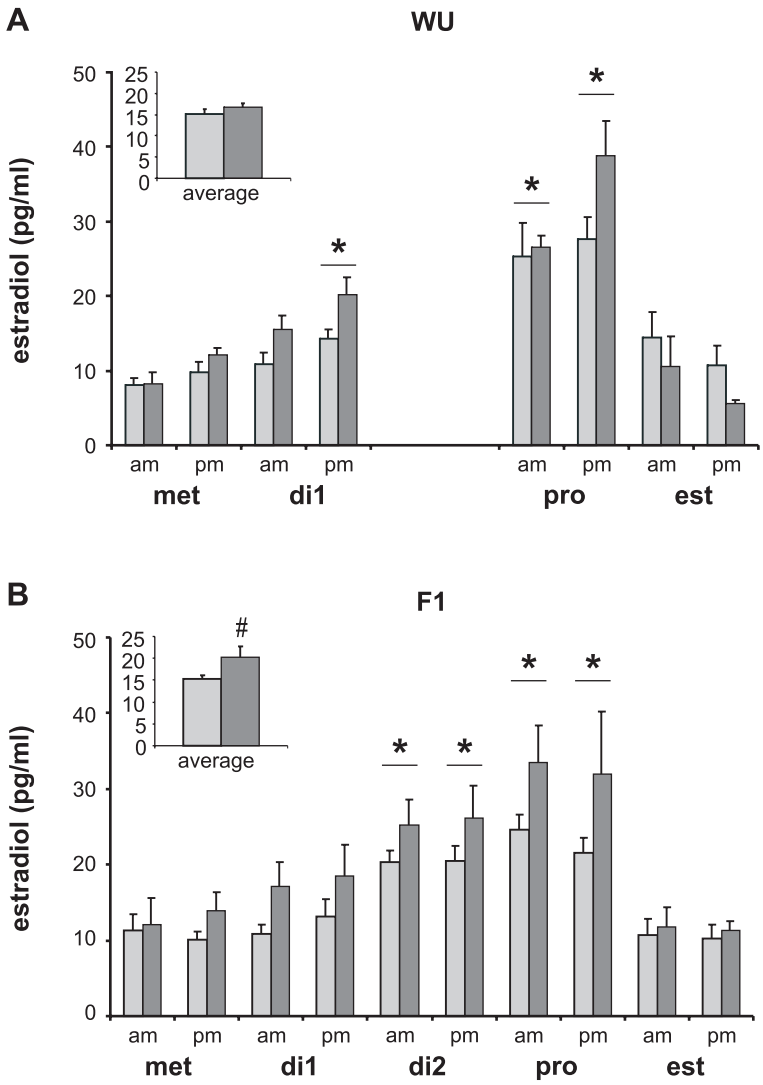

Figure 1. Plasma estradiol levels (pg/ml) during the estrous cycle of 4-day cyclic WU (panel A) and 5-day cyclic F1 (panel B) rats at 4.5 (light gray bars; n=7 WU, n=19 F1) and 9 (dark gray bars; n=7 WU, n=7 F1) months old. Bars represent mean ± SEM. est, estrus; met, metestrus; di1, diestrus I; di2, diestrus II; pro, proestrus; am, ZT 5; pm, ZT 11; *, significantly different from est-am; #, significantly different from the age of 4.5 months ($p<0.05$). Insert: the average estradiol concentration (pg/ml) during the estrous cycle in young and middle-aged WU (panel A) and F1 (panel B) females.

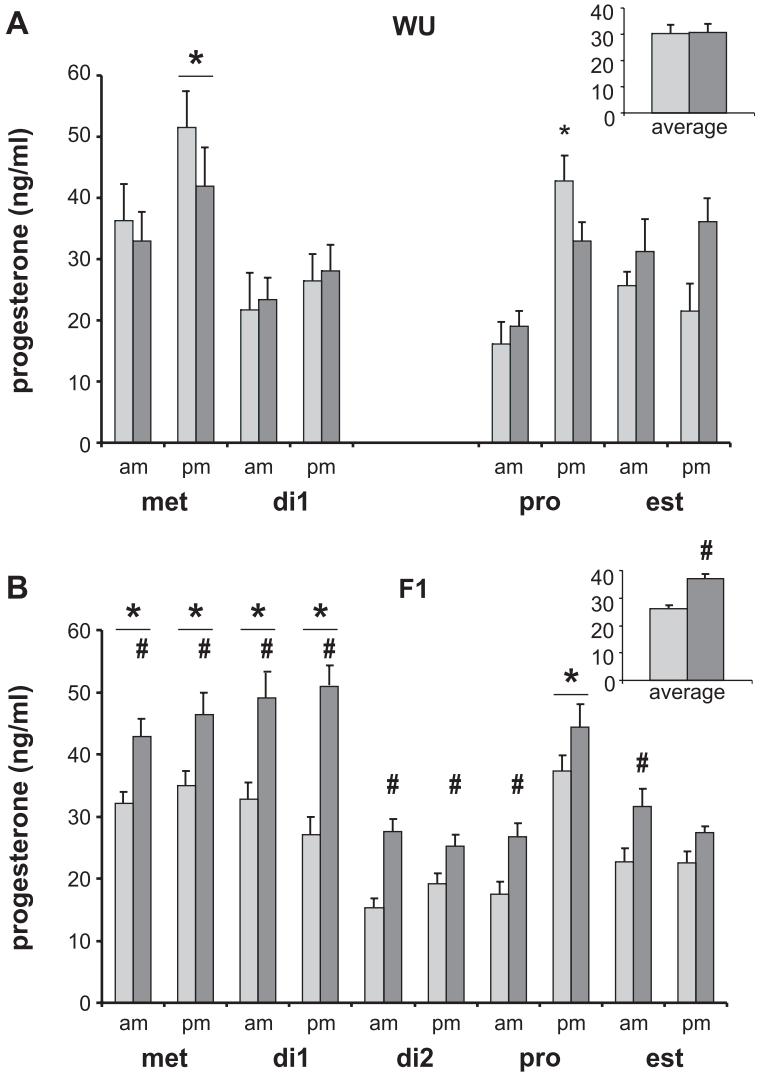

Figure 2. Plasma progesterone levels (ng/ml) during the estrous cycle of 4-day cyclic WU (panel A) and 5-day cyclic F1 (panel B) rats at 4.5 (light gray bars; n=5 WU, n=23 F1) and 9 (dark gray bars; n=7 WU, n=8 F1) months old. Bars represent mean ± SEM. est, estrus; met, metestrus; di1, diestrus I; di2, diestrus II; pro, proestrus; am, ZT 5; pm, ZT 11; #, significantly different from the age of 4.5 months ($p<0.05$). Insert: the average progesterone concentration (ng/ml) during the estrous cycle in young and middle-aged WU (panel A) and F1 (panel B) females.

Plasma P levels changed significantly over the estrous cycle: P concentrations were high on metestrus, were lower on diestrus (diestrus-I morning in 4-day cyclic WU and diestrus II morning in 5-day cyclic F1 rats), higher on proestrus afternoon and lower again on estrus (Figure 2). In F1 rats, P levels were significantly higher in 9-month-old compared to 4.5-month-old females ($p<0.001$) on every time point measured, except on proestrus-pm and estrus-pm. The average P level during the estrous cycle was increased with age in 9-month-old F1 rats ($p=0.002$). In the WU strain there were no significant changes in P levels with age.

Cell nuclei of PR-IR positive cells were densely stained, although a variation in staining intensity between nuclei existed, and no cytoplasmic staining was detected. The distribution of PRs in the AvPv and MPO was comparable to that previously described for PR protein (18,273) and PR mRNA (25,231,289). The number of PR-IR positive cells in the POA was higher on proestrus than on metestrus (Figure 3). This increase was significant for the AvPv and MPO in young and middle-aged F1 rats ($p=0.001$ and $p=0.007$, respectively) and in middle-aged WU rats ($p=0.003$ and $p=0.001$, respectively). We found no significant increase in PR over the estrous cycle in young WU rats, probably due to the large variation found on metestrus, although the difference between metestrus and proestrus was significant in a previous study in our lab (van der Beek, unpublished data). In F1 rats, the number of PR-IR positive cells at metestrus was significantly lower than the number on diestrus II in both the AvPv and MPO ($p=0.002$ and $p=0.043$, respectively), while diestrus II and proestrus numbers were comparable. We found no significant differences in the number of PR-IR positive cells of the AvPv and MPO on metestrus-morning and proestrus-afternoon between rat strains. The number of PR-IR positive cells was decreased with age in both rat strains: PR numbers were significantly lower in 9-month- compared to 4.5-month-old females in the AvPv and MPO of WU ($p<0.001$ and $p=0.003$, respectively) and F1 rats (both $p<0.001$; Figure 3). These age-related differences were significant for both AvPv and MPO on metestrus in WU females ($p=0.003$ and $p=0.015$, respectively) and on diestrus II ($p<0.001$ and $p=0.003$, respectively), and proestrus ($p=0.022$ and $p=0.021$, respectively) in F1 females (Figure 3).

Experiment 2: ovariectomized EB-treated female rats

E_2 levels in the OVX+EB-treated females were comparable between all groups (rat strains, ages). At 2 and 8 h after EB injection the average plasma E_2 concen-

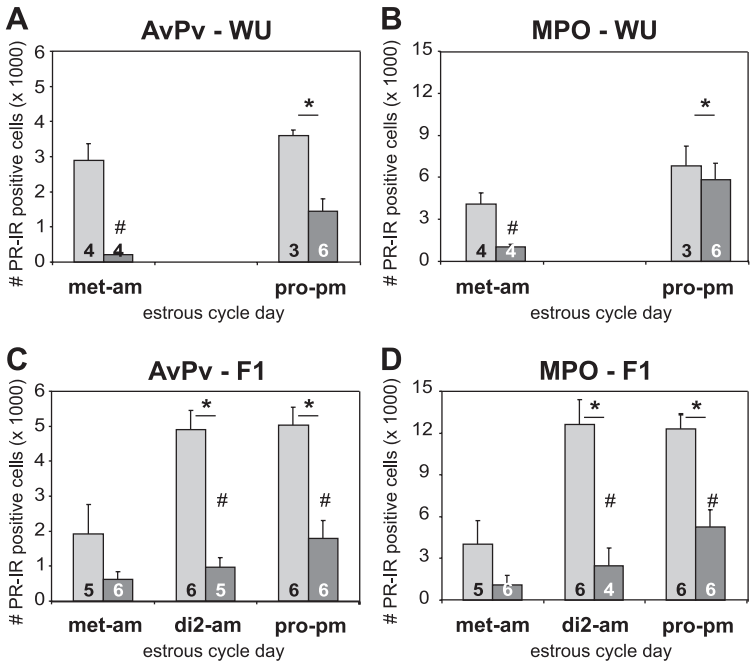

Figure 3. The number of PR-IR positive cells during the estrous cycle in the AvPv (panels A and C) and MPO (panels B and D) of 4-day cyclic WU (panels A and B) and 5-day cyclic F1 rats (panels C and D) at the age of 4.5 (light gray bars) and 9 (dark gray bars) months. Bars represent mean ± SEM. The number of animals per group is depicted at the bottom of the column. met-am, perfused on metestrus at ZT 5; di2-am, perfused on diestrus II at ZT 5; pro-pm, perfused on proestrus at ZT 11. *, significantly different from met-am; #, significantly different from the age of 4.5 months ($p<0.05$).

tration was 78 ± 7 pg/ml and 73 ± 5 pg/ml, respectively. At 26 h after EB injection, E_2 levels were significantly lower (45 ± 4 pg/ml) and at 32 h after EB injection E_2 levels were further decreased to 34 ± 2 pg/ml. Rats perfused at 32 h after EB injection which also received an injection of P at 4.5 h before perfusion, showed E_2 levels (43 ± 3 pg/ml) that were not significantly different from those in rats not receiving P that were perfused 26 or 32 h after EB injection. E_2 levels in the additional group of young WU females included in the second part of the experiment perfused 2 and 32 h after EB injection were comparable to the

corresponding groups in the first part of the experiment.

P levels in OVX+EB-treated females which received a P injection or not and were perfused 32 h after EB injection were comparable (Nonparametric Kruskal-Wallis test; data not shown).

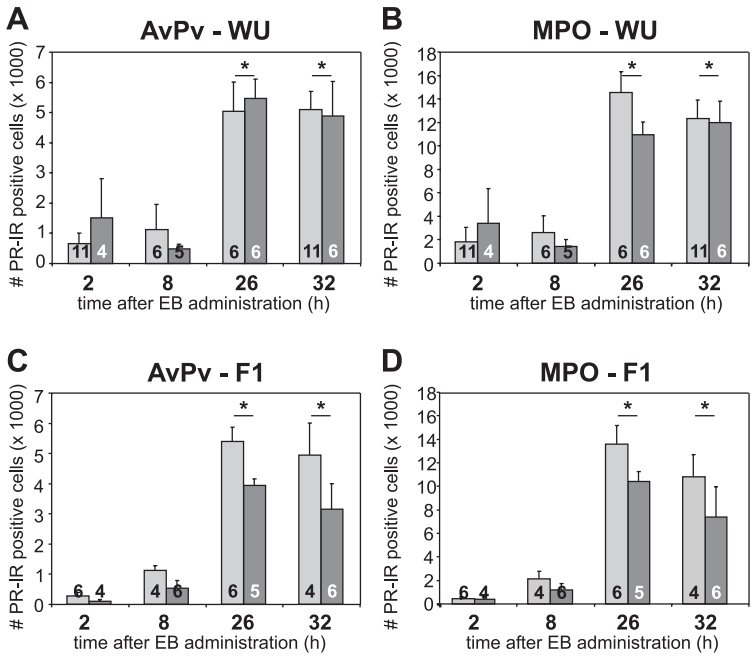

Figure 4. The number of PR-IR positive cells in the AvPv (panels A and C) and MPO (panels B and D) of OVX+EB-treated WU (panels A and B) and F1 rats (panels C and D) at the age of 4.5 (light gray bars) and 9 (dark gray bars) months old. Bars represent mean ± SEM. The number of animals per group is depicted at the bottom of the columns. *, significantly different from 2 h after EB. A significant age-related decrease in the number of PR-IR positive cells was found in the AvPv and MPO of F1 rats ($p<0.05$).

The POA of OVX females with short-term exposure to EB (2 h and 8 h) contained very few PR-IR positive cells in F1 as well as WU rats (Figure 4). At 26 and 32 h after EB injection, the number of PR-IR positive cells was significantly increased in the AvPv and MPO of both rat strains. No differences in PR numbers were present between rat strains. Statistical tests per rat strain revealed a

decrease in PR numbers with age in both the AvPv (p=0.005) and MPO (p=0.037) of F1 rats, but not in WU rats. PR numbers were comparable in OVX+EB-treated rats with or without an additional P injection (data not shown).

Discussion

To our knowledge, this is the first study in which a clear decrease in the number of PR protein containing cells in the POA with age has been demonstrated. In addition, the present study clearly shows that the number of PR-IR positive cells in the POA is decreased in middle-aged rats without concurrent changes (WU), or paralleled by an increase (F1) in E_2 and P hormone concentrations over the estrous cycle. Since the decrease in PR-IR positive cells between young and middle-aged cyclic females was consistent between the two rat strains, we believe that it may represent a general feature in reproductive aging.

We found that E_2 and P hormone profiles of regular cyclic rats are unchanged (4-day cyclic WU rats) or increased (5-day cyclic F1 rats) in 9-month-old females compared to 4.5-month-old females. The increase in E_2 with age as found in our F1 rats is in agreement with a previous study (181), and suggests alterations in follicular development and/or steroid secretion in these females. Since these hormonal changes were only observed in F1 rats, this supports the hypothesis that F1 rats may be more reproductively aged at 9 months than WU rats. However, the marked increase in circulating P levels with age in our cyclic F1 females, which is a novel finding, also suggests alterations in steroid secretion and/or corpus luteum formation and activity. Since it was only seen in F1 and not in Wistar rats, this increase in P might be strain specific, thereby supporting the notion that marked differences can exist between rat strains, and even between individuals of the same strain at various levels in the regulation of the estrous cycle (67). Alternatively, the age-related increase in P may previously have gone undetected, because it may only occur for a relatively brief period in life. Indeed, most studies so far have focused on a more advanced stage of reproductive aging, i.e. (just before) persistent estrus. We found no differences between rat strains in E_2 and P exposure during the estrous cycle when expressed as average concentration of hormone per day (Figure 1 and 2) or as E_2/P ratio (data not shown). The duration

of elevated E_2 concentrations during the cycle, however, was longer in F1 rats (~2 days) than in WU rats (~1.5 days). Thus, the difference in rate of reproductive aging between WU and F1 rats (270) can not simply be explained by exposure to different E_2 and/or P concentrations per se, but might be the result of differences in the duration of exposure to elevated E_2 concentrations.

We found that the number of PR-IR positive cells in the AvPv and MPO changed significantly over the estrous cycle irrespective of the age of the animal: PR numbers were low on metestrus and consistently elevated on proestrus. This pattern was comparable to that found in WU rats in a previous study in our lab (van der Beek *et al*., unpublished data). Interestingly, cyclic rats of both strains showed a significant decrease in the number of PR-IR positive cells in the AvPv and MPO with age. Given the unchanged hormone levels in 9-month-old WU females, these data suggest that the reduction in number of PR-containing cells with age might be the result of a change in hypothalamic responsiveness to estrogen at middle age.

In our study, we measured PR protein positive cells based on staining intensity, and not PR mRNA, and only cells with a concentration of PR protein 'above threshold' were counted. This approach may explain differences in results between our study and previous reports by others in which no clear changes in PR were reported with age (25,77,93,231). Furthermore, the antibody we used does not discriminate between (un)occupied or functional forms of the receptor, or between PR-A or PR-B isoforms. PR-A and PR-B isoforms may have different functions (40) and in the POA only PR-B appears to respond to E_2 and P after ovariectomy (31). It is possible, for instance, that the amount and/or ratio of PR-A to PR-B changes during the aging process, and this could influence hypothalamic responsiveness to estrogen.

The age-related decrease in PR protein found in the present study accords with a recent study in which a decrease in PR mRNA was found in the AvPv, though not in the MPO, on proestrus in 10-13 month-old Long-Evans females (172). In contrast to our study, these Long-Evans rats were in early persistent estrus (acyclic for 30 days), a state in which the female appears to be unresponsive to the positive feedback of E_2. Since we also found a decrease in PR protein in WU rats, we can now conclude that during reproductive aging the number of hypothalamic PR decreases before any changes in steroid hormone concentrations or steroid responsiveness are apparent.

The reduced number of PR-IR positive cells in middle-aged female rats could

be responsible for a change in the GnRH surge and subsequently the LH surge. The importance of PR for the occurrence of the LH surge is illustrated by the fact that injection of a PR antagonist prevents the increase in GnRH pulse amplitude, and the occurrence of GnRH- and LH surges in OVX+EB-treated rats (35), and by the fact that PR knockout mice display no E_2 induced LH surge (36). A mechanism was postulated in which PRs play a crucial role in the induction of the preovulatory LH surge (151). It was hypothesized that rising E_2 levels during the estrous cycle induce an increase in PR concentration in the AvPv. A daily neural signal from the SCN activates these PRs, which eventually leads to the initiation of the GnRH and subsequently the LH surge. Our data as well as a previous report (172) suggest that the ability of estrogen to induce hypothalamic PRs diminishes with age. When the number of cells in the AvPv that contain PRs is thus reduced with age, the SCN signal may activate less cells (144), and this may then result in a diminished stimulation and activation of GnRH neurons (144,213), an attenuated GnRH surge and consequently an attenuated LH surge. Indeed, our regularly cyclic WU and F1 rats already display an attenuated LH surge on proestrus at 9 months of age (67). In addition, we recently found that the concentration of ERα, which is involved in the E_2-induced increase in PRs, is also decreased with age in regularly cyclic 9-month-old WU and F1 rats (69).

Besides at the level of the hypothalamus, the PR also plays an important role at the level of the pituitary gland. The expression of PRs in the pituitary gland is indispensable for the GnRH priming mechanism (274), i.e. the mechanism by which repeated stimulation with the same amount of GnRH results in increased LH release. Based on the significant decrease in PR-IR positive cells in the POA with age, it is feasible to suggest a similar age-related decrease in PR protein in the pituitary gland. Consequently, the GnRH priming mechanism may be impaired at the level of the pituitary. Indeed, a previous study in our lab showed that the GnRH priming mechanism is already impaired in 9-month-old WU rats (117). In addition, the amount of LH released per burst is decreased during the LH surge in 10-12 months old Long-Evans females (161). Altogether, the evidence suggests that the age-related attenuation of the LH surge is the result of a deficient GnRH drive and/or a reduced responsivity of the pituitary gland to GnRH, possibly as a result of a decrease in hypothalamic and pituitary PR protein concentrations.

Although a decreased PR number with age was found in the hypothalamus of cyclic WU and F1 rats, only OVX+EB F1 females showed a decrease in PR-IR

numbers. This further supports our idea that F1 rats may be more reproductively aged at 9 months of age than WU rats. Yet, the magnitude of the decrease in PR numbers with age was much smaller in OVX+EB compared to cyclic F1 rats. This difference may result from differences in (previous) ovarian hormone exposure. Cyclic females are not only exposed to E_2 but also to P and other hormones of ovarian origin. P normally down regulates its own receptor, and may therefore counteract the induction of PRs by E_2 in cyclic compared to OVX+EB-treated females. Furthermore, rats were ovariectomized 13 days before EB administration, and had thus been deprived of steroids for 13 days, which was reflected by the paucity of PRs in the POA at 2 and 8 h after EB administration. As a result, the sensitivity of the hypothalamus to E_2 feedback could have been increased, leading to maximal PR induction after EB exposure. Taken together, the data from OVX+EB rats at least support the notion that hypothalamic responsiveness to E_2 is decreased with age.

In conclusion, we demonstrated that during early reproductive aging, i.e. in regular cyclic rats, the number of PRs in the preoptic area decreases with age. The fact that we were able to demonstrate this in cyclic females of two rat strains as well as in OVX+EB-treated females, suggests that the decrease in preoptic area PRs with age is not related to differences in E_2 concentrations between strains or ages. Therefore, we hypothesize that during early reproductive aging the induction of PR by E_2 is impaired in the AvPv and MPO, and that this results in an attenuated LH surge and subsequently to a reduced fertility.

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Chapter 4

Early reproductive aging is associated with a decrease in ERα protein containing cells in the preoptic area

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(to be submitted)

Abstract

For the induction of the luteinizing hormone (LH) surge, estradiol (E_2) feedback on the medial preoptic nucleus (MPO) and anteroventral periventricular nucleus (AvPv) is important. Since the LH surge attenuates with age, the aim of the present study was to investigate the role of life-time E_2 exposure and E_2 feedback on estrogen receptor-α (ERα) concentrations in the AvPv and MPO of rats during reproductive aging.

Using 4.5- (young) and 9-month-old (middle-aged) Wistar and (UxRP)F1 rats that displayed respectively 4- or 5-day estrous cycles, the relation between E_2 and progesterone (P) levels and ERα-immunoreactive (IR) cell numbers was investigated in cyclic females, while the sensitivity of ERα to E_2 and P feedback was studied in ovariectomized (OVX) females. Plasma E_2 and P levels as well as ERα-IR cell numbers in the AvPv and MPO significantly changed over the estrous cycle. There were no difference in E_2 exposure between ages or strains, although E_2 and P levels were significantly increased with age in (UxRP)F1 rats. ERα concentrations decreased dramatically with age in the AvPv and MPO of both strains. In OVX rats, the administration of estradiol benzoate resulted in a downregulation of ERα between 2 and 32 h, and appeared to decrease with age at 32 h in (UxRP)F1 rats. P decreased the number of ERα-IR cells in the MPO of 9-month-old Wistar rats.

In conclusion, since E_2 exposure was comparable between strains and ages, the dramatic decrease in ERα protein as observed in the AvPv and MPO between 4.5- and 9-month-old rats probably results from a change in sensitivity of ERα to E_2 and/or P feedback with age. Since the induction of PRs, a key event in the generation of LH surges, involves ERα, a decrease in ERα may eventually result in an attenuated LH surge with age.

Introduction

In female mammals, the sequence of events leading to preovulatory GnRH and LH surges and ovulation is highly coordinated and involves several brain areas that express the estrogen receptor (ER). Two forms of the estrogen receptor have been described in mammals, ERα and ERβ, and these forms display different expression

patterns and functions throughout the body (168,230). While both ERα and ERβ are downregulated by estrogen in some, but not all brain areas (89,201,299), it is thought that only ERα induces the expression of the hypothalamic progesterone receptor (PR) (18,89). Knockout studies in mice (133,188) have suggested that ERα plays a key role in the regulation of female reproductive physiology (168). Interestingly, GnRH neurons express ERβ mRNA (100) and ERβ protein, but not ERα (106).

For the induction of the LH surge during a reproductive cycle, the feedback effects of estradiol (E_2) on the medial preoptic nucleus (MPO), and in particular on the anteroventral periventricular nucleus (AvPv), are of crucial importance. Both areas express the ERα and ERβ (237) and the progesterone receptor (PR) (25). The AvPv projects to the MPO, an area rich in GnRH neuronal cell bodies, and to the arcuate nucleus (ARC)-median eminence (ME) complex and the organum vasculosum of the lamina terminalis (OVLT), both containing many GnRH terminals (92). Within the preoptic area (POA; includes the AvPv and MPO), there appears to be a regulatory feedback loop: ERα-containing cells located in the AvPv and MPO strongly innervate GnRH neurons (235), while GnRH neurons have been shown to innervate ER-containing cells of the AvPv and MPO (137). Furthermore, neurons within the AvPv that are activated during the LH surge appear to project directly to GnRH neurons (143).

Fundamental to the generation of an LH surge is the secretion of increasing amounts of E_2 by the growing ovarian follicles. The resulting rise in circulating plasma E_2 concentrations is thought to induce the expression of PRs in the AvPv via actions involving ERα (151). Furthermore, a daily neural signal from the suprachiasmatic nucleus (SCN) activates the PR-containing AvPv neurons through a ligand-independent mechanism (i.e. via cAMP). This activation of PRs stimulates the synthesis and/or release of neurotransmitters acting on GnRH neurons, which eventually results in the initiation of GnRH release. This so-called GnRH surge leads to the LH surge and finally ovulation.

Fertility decreases with age in most (if not all) mammals including women (247) and rats (163,270). One of the first signs of reproductive aging in the female rat is an attenuation of the LH surge (67,161,181,285). In the rat, attenuated LH surges can be demonstrated even before cycles become prolonged, and are, in analogy with those in humans, associated with a reduced probability of conception (9,269). During an attenuated LH surge, the number of GnRH neurons (144,153,212) and

AvPv neurons (144) that express Fos are reduced. Preovulatory progesterone (P) levels enhance ongoing LH secretion by increasing Fos activation of GnRH neurons (146) and pituitary gonadotrophs (61). Proestrous levels of P (170,284) as well as E_2 (284) have been shown to change with advancing age. We recently showed that the number of PR containing cells of the AvPv and MPO is decreased in 9- compared to 4.5-month-old female rats (70). Since estrogen feedback on hypothalamic PRs plays a crucial role in the generation of the LH surge and only ERα appears to be capable to induce expression of the hypothalamic PR, we hypothesized that an altered feedback of E_2 on ERα in the AvPv and MPO may underlie the decrease in PR expression and the attenuation of the LH surge with age.

Although E_2 plays a crucial role in the regulation of the reproductive cycle, perinatal E_2 exposure (197,207) as well as cumulative E_2 exposure during adulthood (254) results in advanced reproductive aging. It is thought that exposure to high E_2 levels damages the GnRH releasing mechanism (97), and that the onset of reproductive senescence relates to the dose and duration of E_2 exposure. Based on these findings, we hypothesized that differences in reproductive aging between individuals and rat strains (270) may be explained by differences in life-time E_2 exposure.

Therefore, the aim of the present study was to investigate (I) if the feedback of E_2 on the number of ERα expressing cells in the POA changes with age, and if so, (II) whether the extent of the change in ERα numbers with age is related to life-time cumulative E_2 exposure. To this end we performed an experiment in 4.5 (young) and 9 (middle-aged) month-old female rats of two rat strains, i.e. the Wistar (HsdCpb:WU) and the (UxRP)F1 strain. Wistar females predominantly display 4-day estrous cycles while (UxRP)F1 females predominantly display 5-day estrous cycles. The ‘extra’ cycle day in 5-day cyclic (UxRP)F1 females is due to a lengthening of late diestrus, resulting in two days with elevated E_2 levels instead of one. Indeed, a decline in fertility (at age 7-9 months) (163) and a cessation of reproductive cycles (at age 9-12 months) (270) have been demonstrated at a relatively early age in these (UxRP)F1 females. Thus, by comparing the feedback effects of E_2 on its receptor in these two rat strains, we expected to gain more insight into the possible mechanisms underlying reproductive aging.

Materials and Methods

Animals

Virgin female F1 rats, a locally bred hybrid of two Wistar substrains (RP-inbred albino females and U-inbred brown hooded males, abbreviated as F1) (163), were obtained from the university animal care facility at 9-10 weeks of age. Virgin female and male Wistar rats (HsdCpb:WU, Wistar Unilever; abbreviated as WU) were obtained from Harlan (Horst; The Netherlands) at 9 weeks of age (females) or at 12 weeks of age (males). Rats were housed five per cage under regular light-dark conditions (L/D 12:12, experiment 1: lights on at 5:00 h ('zeitgeber time 0' or ZT 0), and experiment 2: lights on at 3:00 h (ZT 0)), and had free access to standard pelleted food (Hope Farms B.V., Woerden, The Netherlands) and tap water.

Estrous cyclicity was monitored by daily vaginal lavage from one week after arrival until the end of the experiment or until ovariectomy (young females; 4.5 months old at the end of the experiment; n=70 for the F1 strain and n=62 for the WU strain), or from the age of 7.5 months until the end of the experiment or the moment of ovariectomy (middle-aged females; 9 months old at the end of the experiment; n=70 for each rat strain). In addition to vaginal lavage, receptive behavior was monitored daily. To this end, a naive male WU rat was introduced to the female rat's home cage one hour before lights off. Display of hopping and darting, ear wiggling, and lordosis posture by the female was checked. One week before cannulation, each female was housed individually until the end of the experiment. The experiment was approved by the animal experimental committee of Wageningen University.

Experimental design

The experimental setup was exactly the same as previously reported (70), except that in this study the brain slices were immunocytochemically stained for ERα.

Experiment 1: cyclic female rats

To study changes in the feedback of gonadal hormones on hypothalamic estrogen receptors (ERα) between young and middle-aged regular cyclic rats, we measured steroid hormone levels (E_2 and P) over the estrous cycle. In addition, the number of ERα-expressing cells in the hypothalamic POA was measured at specific time points during the estrous cycle.

To this end, young female WU (n=20) and F1 (n=40), and middle-aged female WU (n=40) and F1 (n=40) rats were used. Rats were cannulated at 11 weeks (young), or at 36 weeks (middle-aged) old. After a recovery period of at least 5 days, one blood sample of 300 μL was taken twice a day at 10:00 h (ZT 5) and 16:00 h (ZT 11) during one complete estrous cycle. Only females with a distinct and regular 4-day (WU; n=14) or 5-day (F1; n=32) estrous cycle were included in this experiment. Immediately after the last blood sample was taken, each animal was perfused. Perfusions were performed at specific time points of the estrous cycle: i.e. on metestrus (10:00 h = ZT 5), diestrus II (10:00 h = ZT 5), or proestrus (16:00 h = ZT 11). Each group contained 4 to 6 animals. Brains were collected and 40 μm vibratome sections of the POA were immunocytochemically stained for ERα.

Experiment 2: ovariectomized EB-treated female rats

To study age-related changes in the sensitivity of hypothalamic ERα to E_2 feedback in more detail, we used ovariectomized 4- and 8.5-month-old WU and F1 rats (n = 30 for each group) that were subcutaneous (sc.) injected on day 13 after ovariectomy with a physiological dose estradiol benzoate (EB; 6.25 μg) at ZT 3. In addition, we tested whether P influenced the number of ERα-expressing cells in the POA in the time course normally found to enhance the ongoing LH surge (146,256).

All young and middle-aged female WU and F1 rats were randomly divided over five experimental groups: four groups only received EB and were perfused 2, 8, 26 or 32 h (ZT 5 and ZT 11) after EB administration, and one group exposed to EB for 32 h received an additional P injection 4.5 h before perfusion. Because the experiment was split into two parts, i.e. young and middle-aged females, an additional group of 12 young WU females was included in the second part of the experiment. These females were perfused 2 h (n=6), or 32 h (n=6) after EB administration. One blood sample was taken via cardiac puncture to measure E_2 and P concentrations just before perfusion. Hypothalami of all females were processed for ERα immunocytochemistry.

Cannulation

To obtain stress-free blood samples, the right jugular vein of all females was cannulated according to the method of Steffens (242). Females were

anaesthetized by an intra peritoneal (ip.) injection with a Ketamine/Rompun® mixture, i.e. 60 mg Ketamine (Kombivet, Etten-Leur, The Netherlands) and 3.3 mg Rompun® (Bayer, Leverkusen, Germany) per kg bodyweight, followed by a sc. injection with an antibiotic (Duplocilline®, Mycofarm, De Bilt, The Netherlands) after 5 minutes. The cannula was filled with polyvinyl pyrrolidon solution (PVP-25, Merck, Darmstadt, Germany; 505 w/w, 500 IU/ml heparin in saline). In order to prevent cannulae from clotting, each cannula was flushed with heparinized saline solution twice a week, where after it was filled with fresh PVP solution (242).

Blood samples

Blood samples were collected in heparinized, air-dried vials (25 IU heparin, Leo Pharma BV, Breda, The Netherlands), and kept on ice until centrifugation at 13,000 rpm for 5 minutes. For estradiol measurements, 100 µl pure plasma was collected into glass vials and stored frozen at -20°C until RIA. For P measurements, 20 µl plasma was 1:20 diluted in phosphate buffered saline (PBS; pH 7.0) containing 0.1% bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO, USA) in polystyrene vials, where after the diluted plasma was stored frozen at –20°C until RIA.

Ovariectomy and steroid injections

All females were bilaterally ovariectomized under Ketamine/Rompun® anesthesia (60 mg Ketamine/kg and 3.3 mg Rompun®/kg bodyweight; ip. injected). Visagel (Eurovet, Bladel, The Netherlands) was applied to the rat eyes to prevent them from dehydration. On day 13 after ovariectomy, each female was injected sc. with 6.25 µg EB in 0.2 ml cottonseed oil at ZT 3. On day 14 after OVX, six females per group received a sc. P injection (0.5 mg P in 0.2 ml cottonseed oil) at ZT 6.5. Plasma E_2 and P levels were determined as described above.

Radio immuno assays (RIA)

E_2 and P levels were determined by validated RIA's that are routinely used in our department.

E_2 levels were determined by extraction using dichloromethane and were expressed in terms of pg/ml E_2 (E-1132; Sigma Chemical, St. Louis, MO, USA). The assay sensitivity was 5 pg/ml for E_2 at 90% of the maximal binding. The intra- and inter-assay variation coefficient was determined using pooled rat serum and

amounted to 10.3% for the intra-assay variation and to 12.5% for the inter-assay variation in the E_2 assays at 50% of the maximal binding (263).

P plasma levels were determined using a single-antibody RIA as previously described (262). A specific rabbit antiserum against 4-pregnene-6β-ol-3,20-dione-hemisuccunate-BSA was used in a dilution of 1:15,000 with PBS containing 0.1% BSA. Progesterone (P-9776, Sigma Chemical, St. Louis, MO, USA) was used as a reference standard and [1,2,6,7-^{3}H]Progesterone (TRK 413, specific activity 87.0 Ci/mmol, Amersham, UK) was used as the tracer. P levels were determined in 14 separate assays. Assay sensitivity was 2 ng/ml for P at 90% of the maximal binding. The intra- and inter-assay coefficients of variation were determined using pooled rat serum and amounted to respectively 15.2% and 12.0% at 50% of the maximal binding.

Perfusion and sectioning of the brain material

Experiment 1. Each animal was deeply anesthetized by an intravenous (iv.) or sc. (in case the cannula was blocked) injection with an overdose of Nembutal® (CEVA sante animale BV, Maassluis, The Netherlands; 60 mg/ml; 1.5 to 3 times bodyweight (kg) in ml). A blood sample was taken via cardiac puncture to measure E_2 and P concentrations (last blood sample of hormone profile). Thereafter, each animal was transcardially perfused with 250-350 ml 0.9% NaCl solution followed by 200-300 ml 4% paraformaldehyde (PAF) in 0.1M phosphate buffer (pH 7.4). After perfusion, brains were removed from the scull and postfixed for 25 h in the same fixative. Brains were stored at 4°C in Tris-buffered saline (TBS; pH 7.4) containing 0.1% sodiumazide until further processing. Each brain was embedded in 20% gelatin in TBS and postfixed for 4-6 h (91). Subsequently, 40 μm thick brain sections were cut on a vibratome (1000 classic, Vibratome Company, St. Louis, MO, USA) from the coronal level containing the medial septum and the diagonal band of Broca to the posterior hypothalamus. Sections were stored in TBS containing 0.1% sodiumazide at 4°C until immunocytochemistry.

Experiment 2. Protocols for perfusion of the animals and sectioning of the brain material were as described for experiment 1 (see above), except that in this experiment the hypothalamic sections were stored at 4°C in cryoprotectant buffer (500 ml 0.1 M phosphate buffer, 300 g sucrose, 5 g polyvinyl pyrrolidon (MW 40,000; PVP-40), 300 ml ethylene glycol per liter) (276). One month before staining, the sections were placed in TBS containing 0.1% sodiumazide.

Immunocytochemistry

One third of the sections (every 120 μm) was stained for ERα. In brief, free-floating sections were incubated for 20 minutes in TBS containing 0.1% sodiumborohydrate, washed in TBS several times and incubated in TBS containing 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity (243). Thereafter, sections were washed three times in TBS and incubated in monoclonal anti-ERα antibody (mouse anti-human, M7047, DAKO Inc., Glostrup, Denmark; dilution 1:1000) for 4-6 h at room temperature, followed by four nights at 4°C. Then, sections were washed and incubated in biotinylated Horse-anti-Mouse immunoglobulins (1:250; Vector Laboratories, Inc., Burlingame, CA, USA) for 30 minutes at room temperature followed by one night at 4°C. Sections were washed with TBS thoroughly again and incubated in Vector Elite Avidin-Biotin-peroxidase complex (1:750-1000; Vector Laboratories, Inc., Burlingame, CA, USA) for 2 h at room temperature. After washing in TBS, immunoreactivity was visualized by 3,3'-diaminobenzidine (DAB; 5 μg in 10 ml) in a TBS solution containing nickel (2% nickelammoniumsulfate; 1 ml in 10 ml) and H_2O_2 (30%; 3 μl in 10 ml) for 20-30 minutes. Primary and secondary antibodies were diluted in TBS solution containing 0.3% Triton X-100 and 0.25% gelatin. Sections were mounted on glycerin albumin-coated slides, air dried, dehydrated, and coverslipped using Depex mounting medium (Gurr®, BDH, UK).

Image analysis

Images of the right side of the brain containing the AvPv (standardized to 3 images per rat brain) and MPO (standardized to 5 images per rat brain) were captured using a microscope (10 × 10 magnification) with a digital black-and-white CCD camera (Sony, XC-77CE) coupled to a PC. The eight consecutive sections (80 μm apart) containing the AvPv and MPO were selected using several criteria, including the shape of the third ventricle and the optic chiasm and the staining pattern. The second section of the AvPv and the third section of the MPO corresponded to levels of the rat brain atlas of Swanson (level 18 and 20 respectively) (245). The size of the captured images was 530 x 795 μm (horizontal x vertical). By capturing images immediately adjacent to the third ventricle, just above the optic chiasm or optic nerves, we captured the entire AvPv. In case of the MPO, a selection of the total staining area containing the medial preoptic nucleus (MPN) completely, but not the more lateral located nuclei, was captured.

Images were analyzed using an image analysis program (Scion Image Beta, version 4.02; Scion Corporation, Maryland, USA) using gray level threshold discrimination as previously described (14). To be able to compare ERα data of multiple stainings, each section was captured and analyzed with standardized procedures. A background correction was made by capturing each image of the POA at the same gray level (i.e. the average gray level of the entire image). After capturing all the images of one staining procedure, the mean background was calculated by measuring the gray level in a selection of the total area that did not contain ERα-positive nuclei in a representative selection of the images. The gray level threshold was determined using the background: mean maximal gray level in background + mean gray level in background + 3 x mean S.D. of the background, which theoretically results in a $p<0.001$ to include false positive nuclei in the final counting procedure. With this threshold, the image was converted in a binary image in which gray levels above threshold were colored red (i.e. the positive staining), and particles smaller or larger than a requisite number of pixels (n = 3) were excluded. Artifacts that were also colored red were excluded by hand. Finally, individual ERα-positive nuclei were counted automatically. Using this method, only dark stained cells were counted, i.e. only cells with a high concentration/density of ERα. Cells with a lower concentration of nuclear ERα from which the staining intensity did not reach threshold levels were not included in the counting. Only one third of the brain sections was stained for ERα and only the right side of the brain was analyzed. Since we found no significant differences in total number of ERα-positive cells between the left and right side of the brain for the AvPv and MPO (van der Beek and Franke, unpublished data), we calculated the total number of ERα-positive cells per brain area by multiplying the counted nuclei by six.

Statistics

Data were expressed as mean ± SEM and analyzed using SPSS 10.1. To determine the effect of age on hormone profiles of E_2 within a rat strain in experiment 1, data were LN transformed and tested using a general linear model (GLM) for repeated measurements. For P data, ANOVA was used per experimental group (age and strain), because an interaction between age and estrous cycle day was present. Post hoc tests were performed (Bonferroni, Dunnett T3) for E_2 and P data to test for significant differences between estrous cycle days. In F1 rats, significant differences in E_2 and P levels with age were tested for each

day of the estrous cycle using an independent-samples t-test. In addition, the average hormone concentration of an estrous cycle was calculated and tested for age and strain differences using a GLM – univariate test. A one-way ANOVA per rat strain was used to test if the difference with age was significant for F1 and/or WU rats. To determine the effect of age on the number of ERα-IR cells in the AvPv and MPO, data were LN transformed and a GLM – multivariate test was performed for each rat strain. In case of WU rats, data were analyzed per age or estrous cycle day. Furthermore, the existence of strain differences in number of ERα-IR cells in the AvPv and MPO of young and middle-aged rats was investigated by comparing ERα data of metestrus-morning and proestrus-afternoon of both rat strains using one-way ANOVA.

In experiment 2, we checked if E_2 concentrations on the different perfusion time points were comparable between groups with a GLM-univariate test. A total of nine females were excluded from ERα data analysis, seven were outliers ('extremes' as revealed by boxplots) and two were excluded, because extreme low ER numbers in addition to high PR numbers at 2 or 8 h after EB administration suggested that these females were not completely ovariectomized. ERα data of OVX+EB-treated females (AvPv and MPO) were compared per rat strain using GLM – multivariate test (EB-time 2-32 h) and one-way ANOVA tests (EB-time 32 h only). Post hoc tests were performed for EB-time (Dunnett T3). In addition, the existence of a strain difference in the number of ERα-IR cells in the AvPv and MPO was investigated by comparing ERα data of both strains using a GLM-multivariate test. ERα data of females perfused 32 h after EB injection with or without P treatment were compared to test the effect of P on the number of ERα-IR cells in the AvPv and MPO (GLM – multivariate test). A $p<0.05$ was considered to be significant.

Results

Estradiol and progesterone

The results of the E_2 and P plasma concentrations over de estrous cycle have been graphically presented previously (70), and are summarized in Table 1.

Plasma E_2 levels varied significantly over the estrous cycle. They were low during estrus and metestrus, and gradually increased on diestrus to reach peak levels on proestrus.

	age (months)	N	hormone	est-am	est-pm	met-am	met-pm	di1-am	di1-pm	di2-am	di2-pm	pro-am	pro-pm
WU	4.5	7	E_2	**14.4**±3.5	**10.6**±2.7	**8.1**±0.9	**9.8**±1.4	**10.8**±1.6	**14.3**±1.2*	X	X	**25.3**±4.5 *	**27.6**±3.0*
	9	7		**10.6**±4.0	**5.5**±0.6	**8.2**±1.5	**12.1**±1.0	**15.5**±1.9	**20.2**±2.3*	X	X	**26.5**±1.7 *	**38.8**±4.7*
	4.5	5	P	**25.7**±2.3	**21.5**±4.5	**36.3**±6.0	**51.5**±6.0*	**21.7**±6.1	**26.4**±4.4	X	X	**16.1**±3.6	**42.8**±4.6*
	9	7		**31.2**±5.3	**36.1**±3.7	**33.0**±4.8	**41.9**±6.3*	**23.4**±3.6	**28.1**±4.3	X	X	**19.0**±2.5	**33.0**±3.1
F1	4.5	19	E_2	**10.8**±2.1	**10.2**±1.8	**11.3**±2.1	**10.1**±1.1	**10.9**±1.3	**13.2**±2.2	**20.4**±1.6*	**20.5**±1.9*	**24.6**±2.0 *	**21.6**±2.0*
	9	7		**11.7**±2.7	**11.3**±1.3	**12.1**±3.4	**13.9**±2.4	**17.1**±3.2	**18.5**±4.0	**25.2**±3.5*	**26.2**±4.2*	**33.5**±4.8 *	**32.0**±8.3*
	4.5	23	P	**21.9**±2.2	**22.1**±1.9	**31.6**±1.9*	**34.2**±2.4*	**32.4**±2.6*	**26.9**±2.7*	**14.9**±1.5	**18.7**±1.6	**17.2**±1.9	**37.3**±2.5*
	9	8		**31.6**±2.8#	**27.4**±1.0	**42.8**±2.8*#	**46.4**±3.6*#	**49.1**±4.0*#	**51.0**±3.2*#	**27.6**±2.0#	**25.3**±1.8#	**26.7**±2.3 #	**44.4**±3.6*

Table 1. Plasma E_2 (pg/ml) and P levels (ng/ml) during the estrous cycle of 4.5- and 9-month-old 4-day cyclic WU and 5-day cyclic F1 rats. N, number of animals; est, estrus; met, metestrus; di1, diestrus I; di2, diestrus II; pro, proestrus; am, ZT 5; pm, ZT 11; *, significantly different from est-am; #, significantly different from the age of 4.5 months ($p<0.05$).

No differences in E_2 concentrations were found with age in 4-day cyclic WU rats, while E_2 concentrations were higher at the age of 9 compared to 4.5 months in 5-day cyclic F1 rats ($p=0.033$). Post-hoc T-tests revealed that the difference almost reached significance on diestrus-I ($p=0.060$) and proestrus morning ($p=0.087$). The average E_2 concentration during the estrous cycle was comparable between groups. Yet, E_2 concentrations were elevated for a longer period in F1 females (~2 days) compared to WU females (~1.5 day). In the OVX+EB-treated females, E_2 levels were comparable between the experimental groups and decreased in time. They averaged 84 ± 9 pg/ml at 2 h after injection of EB, were significantly lower at 26 h (45 ± 4 pg/ml), and further decreased at 32 h after EB injection (33 ± 2 pg/ml). In addition, the control groups that were included to check for possible differences between the first and second part of experiment 2 (i.e. young WU females perfused 2 and 32 h after EB injection) showed comparable E_2 levels.

Plasma P levels also varied significantly over the estrous cycle: P concentrations were low during estrus, increased and reached peak values at metestrus, were lower on diestrus (diestrus-I morning in 4-day cyclic WU and diestrus-II morning in 5-day cyclic F1 rats), and higher again on proestrus afternoon (Table 1). The average P concentration during the estrous cycle was significantly increased in 9- compared to 4.5-month-old rats in the F1 ($p<0.001$), but not in the WU strain. P levels in F1 females were elevated with age during almost the entire estrous cycle (Table 1).

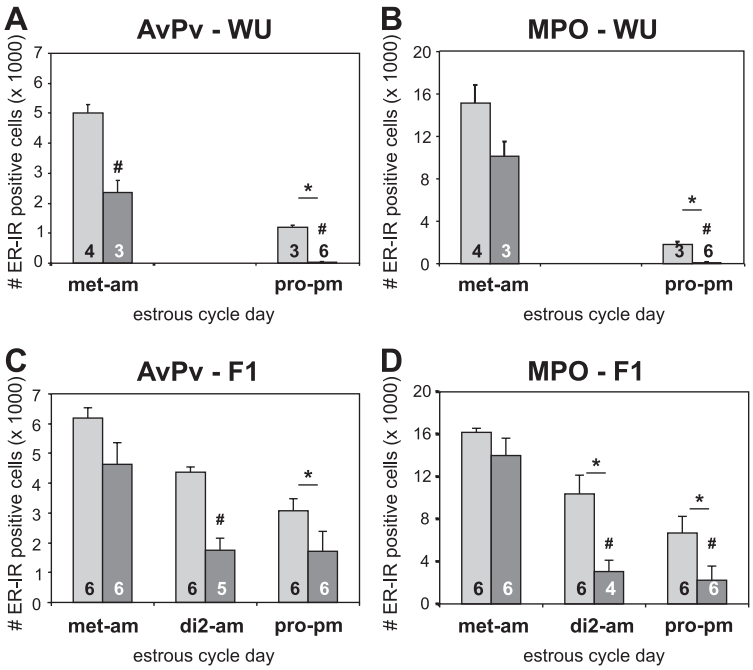

Figure 1. The number of ERα-IR positive cells during the estrous cycle in the AvPv (panels A and C) and MPO (panels B and D) of 4-day cyclic WU (panels A and B) and 5-day cyclic F1 rats (panels C and D) at the age of 4.5 (light gray) and 9 (dark gray) months. The number of animals per group is depicted at the bottom of the columns. met-am, perfused on metestrus at ZT 5; diII-am, perfused on diestrus II at ZT 5; pro-pm, perfused on proestrus at ZT 11. *, significantly different from met-am; #, significantly different from the age of 4.5 months ($p<0.05$).

ERα in cyclic females

Cell nuclei of ERα-IR cells were densely gray-to-black stained and the distribution of IR-nuclei corresponded to that previously described for ERα protein (51,235,273) and ERα mRNA (191,229,232).

We found that the number of ERα-IR cells in the AvPv and MPO of rats of both strains was high on metestrus and low on proestrus (Figure 1), and this difference was significant for the AvPv ($p<0.001$) and MPO ($p<0.001$) of WU rats. The AvPv of F1 rats also showed a significantly lower number of ERα cells on proestrus compared to metestrus ($p=0.030$), while in the MPO the number of ERα-IR cells was significantly lower both on proestrus ($p=0.007$) and diestrus-II ($p=0.019$) compared to metestrus.

In young as well as middle-aged females, we found significant strain differences in the number of ERα-IR cells on metestrus-morning in the AvPv ($p=0.034$ (young) and $p=0.014$ (middle-aged)), and on proestrus-afternoon in the AvPv ($p=0.011$ (young) and $p=0.001$ (middle-aged)) and MPO ($p=0.017$ (young)). In both areas, the number of ERα-IR containing cells was higher in F1 compared to WU females. In addition, the number of ERα-IR cells was significantly lower in 9- compared to 4.5-month-old females in the AvPv and MPO of F1 as well as WU rats. In F1 rats, the number of ERα-IR cells was decreased with age on diestrus-II in both AvPv ($p=0.002$) and MPO ($p=0.005$), and on proestrus in the MPO ($p=0.037$). In WU rats, this decrease with age was significant on metestrus in the AvPv ($p=0.005$), and on proestrus in AvPv ($p<0.001$) and MPO ($p=0.005$).

ERα in ovariectomized EB-treated females

In young and middle-aged females of both rat strains the number of ERα-IR cells in the AvPv and MPO gradually decreased between 2 and 32 h after EB administration ($p<0.001$; Figure 2). This decrease was significant at 32 h after EB administration in the AvPv of WU and F1 rats, and at 26 h and 32 h after EB administration in the MPO of WU and F1 rats and, in addition, at 8 h after EB administration in the MPO of the F1 strain. We found a strain difference in the MPO with a higher number of ERα containing cells in WU than in F1 females ($p<0.004$). In addition, no significant changes in the number of ERα containing cells were found between 4.5- and 9-month-old OVX+EB-treated females, although a slightly lower number was present in the MPO of WU females (trend: $p=0.064$). If only data of 32 h after EB administration were used in the analysis, a significant decrease in

ERα containing cells with age was found in the AvPv (p=0.043) and MPO (p=0.030) of F1 females, but not WU females.

In rats that had received the additional P injection, the number of ERα-IR cells at 32 h after EB administration was significantly decreased in the MPO of middle-aged WU rats (p=0.030), but not in young WU rats, F1 rats, or the AvPv (Figure 3).

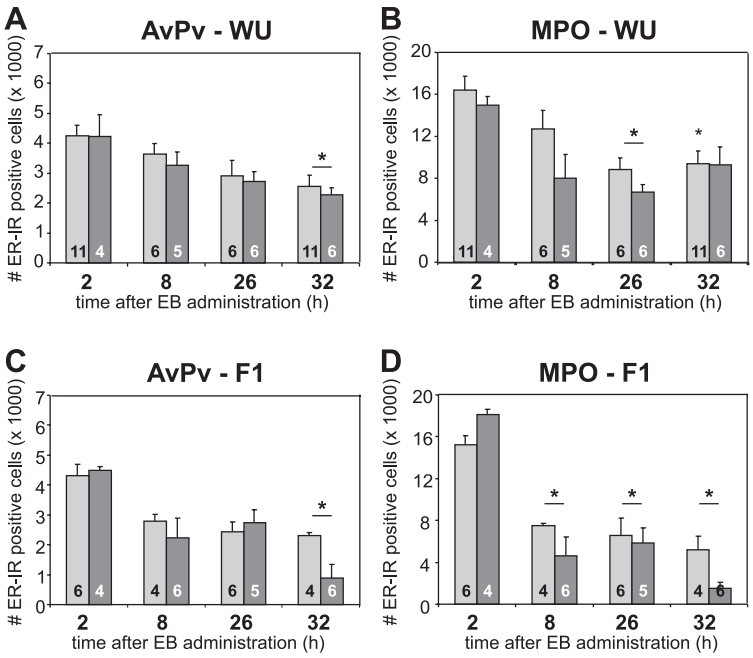

Figure 2. The number of ERα-IR positive cells in the AvPv (panels A and C) and MPO (panels B and D) of OVX+EB-treated WU (panels A and B) and F1 rats (panels C and D) at the age of 4.5 (light gray) and 9 (dark gray) months. The number of animals per group is depicted at the bottom of the columns. *****, both 4.5 and 9 months are significantly different from 2 h after EB ($p<0.05$); * only 4.5 months significantly different from 2 h after EB ($p<0.05$).

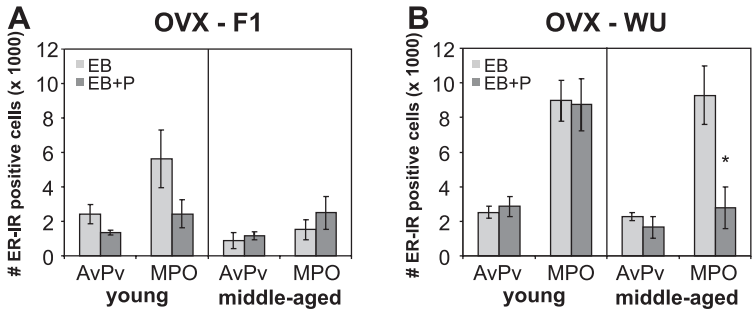

Figure 3. The number of ERα-IR positive cells in the AvPv and MPO of 4.5- (light gray) and 9- (dark gray) month-old OVX+EB-treated rats of the WU (panel A) and F1 (panel B) strain with (EB+P) or without (EB) additional P administration. *, significantly different from OVX+EB-treated rats ($p<0.05$).

Discussion

Our results clearly show that the number of ERα protein containing cells of the POA is dramatically decreased in regularly cyclic 9-month-old compared to 4.5-month-old cyclic female rats. Since this decrease was found in cyclic WU as well as F1 females, it likely represents a general phenomenon that contributes to the decline in fertility with advancing age. Furthermore, we showed that E_2 levels over the estrous cycle were unaltered (WU) or increased (F1) in middle-aged compared to young females. The decrease in ERα protein containing cells thus apparently occurs irrespective of increased steroid hormone concentrations, and based on the present findings, we suggest that it might be the result of increased sensitivity of ERα to steroid feedback.

The estrous cycle

The results in the present study clearly showed that during the normal reproductive cycle of young rats, E_2 and P hormone concentrations as well as the number of ERα-IR cells in the POA changed significantly over the cycle. We found that ERα cell numbers were lower on proestrus compared to metestrus, which is in agreement with previous reports on ERα protein (68) and ERα mRNA (228,233) in the POA during the estrous cycle. Since E_2 levels were higher on proestrus compared to metestrus, these data suggest a downregulation of ERα protein by E_2.

This downregulation of ERα by E_2 accords with previous reported data on the effect of E_2 on ER protein (299) and ER mRNA levels (231,233) and are in line with our finding that E_2 exposure in OVX rats also resulted in a decrease in ERα-IR positive cells within 32 h after EB injection. Thus, during the normal reproductive cycle the growth of the follicles with the resulting increase in E_2 levels leads to a decrease in cells in the AvPv and MPO that express ERα protein.

Reproductive aging

One of the aims of the present study was to investigate the feedback of E_2 on the number of ERα expressing cells in the POA (AvPv and MPO) with age. We found a clear decrease in ERα with age in both strains, while E_2 levels were unaltered in WU rats. In addition, at 32 h after EB administration the number of ERα cells was decreased to a further extend in middle-aged compared to young females, but only in the F1 strain. These data strongly suggest that the negative feedback of E_2 on the expression of ERα protein is increased in 9-compared to 4.5-month-old rats. In addition, since ERα mRNA levels do not change with age in the POA of OVX+E_2 treated Fischer 344 rats (171), or decrease only in the AvPv of 19-24 month-old female Sprague-Dawley rats (283), this may indicate that during reproductive aging alterations at the protein level occur before any changes in ERα mRNA levels become evident.

Furthermore, our results clearly showed that P is also able to affect the number of ERα-IR positive cells in the POA: an additional P injection 4.5 h prior to perfusion significantly decreased the number of ERα-IR positive cells in the MPO of OVX+EB-treated rats. Since the effect of P on ERα was only apparent in middle-aged WU females, P exposure may have been too short to reduce the number of ERα-IR neurons effectively in the other experimental groups. The possibility that P reduces hypothalamic ER levels has been postulated previously (158) and has been demonstrated in other tissues, e.g. rat uterus (5), human endometrium (81), monkey corpus luteum (54), and hamster ovaries (298). However, P did not affect nuclear estradiol binding in the POA (5,24), and appeared to decrease cytosolic estrogen binding (24) in the POA and the proportion of darkly stained ER-IR cells in the bed nucleus of the stria terminalis (50). In addition, treatment with estrogen and P, but not estrogen alone, decreased the ER staining intensity in the AvPv of female guinea pigs (50).

Based on these findings, we suggest that the decrease in ERα concentrations

in the AvPv and MPO of 9- compared to 4.5-month-old cyclic rats may partly be the result of an age-related change in the sensitivity to E_2 and P feedback.

Differences between cyclic and OVX+EB-treated rats

The fact that decrease in ERα was found in cyclic rats in both strains, but only in the F1 in OVX+EB-treated rats, suggests that the increase in sensitivity of ERα to estrogen feedback with age is subtle. Therefore, it may be detectable only after a certain time to E_2 exposure (> 32 h) under OVX conditions at 9 months of age. Indeed, in slightly older (10-12 month-old) OVX+E_2 treated Sprague-Dawley rats a decrease in ER concentration in the POA was found compared to young rats at two days after E_2 implantation (289). Also, the age at which cell nuclear estrogen binding capacity is decreased in the MPO differs between cyclic (8-11 months) (287) and OVX+EB-treated (19 months) (25) Sprague Dawley rats.

The most obvious explanation for the differences results between cyclic and OVX+EB-treated rats is a difference in hormone exposure in the days prior to perfusion. Firstly, OVX rats were deprived of (high) estrogen levels for a period of 13 days after OVX and this may lead to sensitization of the hypothalamus to E_2 feedback that may mask subtle changes in the sensitivity of ERα to E_2 feedback. Our observations that the number of ERα containing cells was high at 2 h after EB injection and maximally downregulated at 32 h accord with this notion. Secondly, OVX rats lacked exposure to other ovarian hormones, for instance P, which also may affect ERα concentrations as demonstrated in the present study.

Life-time E_2 exposure and aging

Since E_2 exposure is thought to advance reproductive aging, the second aim of our study was to investigate whether the extent of the change in ERα numbers with age was related to life-time cumulative E_2 exposure. To this end, we determined if possible strain differences in (cyclic) E_2 exposure were related to changes in preoptic ERα-IR cell numbers. Since only F1, and not WU, females showed a significant age-related change in both E_2 and P profiles over the estrous cycle, our results suggest that 9-month-old F1 females may be reproductively aged to a further extent than 9-month-old WU females. Nevertheless, the average E_2 concentration per day was comparable between WU and F1 females in young as well as in middle-aged rats, suggesting that there is no strain difference in overall life-time E_2 exposure between these two rat strains. F1 females, however display

two days with high E_2 concentrations (E_2 level above 20 pg/ml), while WU females display only one day with high E_2 concentrations during the estrous cycle. Thus, it is possible that E_2 advances reproductive aging only when concentrations are above a certain threshold. Nevertheless, the changes in ERα-IR cell numbers in the AvPv and MPO with advancing age were more pronounced in WU rats, while F1 rats showed more pronounced changes in PR-IR cell numbers (70). Thus, aging may affect primarily ERα in WU rats, and E_2, P and PR in F1 rats, which leads to the notion that strain differences in preoptic ERα-IR cell numbers are not likely related to the average E_2 exposure per day.

In the present study, we found a clear strain difference in ERα density in the POA of cyclic as well as OVX+EB-treated rats. Yet, cyclic WU rats showed a lower, while OVX+EB-treated WU rats showed a higher number of ERα-IR cells compared to F1 rats. Our data in OVX+EB-treated rats suggest that, while both strains showed the same 'maximal' number of ERα-IR positive cells in the AvPv and MPO (i.e. at 2 h after EB), the strain difference may result from a stronger downregulation of ERα by EB in F1 compared to WU rats. Yet, ERα numbers were consistently lower in cyclic WU compared to F1 rats (i.e. on metestrus and proestrus), resulting in a lower peak number of ERα-IR positive cells on metestrus in WU rats. Differences in total number of ERα containing cells or E_2 exposure do not appear to underlie the observed strain difference in ERα-IR cells. Firstly, the average E_2 concentration was comparable between cyclic WU and F1 rats and 'maximal' ERα-IR numbers were comparable (OVX). Secondly, exposure to elevated E_2 concentrations was longer in cyclic F1 females (two instead of one day high E_2 levels) and F1 females may have a stronger downregulation of ERα by E_2 (OVX). Thus, the strain difference in ERα numbers in the AvPv and MPO likely result from another factor than E_2. Since we showed that P decreased ERα numbers only in middle-aged WU females, this may suggest that WU females are more sensitive to P feedback. Consequently, a strong P-mediated downregulation of ERα may explain the lower ERα numbers in cyclic WU females, despite the fact that P levels are elevated in middle-aged F1 females. Also, in the absence of P (OVX+EB), a stronger E_2-mediated downregulation of ERα in F1 rats may explain the lower ERα numbers in those females.

Conclusion

We found that the number of ERα protein containing cells in the AvPv and MPO was decreased in 9- compared to 4.5-month-old cyclic rats in two rat strains. Our data also suggest that it is highly unlikely that the change in ERα-IR positive cells in the POA is related to changes in E_2 exposure. Based on these data, we hypothesize that the sensitivity of ERα to estrogen feedback may increase with age. Because P appears to be able to reduce the number of ERα-IR positive cells in the POA, the sensitivity of ERα to P feedback may also contribute to the decrease in ERα with age. Since ERα is involved in the stimulating effect of estrogen on the induction of PRs, which is thought to be a key event in the generation of the GnRH and LH surges, a decrease in ERα may eventually result in attenuation of the LH surge and reduced fertility with advancing age.

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Chapter 5

Aging alters the sensitivity of arcuate neurons to gonadal steroid feedback in cyclic female rats

Annelieke N. Franke, Victor M. Wiegant, Hans J.M. Swarts, Jan Kastelijn, Eline M. van der Beek

Abstract

The feedback of gonadal hormones on the arcuate nucleus (ARC) plays a crucial role in the regulation of estrous cyclicity in females, and both ARC functions and fertility show age-related deficits. We hypothesize that these changes result from an altered feedback of gonadal hormones on ARC neurons. To test this hypothesis, we investigated the relation between the plasma estradiol (E_2) and progesterone (P) concentrations and the concentrations of gonadal hormone receptors, i.e. estrogen receptor (ER) and progesterone receptor (PR), in hypothalamic ARC neurons in young and middle-aged cyclic rats of two strains that differ in rate of reproductive aging.

To this end, a blood sample was taken at metestrus morning or proestrus afternoon in young (4.5 months) and middle-aged (9 months) females of the 5-day cyclic (UxRP)F1 and the 4-day cyclic Wistar strain to measure plasma E_2 and P concentrations, whereafter all females were sacrificed and perfused for immunocytochemical staining of the brain slices for ERα and PR.

Both rat strains displayed low plasma E_2 on metestrus and high concentrations on proestrus. No significant differences in plasma P concentrations were found between these days, but P levels were significantly increased with age in F1 females. In the ARC, the number of ERα-IR cells was markedly decreased and of PR-IR cells increased from metestrus to proestrus in young and middle-aged females. Both ERα and PR protein concentrations showed differences between strains and were significantly decreased with age. E_2 levels correlated negatively with ERα-IR in WU rats, and positively with PR-IR in F1 rats.

Since the marked changes in ARC steroid receptors between young and middle-aged cyclic females was found in both rat strains, we hypothesize that an altered feedback of E_2 on ERα and PR concentrations in the ARC represents one of the first signs of reproductive aging. The fact that we found a number of differences between the two strains including strain specific correlations between hormone and receptor (the hormonal changes were not comparable between rat strains), supports the notion that strain differences exist in the regulation of the reproductive axis and in the rate of reproductive aging.

Introduction

The arcuate nucleus of the hypothalam us (ARC) is a brain area that plays an instrumental role in the regulation of several neuroendocrine systems, including the gonadal-, stress-, and somatotropic-axis (145). ARC neurons project, for instance, to the preoptic area (POA) and to GnRH neurons in order to regulate neuronal activity (111). In addition, many ARC neurons project to the median eminence (ME) where they modulate the release of neurohormones such as gonadotropin-releasing hormone (GnRH) (111,145).

Previous research in rats has suggested that the ARC is involved in the negative feedback of gonadal steroids on GnRH release (82), since, for instance, estradiol (E_2) injections or implants into the ARC decreased LH secretion, which was even further suppressed by P (82). In addition, the GnRH 'pulse generator', described as a set of neurons that periodically fire a high frequency volley of action potentials which culminate in the neurosecretion of a pulse of GnRH that is involved in the regulation of pulsatile, basal LH release (150), appears to be situated in this area (82,119,150).

Indeed, the release of several ARC neurotransmitters such as β-endorphin (β-END) (198) and neuropeptide Y (NPY) (13,297) changes over the estrous cycle and both neurotransmitters have shown to influence GnRH release (111,238), which is instrumental in the regulation of pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) release (111,150). In addition, E induces synaptic remodeling in the ARC during the estrous cycle (194) and this mechanism is thought to play a role in the induction of the GnRH and LH surge (107,179). The ovarian steroid hormone progesterone (P) has shown to influence proopiomelanocortin (POMC) gene expression in the ARC and is known for its role in the timing of the LH surge and the restriction of the LH surge to proestrus (198).

Therefore, the ARC with its central role in the regulation of neuroendocrine systems is a likely candidate for early changes in the hypothalamus-pituitary system with age. The ARC appears to be affected by age in several ways: not only gene expression of POMC (154) and NPY (216) and synaptic plasticity (107) are changed with age, but also an E-induced selective loss with age of neurons containing β-END has been reported (20,47,48).

Both sexual differentiation and the rate of reproductive aging are affected by the exposure to (high concentrations) of gonadal hormones, particularly E

(49,63,97,155,207). The rate of reproductive aging and the age at which fertility and fecundity start to decline differs between individuals and rat strains. This raises the possibility that individual or strain differences in gonadal hormone exposure underlie the differences in rate of reproductive aging. Indeed, there are indications that female fertility declines at a relatively early age in the (UxRP)F1 strain compared to other rat strains (96,270). These females predominantly display 5-day estrous cycles and are exposed to high E_2 concentrations for two days during one estrous cycle. In contrast, cyclic Wistar rats, that predominantly display 4-day estrous cycles, are exposed to only one day of high E_2 concentrations per estrous cycle (70).

We hypothesize that the change in ARC activity with age may result from an altered feedback of gonadal steroids. This assumption is based on the fact that the feedback of gonadal hormones on the ARC plays a crucial role in the regulation of ARC function and consequently estrous cyclicity in females, and that the activity of ARC neurons appears to change with age. Therefore, the aim of the present study was to investigate the relation between plasma gonadal hormone concentrations (E_2 and P) and the concentration of gonadal hormone receptors (ER and PR) in ARC neurons with age within two rat strains. To this end, two time points during the estrous cycle were examined, i.e. metestrus-morning and proestrus-afternoon. At these time points, a blood sample was taken from young (4.5 months) and middle-aged (9 months) females of the (UxRP)F1 and Wistar strain. Thereafter all females were sacrificed and perfused to stain brain slices for ER and PR.

Materials and Methods

Animals

Virgin female F1 rats, a locally bred hybrid of two Wistar sub strains (RP-inbred females (albino) and U-inbred males (brown hooded); abbreviated as F1), were obtained from the university animal care facility at 9 weeks old. Virgin female and male Wistar rats (HsdCpb:WU, Wistar Unilever; abbreviated as WU) were obtained from Harlan (Holst, The Netherlands) at 9 weeks old (females) or at 12 weeks old (males). Rats were housed five per cage under regular light-dark conditions (L/D 12:12, experiment 1: lights on at 5:00 h (‘zeitgeber time 0’ or ZT 0) and had free access to standard pelleted food (Hope Farms B.V., Woerden, The Netherlands) and tap water.

Estrous cyclicity was monitored by daily vaginal lavages from one week after arrival until the end of the experiment (young females; 4.5 months old at the end of the experiment) or from the age of 7.5 months until the end of the experiment (middle-aged females; 9 months old at the end of the experiment). In addition to vaginal lavages, receptive behavior was monitored daily. To this end, a naive male WU rat was introduced to the female rat home cage one hour before lights off, where after display of hopping and darting, ear wiggling, and lordosis posture by the female was checked. The experiment was approved by the animal experimental committee of the Wageningen University.

Experimental design

To study changes in the feedback of gonadal hormones on hypothalamic ER and PR between young and middle-aged cyclic rats, we measured steroid hormone levels (E_2 and P) in relation to the number of ER- and PR-expressing cells in the ARC at specific estrous cycle days. Only regularly 4-day (WU) and 5-day (F1) cyclic females were used at the age of 4.5 and 9 months. Just before perfusion, a blood sample was taken via cardiac puncture in young WU (n=7) and F1 (n=18), and middle-aged WU (n=9) and F1 (n=15) rats. Blood samples were collected and assayed for E_2 and P measurements as previously described in detail (69,70). Perfusions were performed at two specific time points of the estrous cycle: on metestrus morning (10:00 h = ZT 5) or proestrus afternoon (16:00 h = ZT 11). Each group contained 3-6 animals. Brain sections containing the ARC were immunocytochemically stained for ER or PR.

Perfusion and sectioning of the brain material

Each animal was transcardially perfused under Nembutal® anesthesia (CEVA sante animale B.V., Maassluis, The Netherlands; 60 mg/ml) (69,70) with 250-350 ml 0.9% NaCl solution followed by 200-300 ml fixative consisting of 4% paraformaldehyde in 0.1 M PBS (pH 7.4) and brains were postfixed for 25 h in the same fixative. Brains were stored at 4°C in Tris-buffered saline (TBS; pH 7.4) containing 0.1% sodiumazide until further processing. Each brain was embedded in 20% gelatin in TBS and postfixed for 4-6 h (91). Subsequently, 40 µm thick brain sections were cut on a vibratome (1000 classic, Vibratome Company, St. Louis, MO, USA) from the coronal level containing the medial septum and the diagonal band of Broca to the posterior hypothalamus. Sections were stored in TBS

containing 0.1% sodiumazide at 4°C until immunocytochemistry.

Immunocytochemistry

Every third section was used for PR or ER immunocytochemistry as previously described in more detail (69,70). Briefly, free-floating sections were immunocytochemically stained by incubating them into the first antibody against PR (rabbit anti-human; A0098, DAKO Inc., Glostrup, Denmark; dilution 1:500-600) or ERα (mouse anti-human; M7047, DAKO Inc., Glostrup, Denmark; dilution 1:1000) and a second biotinylated antibody (Goat-anti-Rabbit (PR; 1:400) or Horse-anti-Mouse (ER; 1:250)). Thereafter, sections were incubated in a Vector Elite Avidin-Biotin-peroxidase complex (PR: 1:1200; ER: 1:750-1000) and immunoreactivity was visualized by 0.05% 3,3'-diaminobenzidine (DAB) with nickelammoniumsulfate and H_2O_2. Sections were mounted on glycerin albumin-coated slides, air dried, dehydrated, and coverslipped using Depex mounting medium.

Image analysis

By staining 1-out-of-3 sections, the total ARC consists of 18 sections. The three most anterior sections of the ARC were excluded, since these contain almost no ER- or PR- immunoreactive (IR) positive cells (unpublished data, van der Beek). From the remaining 14 sections every second section was included in the counting procedure, amounting to a total of 7 sections per animal, in which receptor containing nuclei were counted unilaterally. We have shown (van der Beek and Franke, unpublished data) that there are no significant differences in total number of ER-IR or PR-IR positive cells between the left and right side of the ARC, thus only the right ARC was analyzed. If the right ARC of an animal was damaged, the complete left ARC of that animal was analyzed.

Images of the right side of the brain containing the ARC were captured using a microscope (10 × 10 magnification) with a digital black-and-white CCD camera (Sony, XC-77CE) coupled to a PC. Thereafter, images were analyzed using an image analysis program (Scion Image Beta, version 4.02; Scion Corporation, Maryland, USA) using gray level threshold discrimination as previously described (14). Each section was captured and analyzed with standardized procedures using a background correction, i.e. each image of the ARC was captured using the same gray level (the average gray level of the entire image). The mean background was

calculated by measuring the gray level in a selection of the total area that did not contain positive nuclei in a representative selection of the images (10-40%). The threshold was determined using these background measurements: mean 'maximal gray level in background' + 3 x mean 'S.D. of the background' for PR, which theoretically results in a $p<0.001$ to include false positive nuclei in the final counting procedure. For ER, the threshold was based on: mean 'maximal gray level in background' + mean 'mean gray level in background' + 3 x mean 'S.D. of the background', since the background in general was very light in ER compared to PR stainings. With this threshold, the image was converted in a binary image in which gray levels above threshold were colored red (i.e. the positive staining), and particles smaller than a minimal number of pixels (n=3) were excluded automatically. Artifacts that were colored red were excluded by hand. Finally, individual ER- and PR-positive nuclei were counted automatically. Using this method, only dark stained ER or PR cells were counted, which corresponds to cells with a high concentration/density of ER or PR (18). Cells with a lower concentration of nuclear ER or PR, from which the staining intensity did not reach threshold levels, were not included in our counting result. The total number of PR-positive cells per brain area of an animal was calculated by multiplying the counted nuclei by 15.4 (i.e. 3 (every third section stained) * 2 (bilateral) * 18/7 (distance between section)).

Statistics

Data were analyzed using SPSS 10.1 and expressed as mean ± SEM. To determine the effect of estrous cycle day or age on plasma E_2 and P concentrations, we used the nonparametric Kruskal-Wallis test in young and middle-aged WU and F1 rats.

To determine the effect of age on the number of ER- and PR-positive cells in the ARC for each rat strain, data were LN transformed and for F1 rats a GLM – multivariate test was performed (estrous cycle day, age). Age differences were further analyzed by comparing ER and PR data between young and middle-aged females using an independent-samples t-test. In case of the WU strain, data were analyzed for effects of age or estrous cycle day using the nonparametric Kruskal-Wallis test. In addition, the existence of a strain difference in the number of ER- and PR-IR positive cells in the ARC was investigated by comparing ER and PR data of metestrus morning and proestrus afternoon of both rat strains per age using

the nonparametric Kruskal-Wallis test. To investigate the relation between gonadal hormone concentrations and the number of cells containing ER and PR, we used the Pearson correlation test. A $p<0.05$ was considered significant.

Results

Ovarian hormone concentrations

Plasma E_2 levels changed significantly between metestrus morning and proestrus afternoon: E_2 concentrations were low on metestrus and high on proestrus (Figure 1A). This increase reached significance in young ($p=0.050$) and middle-aged ($p=0.046$) WU and in middle-aged F1 ($p=0.004$) females, while in young F1 females there was a trend ($p=0.088$). In addition, proestrous E_2 levels were significantly higher ($p=0.039$) in middle-aged compared to young WU females. No differences were found in E_2 levels between WU and F1 females.

Plasma P levels were comparable between metestrus morning and proestrus afternoon in all groups (Figure 1B). Proestrous P levels were significantly lower in middle-aged compared to young WU females ($p=0.046$), while in middle-aged F1 females P levels were significantly higher on metestrus ($p=0.016$) and proestrus ($p=0.037$) compared to young F1 rats. A significant strain difference was present in proestrous P levels ($p=0.004$) and a trend for metestrus P levels ($p=0.071$) of middle-aged females: P levels were higher in F1 compared to WU rats.

ERα-containing cells in the ARC

Cell nuclei of ERα-IR cells were densely stained and the distribution corresponded to that previously described for ERα protein (51,235,273) and ERα mRNA (191,229,232).

We found that the number of ERα-IR cells in the ARC was high on metestrus-morning and low on proestrus-afternoon (Figure 2). The difference in ERα-IR cells over the estrous cycle was significant for young and middle-aged F1 (both $p<0.001$) as well as WU rats ($p=0.034$ and $p=0.020$, respectively). We found that the number of ERα-IR cells in the ARC was significantly lower in WU compared to F1 rats in young females at proestrus ($p=0.020$) and in middle-aged females at proestrus ($p=0.006$) as well as metestrus ($p=0.020$). In addition, the number of ERα-IR cells in the ARC of both rat strains showed a significant difference with

age. In all cases, the number of ERα-cells was decreased in 9- compared to 4.5-month-old females (Figure 2).

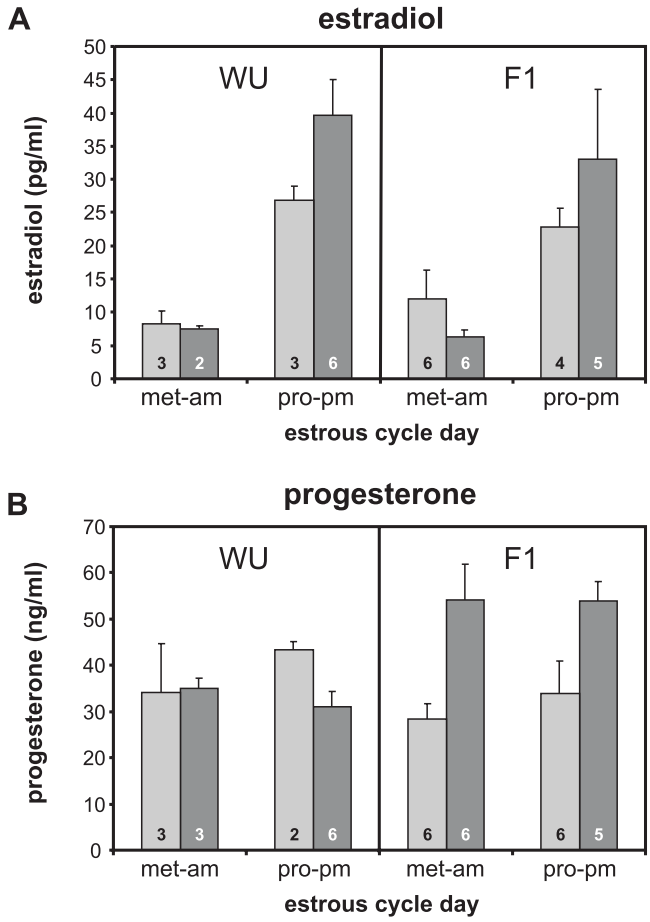

Figure 1. Estradiol (**panel A**) and progesterone (**panel B**) concentrations on two days of the estrous cycle in 4-day cyclic WU and 5-day cyclic F1 females at the age of 4.5 (light gray bars) and 9 (dark gray bars) months. The number of animals per group is depicted at the bottom of the column. met-am, perfused on metestrus at ZT 5; pro-pm, perfused on proestrus at ZT 11.

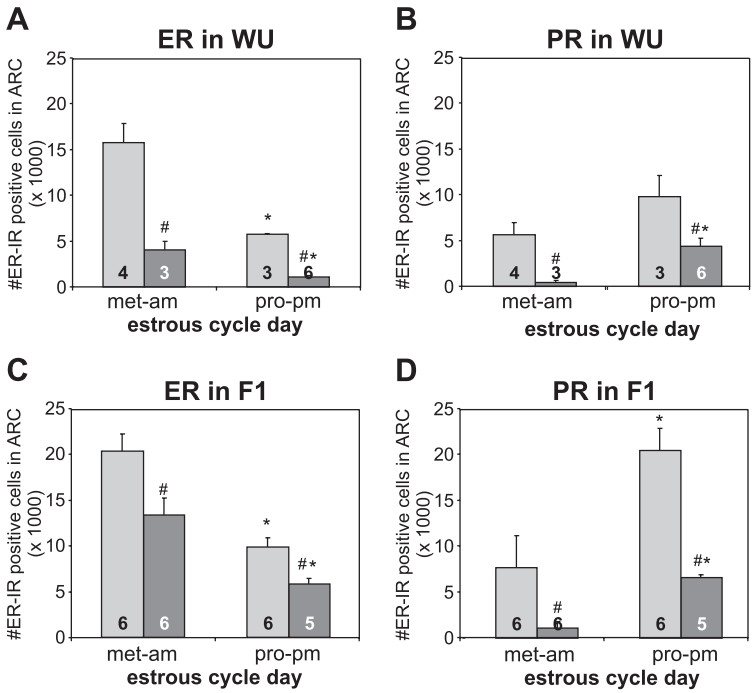

Figure 2. The number of ERα-IR (**panel A and C**) and PR-IR (**panel B and D**) positive cells during the estrous cycle in the ARC of 4-day cyclic WU (**panel A and B**) and 5-day cyclic F1 rats (**panel C and D**) at the age of 4.5 (light gray bars) and 9 (dark gray bars) months. The number of animals per group is depicted at the bottom of the column. met-am, perfused on metestrus at ZT 5; pro-pm, perfused on proestrus at ZT 11. *, significantly different compared to met-am cell numbers within the group, #, significant difference with age ($p<0.05$).

PR containing cells in the ARC

Cell nuclei of PR-IR positive cells were densely stained, although a variation in staining intensity between nuclei was clearly present. The distribution of PRs in the AvPv and MPO was comparable to that previously described for PR protein (18,273) and PR mRNA (25,231,289).

Our PR data clearly showed that the number of PR-IR positive cells in the ARC was low on metestrus and high on proestrus (Figure 2). The increase in PR-IR positive cells was significant in middle-aged ($p=0.020$) WU as well as young ($p=0.016$) and middle-aged F1 ($p=0.014$) rats, but not in young ($p=0.077$) WU rats.

We found a significant strain difference in the number of PR-IR positive cells on proestrus-afternoon only in young rats, e.g. the number was higher in F1 than in WU rats (p=0.020). A significant change with age in the number of PR-IR positive cells of the ARC was found in both strains. The number of PR-IR positive cells was lower in 9-month- than in 4.5-month-old WU rats at metestrus (p=0.034) and proestrus (p=0.039), and in F1 rats at metestrus (p=0.005) and proestrus (p=0.021; Figure 2).

Correlations between ovarian hormone concentrations and ARC receptor numbers

E_2 levels and the number of ER-IR cells in the ARC showed a clear correlation in young (r=-0.89, p=0.018) and middle-aged (r=-0.87, p=0.005) WU females, but not in F1 females. Only PR-IR cells in the ARC of young F1 females correlated with E_2 levels (r=0.81, p=0.001; Figure 3).

Discussion

We found a clear decrease in ERα and PR concentrations with age in the ARC of WU and F1 females, which was comparable in magnitude to the decrease in hormone receptors previously described for the AvPv and MPO of these same females (69,70). However, the role of the ARC in the regulation of the hypothalamus-pituitary-gonadal axis is different from that of the AvPv. Whereas the latter is mainly involved in the positive feedback of E_2, the ARC is thought to play a role in the negative feedback of steroids on the reproductive cycle.

The estrous cycle

In the present study, we found that both rat strains displayed low plasma E_2 concentrations on metestrus and high concentrations on proestrus, while there were no significant differences in plasma P concentrations between these cycle days. This corresponds to previous reports (70). At the level of the ARC, the number of cells containing high concentrations of ERα decreased and that of PR increased from metestrus morning to proestrus afternoon in young F1 and middle-aged WU and F1 rats. In young WU rats only a trend to an increase in PR-IR cells was found (p=0.077), but it did reach significance in a previous study in our lab

(van der Beek, unpublished data). These data may suggest a causal relationship between E_2 concentrations, ERα numbers, and PR numbers. Indeed, previous research showed that E_2 down-regulates its own receptor (89,201,299) and induces PR expression (18,89), and in the present experiment we showed that E_2 concentrations correlated significantly to ERα numbers in the ARC of WU rats and to PR numbers in the ARC of young F1 rats.

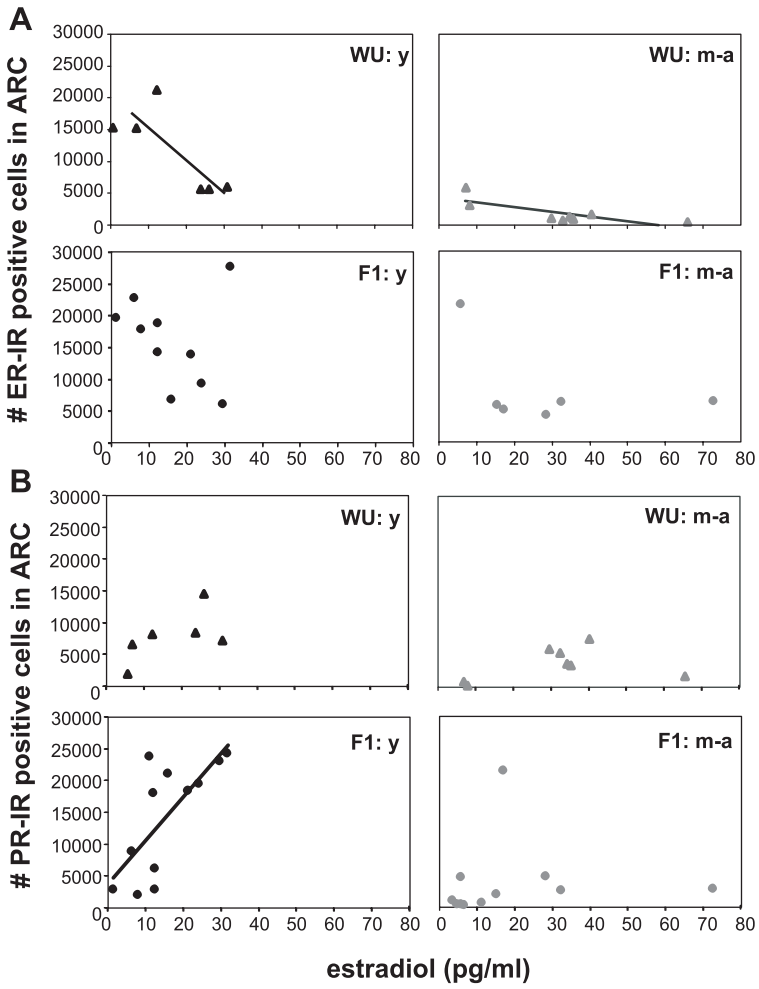

Figure 3. Estradiol levels in relation to the number of ERα- (**panel A**) and PR- (**panel B**) expressing cells in young (y) and middle-aged (m-a) WU (above) and F1 (below) female rats. Significant correlations are indicated by lines. A $p<0.05$ was considered to be significant.

Based on these data, we hypothesize the generation of the GnRH surge, which involves the shift from negative to positive feedback of gonadal hormones on GnRH release, requires a decrease in neurons containing ERα and an increase in neurons containing PR in the brain.

Indeed, previous research showed that E_2 and P feedback is necessary to regulate synaptic remodeling in the ARC during the estrous cycle (194) as well as for estrous cycle related changes in ARC activity. Synaptic remodeling regulates synaptic input and neurotransmitter release in the ARC/ME area (107,174,179). In addition, β-END and NPY neurons in the ARC express ERα (235), and both β-END (198,280) and NPY (111) are influenced by E_2 and/or P. Beta-END has been reported to inhibit GnRH release (111), while NPY stimulates GnRH release directly (235,238), and by increasing the sensitivity of pituitary gonadotrophs to GnRH (111,296). Furthermore, the activation of hypothalamic PRs results in an increase in NPY release and NPY-receptor expression in the hypothalamus (297), NPY neurons innervate β-END neurons (111) and NPY inhibits electrical activity within the ARC itself (135). We hypothesize that the increase in E_2 during the estrous cycle results in a decrease in ERα and an increase in PR expression in the ARC, leading to an activation of the NPY system and an inactivation of the β-END system. In this way, the negative feedback (i.e. β-END) is abolished or diminished on proestrus morning, thereby allowing the generation of the preovulatory GnRH surge. Altogether, these data suggest that E_2 and P feedback on ERα and PR in the ARC play a crucial role in the generation of a GnRH and LH surge on proestrus.

Changes during reproductive aging

In our 9-month-old rats that were still regularly cyclic, we found several changes compared to the 4.5-month-old young rats. P levels were significantly increased in F1 females and proestrous steroid hormone levels were slightly altered in WU rats at the age of 9 months. Interestingly, the changes at the level of the brain between young and middle-aged cyclic females were more dramatic and consistent between rat strains. Both WU and F1 females showed a clear decrease in the number of cells that contain high concentrations of the ERα and PR at metestrus as well as proestrus with age. These findings were similar to those previously reported in the same females for two other brain areas, the anteroventral preoptic area (AvPv) and the medial preoptic area (MPO), and even the magnitude of the decrease with age

appeared to be comparable (69,70). In the ARC, however, ERα and PR dynamics were more pronounced than in the AvPv and MPO: the decrease in ERα and PR was significant on metestrus as well as proestrus of both rat strains in the ARC, but not in the AvPv and MPO. Since a clear decrease in steroid receptors was found, while the changes in hormone levels were less clear, these results suggest an altered sensitivity of steroid receptors to hormonal feedback with age. Therefore, these data suggest that in general the feedback of E_2 and P on their receptor numbers is increased, while the E_2-induced induction of PR expression may be decreased with age, and this change with age may be first detectable in the ARC, even before it becomes evident in the AvPv and MPO. The change in feedback of E_2 on the ERα and PR in the ARC may, for instance, underlie the changes in the gene expression of POMC/β-END (154) and synaptic plasticity (107) as previously reported. E_2 and P feedback on the ERα and PR in the ARC appear to play a crucial role in the inhibition of the β-END system to abolish or diminish the inhibition of the GnRH system on proestrus morning. Consequently, the decrease in ERα and PR concentrations as found in the present study may result in changes in sensitivity of the β-END system to hormonal feedback. This may result in changes in GnRH release, GnRH priming, and the LH surge, thereby affecting fertility. Since the LH surge is significantly attenuated in both rat strain at the age of 9 months (67), the present data support the idea that the decrease in activity of the gonadal axis with age primarily results from changes in the central hypothalamus-pituitary system.

Strain differences in the reproductive axis

Besides the presence of significant correlations between ER and E_2 levels in WU but not F1, two other strain differences were found. Firstly, P levels appeared to be higher in F1 compared to WU females. This strain difference in P levels likely results from the elevated plasma P levels in F1 on both days (i.e. metestrus and proestrus) as well as a decrease in proestrus P levels in middle-aged compared to young WU females. Yet, since proestrous P levels were only measured in two young WU rats, these results should be handled with caution. Indeed, previous research concerning a larger group of WU females failed to demonstrate a significant decrease in proestrous P levels at this age (70). Secondly, WU females showed an increase in proestrous E_2 levels with age, but no age-related changes in E_2 levels were demonstrated in F1 rats. Also, no strain differences in E_2 levels

were found. Thus, strain differences are present in plasma concentrations of ovarian steroid hormones E_2 and P. Since these strain differences were only apparent at the age of 9 months, these may indicate strain differences in (rate of) reproductive aging. Furthermore, because E_2 concentrations correlated to ERα numbers in the ARC of WU rats and to PR numbers in the ARC of young F1 rats, we speculate that the feedback of ovarian hormones on the brain may show some strain specificities. Consequently, estrous cyclicity may be primarily regulated via the E_2-induced down-regulation of ERα in WU females, but via the E_2-induced expression of the PR in F1 females. This speculation requires further investigation.

Conclusions

We showed that differences in ovarian hormone concentration between metestrus and proestrus, i.e. the days of the estrous cycle that are likely associated with respectively a negative and positive feedback of steroid hormones on GnRH and LH release, were related to the number of ARC cells containing high levels of ERα or PR. Since the dramatic changes in ARC steroid receptors between young and middle-aged cyclic females occurred in both rat strains, we hypothesize that one of the first changes during reproductive aging is an altered feedback of E_2 on the ERα and PR on the hypothalamic ARC, which eventually results in a disregulation of estrous cyclicity and decreased fertility. The fact that hormonal changes with age as well as the number of steroid receptors were not comparable between rat strains, may support the presence of strain differences in regulation of and/or the rate of reproductive aging.

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Chapter 6

The decline in follicular reserve between 4 and 8.5 months in cyclic female rats is not accompanied by changes in spontaneous preovulatory and GnRH-induced follicle stimulating hormone (FSH) release

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(to be submitted)

Abstract

Fertility decreases with age in female mammals. One of the first indications of reproductive aging in rats is an attenuation of the LH surge that appears to result from age-related alterations in the hypothalamus-pituitary system. In humans, however, this attenuation appears to be preceded by alterations at the level of the ovary: decreased inhibin B levels resulting in elevated FSH levels and an accelerated loss of primordial follicles. To investigate whether age-related alterations in FSH release may precede the attenuation of the LH surge in rats as well, we performed experiments in 4- and 5-day cyclic Wistar rats and 5-day cyclic (UxRP)F1 rats. In this way, we are able to elucidate general and rat (sub)strain- or cycle length- dependent mechanisms in the aging process.

The preovulatory FSH surge as well as GnRH-induced FSH release was measured in female rats at the age of 4 months (young) and 8.5 months (middle-aged) by taking hourly blood samples on a normal proestrus or after administration of a potent GnRH analog. In addition, estrous cyclicity as well as the ovarian follicular reserve were determined in young and middle-aged females of both rat strains. The present results showed clearly that the profile and timing of the preovulatory FSH surge resembled that of the LH surge. We found, however, no significant changes in the magnitude of the preovulatory FSH surge or the pituitary FSH responsiveness to GnRH with increasing age. Apart from an increase in cycle length, we showed that the ovarian follicular reserve pool significantly decreased with age in both strains. This decrease differed in magnitude between rat strains and was related to the magnitude of the proestrous LH, but not FSH surge.

In conclusion, our results suggest that in aging female rat, changes in the hypothalamus-pituitary system precede changes at the level of the ovary. Differences between individuals, strains and species in the rate of reproductive aging may depend on different limitations and contributions of specific factors to the regulation of the reproductive system.

Introduction

It is well known that fertility and fecundity decline in female mammals long before cyclicity ends. Yet, the relative contribution of the hypothalamus-pituitary

system and the ovary during the initial stages of reproductive aging is unclear. In humans, the favorite point of view is that at a mean age of 37-38 years the number of oocytes in the ovary reaches a minimum, resulting in less preantral follicles that secrete inhibin B, leading to a decrease in circulating inhibin B levels. Since the hormone inhibin acts to inhibit follicle stimulating hormone (FSH) synthesis and secretion, this will result in an elevation of FSH concentrations and increased follicular recruitment from the follicular reserve pool. Eventually, the follicular pool will become exhausted and ovulation and reproductive cyclicity end (27,224,239,248).

In rats, there are indications that hypothalamic aging plays a crucial role (195,210,288,291), since in contrast to humans rodents still posses a significant number of oocytes when fertility ceases (270). Orthotopic ovarian transplantations in young (6 weeks old) and aged (17 months old) C57BL/6J mice support the idea that the aging ovary and uterus play a secondary role in reproductive failure and that the aging hypothalamus-pituitary system is primarily responsible for the loss of fecundity in rodents (195). The percentage of female mice that was pregnant from the four ovarian transfer combinations were: young into young 58%; young into aged 9%; aged into young 50%; and aged into aged 0% (195).

In rats, our working hypothesis is that the hypothalamus will become less sensitive to the positive ovarian steroid feedback with age, resulting in reduced gonadotropin-releasing hormone (GnRH) secretion by the hypothalamus, decreased priming at the pituitary level, and consequently an attenuation of the preovulatory luteinizing hormone (LH) surge (69,70). Consequently, there are two pituitary hormones, LH and FSH, which can be used as an indicator for early reproductive aging. Both LH and FSH are secreted by the anterior pituitary gland in a pulsatile pattern and show a preovulatory surge on proestrus. The preovulatory LH surge is responsible for luteinization of the full-grown follicles, meiotic resumption of the oocyte, and ovulation. Since GnRH also acts as an FSH secretagogue, a preovulatory FSH surge that is involved in follicular rupture accompanies the LH surge. During the LH surge, the granulosa and theca cells of the full-grown follicles start to luteinize. The resulting decrease in inhibin leads to a second, monotropic FSH surge on the morning of estrus that is involved in the recruitment of follicles for the next growth wave (61,73,128).

One of the first indications of reproductive aging in rats is indeed an attenuation of the LH surge around the age of 8-10 months (46,109,285,291). Nevertheless,

alterations in FSH levels with age have been reported in rats and mice, although the results appear somewhat contradictory. Preovulatory FSH surge levels were shown to decrease with (45,46) or without (284,285) a concurrent increase in basal FSH levels, or only an increase in basal FSH levels was reported (109). In some studies, an attenuated LH surge was associated with an attenuated FSH surge (46,109), while in another study it was not (181). A delay in timing of the preovulatory LH surge, however, was always associated with a delay in timing of the preovulatory FSH surge (45,284,285). In addition, although the increase in basal FSH is likely associated with a decrease in inhibin B in humans (239), one study in rats demonstrated an increase in FSH as well as inhibin subunit gene expression with age (109). Altogether, these data suggest that during reproductive aging in the rat, the magnitude of the LH surge is likely to decrease prior to, or concurrent with, the FSH surge. It is not clear if an elevation of basal FSH concentrations may precede the attenuation of the preovulatory FSH surge, and if so, if this elevation is caused by a decrease in inhibin B levels.

The age at which the decline in female fertility becomes evident in mammals shows considerable individual differences (248,270). Genetic factors may explain, at least in part, the age at natural menopause in women (42). A general indication for this decline in fertility is the length of a reproductive cycle. Adult female rats normally display regular 4- or 5-day estrous cycles that become prolonged and irregular with advancing age. Eventually, rats can display periods of pseudopregnancies (PP) or persistent estrus (PE) followed by a state of persistent diestrus (PD) or anestrus (148,270). In women, the length of the menstrual cycle alters from about 28 days at the age of 20 years to 26 days at 40 years old. The variability in cycle length in an individual reaches lowest values around 7 years prior to menopause, whereafter cycle length becomes increasingly variable: cycles can be shortened or lengthened and both are associated with reduced fertility (248).

In regularly cyclic rats, fertility indeed appears to decrease earlier in 5-day cyclic compared to 4-day cyclic Sprague-Dawley rats, although the follicular reserve appeared to be comparable (169). Some rat strains show distinct preferences in cycle length, i.e. predominantly 4-day (i.e. Wistar) or 5-day estrous cycles ((UxRP)F1) (67), which is accompanied by clear differences in circulating hormone concentrations (67,70,96,185). As is the case in the Sprague-Dawley rat, fertility and fecundity appear to decline relatively early in the 5-day cyclic (UxRP)F1 rat

(163,270). Strain differences have also been reported in the number of oocytes present at birth and in follicular growth rate in mice (59). Altogether, these data suggest that strain and/or species differences in the regulation of the reproductive system may underlie differences in the rate of reproductive aging.

The aim of the present study was to gain insight in the changes that occur in the reproductive axis of the aging female rat, specifically changes in FSH levels and the ovarian follicular reserve in relation to the attenuation of the LH surge. We used both 4- and 5-day cyclic Wistar rats and 5-day cyclic (UxRP)F1 rats to elucidate general and rat strain- or cycle length- dependent mechanisms in the aging process. In a previous study, we already demonstrated an attenuated LH surge in 8.5- compared to 4.5-month-old females of both strains (67). By measuring proestrous FSH surge levels at the same time, we were able to determine (I) if the attenuation of the LH surge was accompanied by an attenuation of the FSH surge, and (II) if basal FSH levels preceding the preovulatory surge were increased with age. We measured proestrous FSH levels after administration of a potent GnRH analog to determine (III) if pituitary FSH responsiveness to GnRH was diminished with age. Finally, we determined the ovarian follicular reserve in animals of both strains at 4 and 8.5 months old, to investigate (IV) if the decrease in the size of the follicular reserve differs between rat strains, and (V) if the size of the follicular reserve showed any correlation with proestrous LH or FSH levels.

Materials & Methods

Animals

Virgin female (UxRP)F1 rats (N=60), a locally bred hybrid of two Wistar sub strains (RP-inbred albino females and U-inbred hooded males; abbreviated as F1), were obtained from the university animal care facility at 9-10 weeks of age (163,265). Virgin female (N=60) and male (N=16) Wistar rats (HsdCpb:WU, Wistar Unilever; abbreviated as WU) were obtained from Harlan (Horst; The Netherlands) at respectively 9 or 12 weeks of age. Rats were housed four or five per cage under regular light-dark cycles (L/D 12:12, lights on at 3:00 h defined as ‘zeitgebertime’ 0 or ZT 0) with free access to standard pelleted food (Hope Farms BV, Woerden, The Netherlands) and water. One week before intravenous cannulation, each female was housed individually until the end of the experiment. Blood samples were taken approximately at the age of 4 and 8.5 months, whereafter females were

ovariectomized (i.e. the end of the experiment). The experiment was approved by the animal experimental committee of Wageningen University.

Estrous cycle length

To study the effect of age on cycle length in the two rat strains, estrous cycles were monitored by daily vaginal lavage for a period of approximately 7 weeks. Vaginal lavages were analyzed according to the criteria described elsewhere (73) and were taken from one week after arrival until the end of the experiment (young females; 17 weeks old at the end of the experiment; n=30 for each rat strain), or from the age of 29 weeks until the end of the experiment (middle-aged females; 36 weeks old at the end of the experiment; n=30 for each rat strain). In addition, receptive behavior was monitored daily. To this end, a naive male WU rat was introduced shortly in the female home cage approximately one hour before the lights went off. Display of hopping and darting, ear wiggling, and lordosis posture by the female was checked. Cycle length was determined by the last two monitored cycles, i.e. regular 4-day, regular 5-day, switcher 4-5 day, irregular (one or two times cycle length <3 or >5), or persistent estrus (>10 days cornified epithelium).

Experimental design

To investigate age-related differences in proestrous FSH surge characteristics, 4-month-old ('young') and 8.5-month-old ('middle-aged') female rats with regular 4-day (WU) or 5-day cycles (F1) were used for blood sampling and hormone analyses. Hourly blood samples were taken on proestrus to measure plasma FSH levels. An additional group of 5-day cyclic young (n=5) and middle-aged (n=5) WU rats was included to account for differences in hormone profiles between 4-day and 5-day cyclic rats. To investigate pituitary FSH responsiveness a potent GnRH analog (Ovalyse®; des-Gly^{10}-GnRH-ethylamide, Upjohn, Ede, The Netherlands) was used on the following proestrus. Ovalyse® (400 ng/ml, 0.25 ml injected per rat) was administered via the jugular vein cannula immediately after the first blood sample was drawn.

In addition, the ovarian follicular reserve was measured in young and middle-aged females of both rat strains. To this end, only regular 4-day (WU) or 5-day (F1) cyclic females (n=6 per group) were chosen, from which the ovaries were collected approximately 1-2 weeks after blood sampling for FSH surge measurements.

Cannulation and blood sampling

To obtain stress-free blood samples, the right jugular vein of the females was cannulated. After a recovery period of at least five days, ten hourly blood samples of 170 µl were taken on proestrus from ZT 5.5 to ZT 14.5 in 4- and 5-day cyclic rats. Plasma was 1:4 diluted in phosphate buffered saline (pH 7.5) containing 0.1% bovine serum albumin (Sigma Chemical, St. Louis, MO, USA) and stored at –20 °C until RIA analysis. A more detailed description has been reported previously (67).

Radio Immuno Assay (RIA)

FSH plasma levels were determined by a validated RIA, which is routinely used in our department (164,208). FSH plasma levels were analyzed by a double-antibody RIA. The first antibody used was NIDK-anti-rFSH-S-11 (dilution 1:80,000). The tracer (NIDK-rat FSH-I-7) was iodinated with ^{125}I by the chloramine-T method. The second antibody was a donkey-anti-rabbit antibody (AA-SAC1 Donkey-anti Rabbit) supplied by Wellcome Reagents (Beckenham, UK) and was diluted 1:20 in PBS containing 0.1% BSA (pH 7.5) and 10% polyethylene glycol (PEG 6000, Merck, Hohenbrunn, Germany). Plasma FSH levels were expressed in terms of the reference standard NIDK-rFSH-RP-2 (AFP-4621C). The assay sensitivity was 1.6-2.1 ng/ml FSH at 96% of maximal binding. FSH concentrations were determined in four separate assays. The inter- and intra-assay coefficients of variation were determined using pooled rat serum, and amounted to 12.1% and 10.8%, respectively.

Ovarian histology and ovarian follicular reserve counting

For histological examination of the ovarian follicular reserve pool, the right ovary of young and middle-aged WU and F1 rats was removed. Ovaries were fixed in Bouin's solution for 25 hours, dehydrated and embedded in paraffin. Serial histological sections (10 µm) were mounted on slides and stained with Harris' hematoxylin (Gurr®, BDH, UK) and eosin (Gurr®, BDH, UK), and embedded in Depex mounting medium (Gurr®, BDH, UK).

The ovarian follicular reserve was determined by counting primordial follicles (one layer of flat cells surrounding the oocyte) and very small primary follicles (one layer of cubical cells surrounding the oocyte) in every sixth section of the right ovary. Only those follicles with an oocyte that contained a clearly visible nucleolus were counted. The total ovarian follicular reserve of an animal was calculated by

multiplying the counted number of follicles by twelve, thereby taking intersection width and the left ovary into account. Based on the average total ovarian follicular reserve in WU and F1 rats at 4 and 8.5 months old, we calculated the number of follicles that leave the follicular pool per day and per estrous cycle, in which WU females were supposed to display 4-day estrous cycles and F1 females 5-day estrous cycles during the period between the age of 4.5 and 8 months. In the same way, we were able to predict at what age the follicular reserve in WU and F1 was likely to be exhausted, assuming that the rate of follicular depletion does not change after the age of 8 months.

Data processing and statistics

To determine the effects of age and strain on the FSH surge, several characteristics were defined: peak time, peak height and the total amount of FSH released during the endogenous FSH surge. Peak time of the FSH surge was defined as the sample hour (ZT) at which the highest FSH concentration was measured. The highest amount of FSH measured at that time was defined as the peak height. The total amount of FSH released during the FSH surge was determined in each animal by the cumulative value of FSH levels during the complete sampling period. Since our FSH assay appeared to be relatively insensitive, we were not able to determine basal FSH levels and onset time of the FSH surge. In case samples were undetectable (i.e. the counts were lower than that for 96% binding) a concentration of 2.1 ng/ml FSH was used (i.e. concentration FSH at 96% binding). FSH surge characteristics were also measured after the administration of the GnRH analog. In this case, the total amount of FSH released was calculated as described above. In addition, we calculated total FSH levels from ZT 5.5 until ZT 8.5 ('induced' FSH release) and total FSH levels from ZT 9.5 until ZT 14.5 ('endogenous' FSH release). These time points were based on our previous results on LH release after Ovalyse® administration on proestrus (67), since LH levels showed a clear distinction between 'induced' LH release resulting from Ovalyse® administration and a second endogenous LH surge resulting from endogenous proestrous GnRH release.

Data were analyzed using SPSS (version 10.1) and expressed as mean ± SEM. GLM-repeated measurement tests were performed per rat strain to test for significant changes in FSH hormone concentrations in time (endogenous FSH surge and FSH after administration of Ovalyse®). Two-way analysis of variance

(GLM – multivariate or univariate) was used to test for strain-, age-, and/or cycle-related differences in total FSH levels and peak height during the endogenous FSH surge, total FSH levels after Ovalyse® administration, and ovarian follicular reserve. Note that in all cases of FSH release after Ovalyse® administration, only data of 4-day cyclic WU and 5-day cyclic F1 were compared. When a significant difference was present, an independent sample t-test was performed to investigate where the difference was located. Peak time of the endogenous FSH was analyzed per group (young and middle-aged females with 4-day cycles (WU) or 5-day cycles (WU or F1) by the nonparametric Kruskal-Wallis test. Pearson correlation tests were performed to test for correlations between ovarian follicular reserve, total FSH and LH release (67), and peak levels of FSH and LH (67) during the surges on proestrus. A $p<0.05$ was considered to be significant.

Results

Cycle length

As expected, most young as well as middle-aged rats of the WU strain displayed 4-day estrous cycles, although females that displayed other cycle lengths (4-5 days, 5-day, irregular, or persistent estrous) were also present (Figure 1). The percentage of females that displayed regular 4-day cycles appeared to decrease with age and was associated with an increase in females that displayed 4-5 day or irregular cycles. In F1 rats, many females showed 5-day estrous cycles, although in young females there appeared to be a relative high number of rats with irregular cycles. Most of these females were 6-day cyclic and thus their cycle length was prolonged. In the middle-aged females of the F1 strain, almost all females displayed regular 5-day cycles (Figure 1).

Proestrous FSH release

On proestrus, FSH levels increased slowly and peak levels of approximately 6.8 ± 0.5 ng/ml FSH were reached around ZT 13. This increase in FSH levels was significant in 4-day and 5-day cyclic WU ($p<0.001$) as well as 5-day cyclic F1 ($p<0.001$) females (Figure 2), and there appeared to be no difference in peak time between groups. Peak time, peak levels and total FSH are depicted in Table 1, and statistical analysis revealed a clear effect of cycle length on peak FSH levels ($p=0.004$) as well as total FSH levels ($p=0.006$) during the preovulatory FSH surge.

Both peak and total FSH levels were higher in 5-day cyclic compared to 4-day cyclic females, independent of the genetic background. No significant differences were found between young and middle-aged cyclic rats.

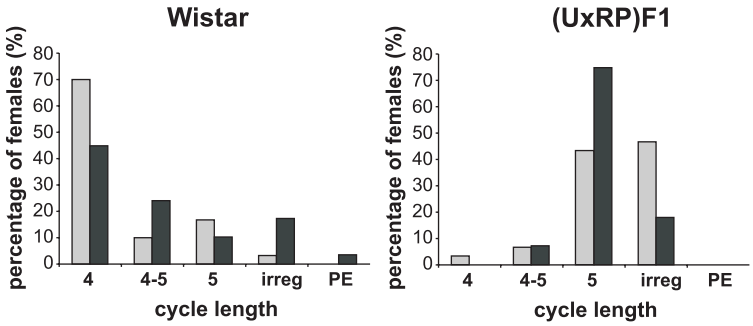

Figure 1. The percentage of young (light gray) and middle-aged (dark gray) females of the WU (A) and F1 (B) rat strain that displayed a specific cycle length during the last two cycles measured. **4,** regular 4-day; 5, regular 5-day; 4-5, switcher 4-5 day; irreg, irregular (one or two times cycle length <3 or >5); PE, persistent estrus (>10 days cornified epithelium).

Table 1. FSH surge characteristics of 4- and 5-day cyclic WU and 5-day cyclic F1 rats: peak time, peak levels, and total FSH levels of the natural FSH surge and total FSH levels of the FSH surge induced with Ovalyse®. *, significant difference with cycle length ($p<0.05$).

strain	cycle	age (months)	peak time (ZT)	peak FSH* (ng/ml)	total FSH* (ng/ml)	total after Ovalyse (ng/ml)
Wistar	**4-day**	4	13.3 ± *0.4*	5.5 ± *0.7*	34.0 ± *3.4*	97.2 ± *5.9*
		8.5	12.2 ± *0.5*	4.5 ± *0.6*	31.1 ± *3.1*	95.2 ± *7.9*
Wistar	**5-day**	4	13.7 ± *0.4*	7.6 ± *1.0*	43.9 ± *6.1*	136.1 ± *35.2*
		8.5	11.9 ± *0.7*	9.7 ± *3.1*	55.4 ± *12.8*	99.4 ± *19.2*
(UxRP)F1	**5-day**	4	12.8 ± *0.8*	8.8 ± *1.0*	46.7 ± *4.9*	121.8 ± *6.4*
		8.5	12.9 ± *0.2*	6.5 ± *0.7*	39.9 ± *4.1*	117.2 ± *7.2*

FSH levels increased directly after Ovalyse® treatment and remained elevated during the entire sampling period (Figure 3). This increase was significant for both WU ($p<0.001$) and F1 ($p<0.001$) females. A significant strain difference was found for total FSH release ($p=0.002$) and 'endogenous' FSH release (from ZT 9.5 until ZT 14.5; $p<0.001$), but not for the 'induced' FSH release (until ZT 8.5). Again, the results were not affected by age.

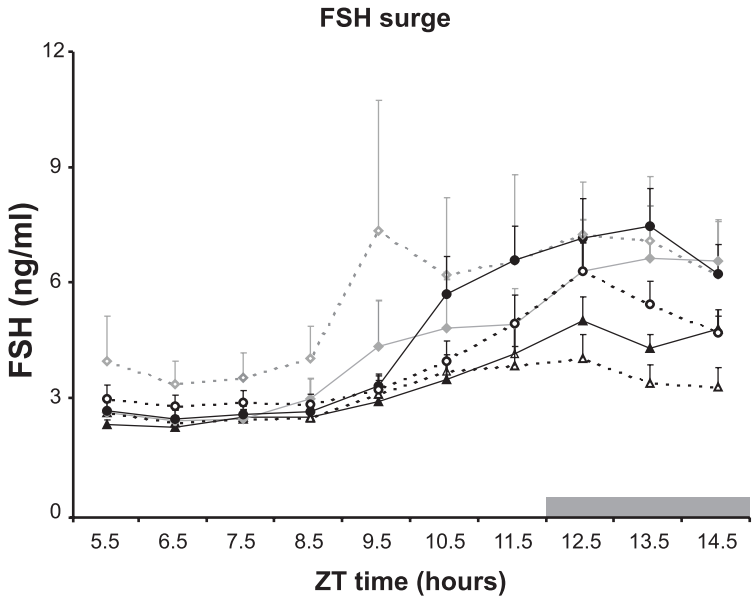

Figure 2. The proestrous FSH surge in young (closed triangles; n=12) and middle-aged (open triangles; n=9) 4- and 5-day (closed diamonds; n=5 and open diamonds; n=5, respectively) cyclic WU rats and in young (closed circles; n=10) and middle-aged (open circles; n=14) 5-day cyclic F1 rats. FSH levels are expressed as group means ± SEM and the black bar on the X-axis indicates the dark period of the light-dark cycle.

Ovarian follicular reserve

The total number of primordial and small primary follicles in young and middle-aged WU and F1 rats is shown in Figure 4. At 4 months old, the average ovarian follicular reserve amounted to 2762 ± 171 follicles. This number was significantly lower at the age of 8.5 compared to 4 months in WU ($p=0.013$) and F1 ($p=0.005$)

females. The size of the ovarian follicular reserve was significantly different between 8.5-month-old WU and F1 rats (p=0.043): WU rats (1520 ± 172 follicles) showed a smaller follicular reserve pool compared to F1 (2104 ± 185 follicles) females. The calculated number of follicles that left the follicular pool per day was higher in WU (7.7 each day) than in F1 (6.3 each day) females, although the number of recruited follicles per estrous cycle was comparable between these strains of rats (i.e. 30.8 for WU and 31.5 for F1). Based on these numbers, we predicted that the follicular reserve pool would theoretically be exhausted in WU rats around the age of 14.9 months, while in F1 rats this would occur much later (approximately at the age of 19.3 months).

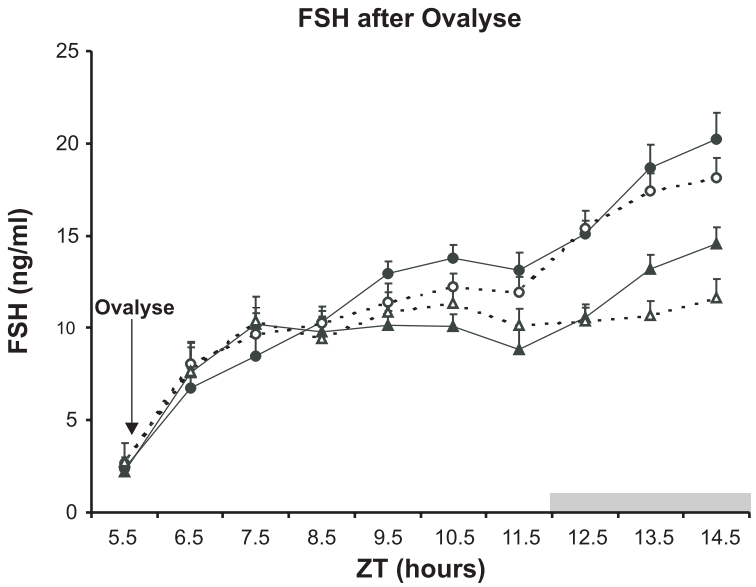

Figure 3. The proestrus FSH surge after Ovalyse® administration in 4-day cyclic young (closed triangles; n=14) and middle-aged (open triangles; n=10) WU rats and in 5-day cyclic young (closed circles; n=8) and middle-aged (open circles; n=12) F1 rats. FSH levels are expressed as group means ± SEM and the black bar on the X-axis indicates the dark period of the light-dark cycle.

In females in which the ovarian follicular reserve was determined as well as LH and FSH surges (n=20, with 4-6 females per experimental group), significant correlations between total FSH levels during the preovulatory surge and peak FSH

levels (r=0.95, p<0.001) or total LH levels (r=0.51, p=0.021) were found. Peak FSH levels were correlated with peak LH levels (r=0.46, p=0.040) and total LH levels (r=0.59, p=0.006). The total number of primordial and small primary follicles correlated with total LH levels during the proestrous LH surge (r=0.44, p=0.045), but not with peak LH or FSH levels or total FSH levels on proestrus.

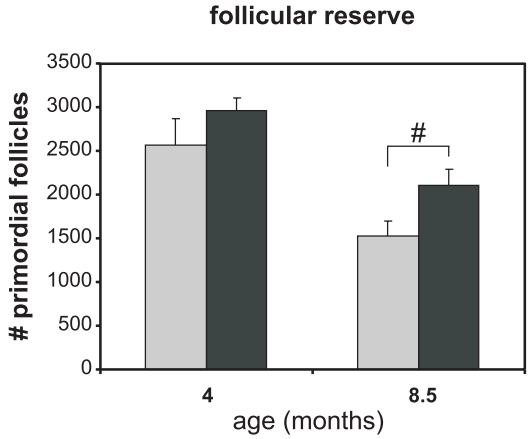

Figure 4. The total ovarian follicular reserve in 4- and 8.5-month-old WU and F1 rats. The total number of primordial follicles per group is expressed as mean ± SEM and was significantly different with age (p<0.05) and between rat strains (p=0.043, indicated with #).

Discussion

In the present study, we have clearly shown that the attenuation of the preovulatory LH surge in 8.5-month-old WU and F1 rats was not accompanied by a significant attenuation of the preovulatory FSH surge or an age-related change in pituitary FSH responsiveness to GnRH. We also demonstrated that the decrease in the ovarian follicular reserve with age differs between rat strains and was related to the magnitude of the proestrous LH, but not FSH surge. Previous studies suggested that age-related changes in LH levels may result from alterations in the hypothalamic-pituitary system, while age-related changes in FSH levels may result from alterations at the level of the ovary. Therefore, these results indicate that in female rats the contribution of the hypothalamus ('hypothalamic aging') appears to

be larger than the contribution of the ovary ('ovarian aging') to reproductive aging.

For the measurements of the FSH surge and ovarian follicular reserve, only regular 4- or 5-day cyclic WU and 5-day cyclic F1 were used in the present study. In general, the profile and timing of the preovulatory FSH surge resembled that of the LH surge in these animals, although the increase in FSH during the time window evaluated was less distinct than for LH (67). Just like in case of the LH surge, the magnitude of the FSH surge (peak FSH as well as total FSH) was higher in regular 5-day compared to 4-day cyclic female rats. These results suggest that it is likely that preovulatory LH and FSH levels are regulated by the same mechanisms, which include GnRH release and pituitary GnRH priming. In contrast to LH (67), however, no significant changes in the magnitude of the preovulatory FSH surge were found with age. This corresponds well with the observation that LH release is thought to be more sensitive to GnRH than FSH release (79), which may lead to more pronounced differences in LH levels when age-related changes occur in GnRH release. Furthermore, since we selected regular 4-day cyclic WU and 5-day cyclic F1 rats for our measurements, these data also suggest that changes in pituitary hormone concentrations (i.e. LH) precede the lengthening of the estrous cycle during early reproductive aging in rats.

We have also investigated the existence of age- and strain-related differences in pituitary responsiveness to GnRH. The administration of the potent GnRH analog Ovalyse® at proestrus just before the natural gonadotropin surges occur, resulted in a rapid increase in plasma FSH concentrations. In contrast to LH levels that decreased two (F1) or three (WU) hours after Ovalyse® administration (67), FSH levels remained elevated. This is probably the result of the higher rate of degradation of LH compared to FSH. After the Ovalyse®-induced FSH release, FSH levels appeared to increase after ZT 8.5 and, unlike LH levels, again slightly after ZT 11.5. It is likely that the first increase after the Ovalyse®-induced FSH (and LH) release results from the endogenous GnRH surge. The presumed second increase that was only found for FSH levels, may result from a decrease in inhibin levels caused by early luteinization of mature follicles. The significant strain difference we found in 'endogenous' FSH release (from ZT 9.5 until ZT 14.5) was most apparent during this presumed inhibin-related increase in FSH levels. If the increase in FSH is caused by the early luteinization of mature follicles, the difference in FSH release suggests a difference in granulosa/theca cell luteinization between strains (F1>WU) and thus in the release of progesterone (F1>WU), the

main steroid secreted by luteinized cells. However, previous results on P levels showed that P levels were slightly but consistently higher in WU compared to F1 rats during the examined time window (67). Thus, we can not explain the strain difference in induced FSH release, but we have shown that there are no age-related changes in cyclic as well as GnRH-induced pituitary FSH release.

As expected, a lengthening of the estrous cycle was found between the age of 4 and 8.5 months in WU females, since the number of females that displayed 5-day cycles increased with age. In F1 females, however, many young females displayed irregular cycles (i.e. 6-day cycles), while most middle-aged females had regular 5-day cycles. The genetic background of these young and middle-aged females was comparable, since the same dams were used for breeding these rats. Yet, a previous study in F1 females did show a clear lengthening of the estrous cycle with age (70): the percentage of F1 rats that displayed 5-day cycles decreased from 53% to 34% between the age of 4.5 and 9 months.

In addition to the increase in cycle length with age, we showed that the ovarian follicular reserve pool significantly decreased between 4 and 8.5 months in both rat strains. This was in accordance with literature (3,59,139,248). Yet, the rate of follicular loss may be strain dependent, since we found a difference in follicular reserve at the age of 8.5 months: ovaries of F1 females had a larger follicular pool than WU females at this age. This suggests that the follicular recruitment may be higher in WU compared to F1 rats. Indeed, we calculated that the number of follicles that left the follicular pool per day was higher in WU (7.7 per day) than in F1 (6.3 per day) females. The number of follicles recruited during each estrous cycle, however, appeared to be comparable between rat strains (approximately 31 follicles). Since we also demonstrated that estrous cycles were predominantly 4 days in WU and 5 days in F1 females, these results may suggest that the decrease in follicular reserve is related to the number of estrous cycles. While the postovulatory FSH surge on the morning of estrus is involved in the recruitment of follicles from the follicular reserve pool, it is likely that exposure to more of these FSH surges due to a shorter cycle length results in an accelerated depletion of the follicular reserve pool.

Interestingly, we observed that the ovarian follicular reserve was significantly correlated with total LH levels, but not with total FSH levels or peak LH and FSH levels on proestrus. Yet, total FSH and LH levels as well as peak LH and FSH levels were significantly correlated. Thus, females with a large magnitude and high

peak levels of preovulatory LH release also appeared to have a large magnitude and high peak levels of FSH release, and, in addition, had a large ovarian follicular reserve pool. Since an attenuation of the LH surge and a decrease in follicular reserve are indicative for reproductive aging (3), individual females with high LH surges appear to have a relatively 'young reproductive system'.

Using the average number of follicles at the age of 4 and 8.5 months in WU and F1 females, we predicted at what age the follicular reserve in WU and F1 is likely to be exhausted. Interestingly, the follicular reserve would theoretically be exhausted much earlier in WU rats (around 15 months of age) than in F1 rats (approximately 19 months of age). Both strains already showed an attenuated preovulatory LH surge (67) as well as changes in hypothalamic steroid receptor concentrations (69,70,Chapter 5) at the age of 8.5-9 months. Based on the literature, we know that cyclicity ends between 12-15 months of age in WU rats and between 8-10 months of age in F1 rats (270). Therefore, these data suggest that the contribution of changes at the level of the hypothalamus to the process of reproductive aging (i.e. hypothalamic aging) is larger in F1 rats than in WU rats.

We have demonstrated that the attenuation of the preovulatory LH surge and decrease in the concentration of hypothalamic steroid receptors with age preceded a lengthening of the estrous cycle as well as possible changes in proestrous FSH levels or in pituitary LH (67) and FSH responses to GnRH in rats. Taken together, the results of these studies suggest that at the age of 8.5-9 months it is not likely that there are changes in factors such as inhibin that regulate FSH release. Since we did find an attenuation of the LH surge (67) and an impaired feedback of gonadal steroids on the hypothalamus (69,70,Chapter 5) at this age, these results strongly suggest that in the female rat changes in GnRH secretion and GnRH priming ('hypothalamic aging') precede a change in inhibin concentrations ('ovarian aging'). It is also clear that based on the above, preovulatory LH levels can indeed be used as an early indicator for reproductive aging in the rat.

Although the LH surge is also attenuated in humans, one of the first indications of reproductive aging appears to be a decrease in inhibin B levels, resulting in an increase in FSH levels and an acceleration in the rate of loss of primordial follicles around the age of 37-38 years ('ovarian aging'). Based on this, the elevation in FSH concentrations at day 3 of the menstrual cycle can be used as an indicator for a declining ovarian reserve, and thus for early reproductive aging in women (118,224). Several other studies have indicated that there are also limitations to the

use of day 3 FSH concentrations (58,118). A decrease in preovulatory LH levels without a change in FSH levels has also been reported in infertile women with apparently regular cycles (269). This raises the possibility that in a subset of women an improper functioning of the hypothalamus-pituitary system, as reflected by a reduced LH surge and the associated decrease in fertility (269), could also underlie a decreased fertility. In general, these data suggest that also in humans, it is likely that changes in the ovary as well as the hypothalamus-pituitary system contribute to reproductive aging, but that their relative contribution may differ between individuals and species.

In conclusion, our results suggest that in aging female rats changes in the hypothalamus-pituitary system, i.e. the attenuation of the GnRH and LH surge, precede changes at the level of the ovary, i.e. a decrease in inhibin levels that would result in an increase in FSH levels. Since the ovarian follicular reserve pool clearly decreased with age and correlated with proestrous peak LH, but not FSH levels, these data suggest that the magnitude of the preovulatory LH surge may be used as an indicator for 'ovarian aging'. Altogether, the present results indicate that differences between individuals and species in fertility and reproductive aging may depend on different limitations and contributions of specific factors, such as the hypothalamus, pituitary, ovarian follicular reserve, and uterus, to the regulation of follicular maturation, ovulation, implantation, and pregnancy maintenance.

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Chapter 7

Summary & General Discussion

Fertility and fecundity decline in female mammals long before reproductive senescence sets in. In the past decades research has focussed on the possible mechanisms that may underlie reproductive aging. Age-related changes that influence fertility and fecundity have been demonstrated at the level of the hypothalamus (131,144,215,219), pituitary gland (45,205), and ovary and uterus (205,224). An attenuation of the preovulatory LH surge, which is responsible for ovulation, appears to be one of the first indications of reproductive aging in rats (134,181,215,264,285,291), but the specific contribution of changes in the hypothalamus, pituitary and ovary to this attenuation is currently unclear.

The present thesis provides further evidence that changes in the hypothalamic-pituitary system may contribute substantially to the age-related decrease in fertility in female mammals. Furthermore, our data strengthen the hypothesis that, at least in rats, hypothalamic changes may precede alterations at the level of the ovary.

In this chapter we aim to (I) summarize and discuss the changes that occur during early reproductive aging in the rat as found in this thesis and in literature, (II) discuss the strain differences in the regulation of the reproductive system as reported in this thesis, (III) propose a general mechanism of reproductive aging in the rat, and (IV) discuss what our results teach us with respect to the reproductive decline with age in women.

7.1. "Early" reproductive aging in rats

To investigate age-related changes that may influence fertility and fecundity, we used female rats that were either young mature (4-4.5 months old) or at a more advanced reproductive age (8.5-9 months old), further referred to as "middle-aged". With respect to aging, these middle-aged females were still in the reproductive phase of their life and displayed regular estrous cycles of normal length (i.e. 4 or 5 days). Also, we compared data of two rat strains that are thought to age at a different rate, i.e. 4- or 5-day cyclic Wistar (abbreviated as WU) and 5-day cyclic (UxRP)F1 (abbreviated as F1) rats (see also Table 1). In this way, we expected to

gain insight in the interaction between the different levels of the hypothalamus-pituitary-gonadal (HPG) axis that assures cyclicity during reproductive life, and in the contribution of each of these levels to the changes that lead to the end of cyclicity.

The ovary

At the level of the ovary we measured in our young and early middle-aged rats the decline of the ovarian follicular reserve and the concentrations over the estrous cycle of two ovarian steroid hormones,E_2 and P, that are predominantly secreted by growing follicles and corpora lutea (CL) (**Chapter 2, 3, 4, and 6**). In this way, we assessed the ability of the ovary to develop follicles and possible changes in follicular development (E_2), CL formation and CL secretory activity (P) with age.

Complying with literature data from rats (139), humans (248), and other mammalian species (270), we found that the ovarian follicular reserve decreased between the age of 4.5 and 9 months in both rat strains (**Chapter 6**; see also table 1). By extrapolating this decrease, we were able to estimate the age when the follicular pool would theoretically be exhausted. This appeared to be at approximately 15 (WU) and 20 (F1) months of age (**Chapter 6**), suggesting that the ability of the ovary to develop follicles is still adequate in both strains at the age of 9 months. Interestingly, an age-related increase in E_2 as well as in P concentrations was found during the estrous cycle in F1, but not WU rats (**Chapter 3 and 4**), while the preovulatory surge in P showed no significant changes with age (**Chapter 2**). The age related increase in E_2 concentrations with age in F1 rats is in agreement with a previous study in 10-12 month-old Long-Evans rats (181), but the increase with of P levels has not been previously reported in other rat strains. However, the responsiveness of the ovary to hCG stimulation was increased in regular cyclic, but not in persistent estrous middle-aged compared to young Long-Evans rats (37), suggesting that an attenuation of the LH surge with age may be accompanied by an increased ovarian sensitivity to LH. Since preovulatory P levels have been shown to decrease in concert with LH levels in 12-month-old Long-Evans rats (170), an attenuated preovulatory LH surge may only be accompanied by an attenuated P surge at a more advanced reproductive age. Thus, the age-related changes in steroid hormone concentrations found in our F1 rats suggest alterations in ovarian steroid hormone synthesis and/or degradation with age. This may result from, or may cause, changes in follicular growth or CL formation and activation,

which are both regulated by timed pituitary gonadotropin secretion.

The anterior pituitary gland

At the level of the anterior pituitary gland, we investigated the magnitude and timing of the preovulatory gonadotropin surges. In addition, the proestrous LH and FSH response to GnRH stimulation was tested to find out if a change in sensitivity of the pituitary gland to GnRH could underlie the expected attenuation of the LH surge with age (**Chapter 2 and 6**).

Indeed, the middle-aged females of both strains showed an attenuation of the proestrous LH (**Chapter 2**), but not FSH (**Chapter 6**) surge compared to young females. No changes in timing (i.e. onset or peak time) of gonadotropin surges were found. Basal LH levels appeared unchanged with age (**Chapter 2**), while we were unable to determine basal FSH levels due to assay limitations (**Chapter 6**).

Table 1. Summary of the age-related changes in parameters measured in this thesis in 4-day cyclic Wistar and 5-day cyclic (UxRP)F1 rats. cyc: over estrous cycle, pro: on proestrus, ovx: in OVX+EB treated females, ova® : after stimulation with Ovalyse®, ↑: increase with age, =: no changes with age, ↓: decrease with age.

	ovary		***pituitary***		***hypothalamus***					
	E_2	***P***	***LH***	***FSH***	***AvPv***		***MPO***		***ARC***	
strain	cyc	cyc, pro	pro, ova®	pro, ova®	PR cyc,ovx	ER cyc,ovx	PR cyc,ovx	ER cyc,ovx	PR cyc	ER cyc
WU	=	= , =	↓ , =	= , =	↓ , =	↓ , =	↓ , =	↓ , ↓	↓	↓
F1	↑	↑ , =	↓ , =	= , =	↓ , ↓	↓ , =	↓ , ↓	↓ , =	↓	↓

An age-related attenuation of the LH surge was previously demonstrated in rats (181,264,285) and humans (269). Interestingly, in humans this attenuation appears to be preceded by changes in FSH levels (27,122,123,224,239,248), while the present data as well as previous research (46,109) suggest that the attenuation of the LH surge precedes changes in FSH concentrations in rats. These data indicate a species difference in the contribution of the different levels of the HPG-axis in the

process of reproductive aging. The present data confirm the idea that the age-related attenuation of the LH surge is indeed one of the first signs of reproductive aging in the female rat, but what is the underlying cause? The magnitude of the LH surge can be influenced by preovulatory P levels, GnRH priming, the capacity of the pituitary gland to secrete LH, and preovulatory GnRH release. Our results show that preovulatory P levels remain unaltered with age (**Chapter 2**), and that *in vivo* stimulation of the pituitary gland with the potent GnRH analog Ovalyse® on proestrus results in a comparable increase in plasma LH and FSH concentrations between young and middle-aged rats (**Chapter 2 and 6**). Thus, the attenuation of the LH surge with age is neither caused by changes in preovulatory P levels nor the secretory capacity of the pituitary gland for LH. Previous research in our lab demonstrated an impairment in GnRH priming in 9-month-old Wistar rats (117), and several other studies suggest a decrease in GnRH secretion as well (211,212). The fact that, despite existing strain differences (see also §7.2.), the age-related changes in pituitary hormones were consistent between the two rat strains further supports a more general role for the hypothalamus-pituitary system in reproductive aging in the female rat.

The preoptic area and hypothalamus

The most consistent and obvious changes with age in the HPG-axis were found at the level of the hypothalamus: a significant decrease in the number of PR- and ERα-IR cells in the AvPv, MPO and ARC of both rat strains (**Chapter 3, 4 and 5**). This decrease was evident in cyclic females, and also in the OVX+EB treated females albeit less dramatic (see Table 1).

Previous reports on age-related changes in PR and ER concentrations in the brain support our results, although the results may differ between brain areas. In general, PR (289) as well as ER protein concentrations (287,289) and E_2-binding capacity (25) appear to decrease with age, while E_2-induced P-binding capacity remains unchanged (25). Since the downregulation of ERα appeared faster in middle-aged compared to young OVX+EB-treated F1 rats, while P appeared to reduce ERα concentrations only in middle-aged OVX+EB-treated WU rats (**Chapter 4**), these data suggest that the decrease in ERα concentrations with age may result from an increased sensitivity of ERα to the E_2- as well as the P- induced reduction in receptor concentrations. The effect of P on ERα in the brain was novel and surprising, although it was previously reported for other tissues like the uterus

and ovary (5,298).

Our results indicate a clear decrease in PR-IR with age (**Chapter 3**). We showed in middle-aged OVX+EB-treated F1 females that this decrease in PRs results from a reduction in E_2-induced synthesis of PR. Since ERα is involved in the E_2-induced synthesis of PRs (132,151), these results suggest that the increased sensitivity of ERα to E_2 in middle-aged compared to young cyclic rats leads to a decreased concentration of ERα, that results in a decreased concentration of PR in several brain areas.

Interestingly, the decrease in PR- and ERα-IR cells appears to be more pronounced in the ARC compared to the AvPv and MPO in both WU and F1 rats, since in the ARC the number of cells containing PR and ERα was decreased on every day of the estrous cycle. This may indicate that during reproductive aging in the rat, the changes in sensitivity to gonadal hormone feedback first become evident in the ARC, even before they become apparent in the AvPv and MPO. Consequently, this would suggest that the changes in pulsatile release of GnRH and the sensitivity to negative feedback of E_2 and P on GnRH release (i.e. the ARC as the 'pulse generator') precede changes in sensitivity to the positive feedback of E_2 and P on GnRH release (i.e. the POA as the 'surge generator'). This issue requires further investigation.

Apart from the changes at the level of the ovary, pituitary gland, and hypothalamus, we found an increase in cycle length with age in both WU (**Chapter 2 and 6**) and F1 rats (**Chapter 2**). Note, however, that all measurements reported in this thesis were performed in rats that were regularly 4- or 5-day cyclic. It is therefore evident that the age-related changes found in this thesis precede the lengthening of the estrous cycle with age.

Correlations between hormone levels and receptor numbers with age

Since several components of the reproductive system (E_2, P, PR and ERα in AvPv, MPO and ARC) were measured within a single animal on a specific day of the estrous cycle, it was possible to search for correlations between these components. In this way, the mechanism(s) involved in the regulation of the reproductive system, in particular in age-related changes in fertility, might emerge. When data of cyclic young and middle-aged WU (n=5-7 and n=8-9, respectively) and F1 (n=14-18 and n=14-17, respectively) females were analyzed, we found a

number of interesting correlations (Table 2).

Positive correlations were found between the AvPv and MPO in all groups for ERα as well as PR cell numbers, except for PR in young WU rats. The correlation found for ERα and PR expression between the AvPv and MPO may suggest that these brain areas are regulated in the same way and may have comparable functions, i.e. the positive feedback of E_2 (and P) on GnRH release. However, no correlations were found between E_2 levels and PR concentrations, with the exception of the ARC of middle-aged F1 females (r=0.585 with p=0.022).

Furthermore, all groups showed a negative correlation between E_2 levels and ERα cell numbers in the ARC (except for young F1 females), but not in the AvPv, nor the MPO. These data suggest that a rise in E_2 levels resulting from the growth of follicles in young females leads to a down regulation of ERα in the ARC. Since the ARC is thought to be involved in the negative feedback of E_2 and P on GnRH release (82), this down regulation of ERα may reduce the negative feedback on GnRH release arising from the ARC.

The most interesting finding was that ERα numbers in the ARC were negatively correlated with PR numbers in the AvPv in young (WU: r=-0.785 with p=0.037; F1: r=-0.684 with p=0.002), but not in middle-aged WU and F1 females. This may suggest that a downregulation of ERα in the ARC may result in an increase in PRs in the AvPv. If this is true, the ARC could also be involved in an increase in positive feedback, since PRs in the AvPv play a crucial role in the generation of the GnRH and LH surge in rats (35,151).

The absence of a correlation between ERα in the ARC and PR in the AvPv in middle-aged females of both rat strains may be illustrative of an age-related impairment in the positive feedback of E_2 on GnRH release as suggested by the decreased PR induction in these females.

→

Table 2. Significant correlations between the concentrations of E_2 and P in plasma and the number of ER-IR and PR-IR cells in the ARC, AvPv and MPO in 4.5- (y) and 9- (a) month-old WU and F1 females. The Pearson Correlation coefficients and p values are given between brackets.

			ARC		AvPv		MPO	
			ER	PR	ER	PR	ER	PR
	E2	y WU	-0.890 (0.018)					
		a WU	-0.870 (0.005)					
		y F1		0.756 (0.001)				
		a F1	-0.722 (0.002)	0.585 (0.022)				
	P	a WU			0.757 (0.018)			
ARC	ER	y WU				-0.785 (0.037)		
		y F1				-0.684 (0.002)		
		a F1					0.657 (0.011)	
	PR							
AvPv	ER	y WU					0.923 (0.003)	
		a WU					0.904 (0.001)	
		y F1				0.508 (0.037)	0.934 (<0.001)	
		a F1					0.857 (<0.001)	
	PR	a WU						0.917 (<0.001)
		y F1						0.837 (<0.001)
		a F1						0.887 (<0.001)
MPO	ER	a WU						-0.721 (0.028)
	PR							

In addition, the results in F1 and WU rats strongly suggest that, in the sequence of events during reproductive aging, age-related changes in ovarian hormone concentrations are secondary to changes at the level of the hypothalamus (ERα and PR) and pituitary gland (LH).

These data suggest a change in the feedback actions of the ovarian hormones E_2 and P to up- and down regulate the number of hypothalamic steroid receptors during reproductive aging. This may lead to impaired preovulatory GnRH secretion and/or pituitary priming, resulting in an attenuation of the preovulatory LH surge with age.

7.2. Individual differences: rat strain and cycle length

Besides the age-related changes summarized above, we also found differences in the regulation of the reproductive axis between rat strains.

Firstly, our results clearly support the idea that the rate of reproductive aging differs not only between individuals, but also between (rat) strains. Based on the age-related changes in the reproductive system (see Table 1), it can now be concluded that 9-month-old F1 females indeed are reproductively aged to a further extent than age-matched WU females.

Secondly, there were significant strain differences in the number of primordial and small primary follicles at the age of 8.5 months but not at 4 months (F1>WU; **Chapter 6**), suggesting that the rate of follicular depletion is higher in WU than in F1 females. Indeed, the number of follicles that enters the growing pool is estimated to be comparable between rat strains when expressed as the number per complete estrous cycle, but not when expressed per cycle day (5-day cyclic F1<4-day cyclic WU; **Chapter 6**).

Thirdly, the number of days with elevated E_2 levels is higher in 5-day cyclic F1 than in 4- and 5-day cyclic WU rats (**Chapter 3 and 4**), which suggests that the follicular growth, as reflected by E_2 levels, may differ between strains. This has not been investigated in the present setup.

Fourthly, the amount of acutely releasable LH after GnRH stimulation was significantly higher in WU than in F1 females (**Chapter 2**), which suggests that the proestrous readily releasable pool of LH is larger in WU than in F1 females.

Finally, the number of ERα-IR positive cells in the AvPv, MPO, and ARC of cyclic (F1>WU) as well as in OVX+EB-treated rats (AvPv and MPO: F1<WU) differed between strains (**Chapter 4 and 5**). Since the total number of cells containing ERα-IR was comparable between rat strains (i.e. ERα cell numbers in OVX females at 2 h after EB administration; **Chapter 4**), the strain difference in ERα cell numbers likely results from a difference in ovarian feedback on ERα concentrations.

The strain differences in follicular depletion and follicular growth could result from differences in basal LH and FSH levels, postovulatory FSH surge concentrations, or follicular atresia as found in mice strains (59). Proestrous basal LH levels were comparable between F1 and WU rats (**Chapter 2**). Yet, the increase in FSH after Ovalyse® administration appeared to be higher in F1 compared to WU rats, suggesting that also the postovulatory FSH surge may be higher in F1 compared to WU rats (**Chapter 2 and 6**). Since a higher postovulatory FSH surge is likely to result in more instead of less follicular growth, both basal LH and postovulatory FSH levels do not appear to underlie the observed strain difference in follicular growth and depletion. Alternatively, strain differences in ERα concentrations and the magnitude of the releasable pool of LH may result from differences in sensitivity to ovarian hormones (ERα-IR, LH) and/or GnRH priming and release (LH). These issues require further investigation.

The estimated age at which the follicular pool is exhausted differed between F1 and WU rats: it appeared to be approximately 15 (WU) or 20 (F1) months (**Chapter 6**). However, cyclicity is thought to end at the age of 8-10 months in F1 and at the age of 12-15 months in WU rats (270). These data suggest that, especially in the F1 strain, changes other than the depletion of the follicular reserve lead to an end of fertility and cyclicity.

Previous research demonstrated that for the initiation of the LH surge the PRs in the AvPv play an important role (35). The number of PR-IR positive cells in the AvPv of young WU females, in contrast to F1 females, did not show a clear change between metestrus morning and proestrus afternoon. This may suggest that the role or contribution of PRs in the initiation of the LH surge is smaller in WU than F1 females. In addition, we showed that in 4-day cyclic WU females of the normal LH surge was smaller in magnitude, while the GnRH-induced LH release was larger (**Chapter 2**) compared to F1 females.

Based on these results, we hypothesize that the magnitude of the LH surge in 5-day cyclic F1 females mainly depends on the hypothalamus (i.e. on the E_2-induced PR expression and the resulting GnRH release), while in 4-day cyclic WU females it depends primarily on the pituitary gland (releasable LH, GnRH priming).

With respect to literature, previous studies have demonstrated changes in the timing of the LH surge with age (173,215,284,285), but only in Sprague-Dawley rats. Therefore, the delay in timing of the LH surge with age may be strain specific or the changes in SCN functioning that appear to underlie the delayed LH surge are expressed at a relatively early age in Sprague-Dawley rats (**Chapter 2**).

Besides these differences between rat strains, also the length of the estrous cycle may be important concerning the differences between 4-day cyclic WU and 5-day cyclic F1 rats as reported in this thesis. The magnitude of the preovulatory LH and FSH surges was larger in 5-day compared to 4-day cyclic female rats (**Chapter 2 and 6**), and the LH and FSH surge profiles were comparable between 5-day cyclic F1 and WU females. Although P levels over the estrous cycle appeared comparable between 5-day cyclic WU and F1 females (unpublished observations), E_2 levels were different: in 4- as well as 5-day cyclic WU rats E_2 levels were elevated for only one day as opposed to two days in 5-day cyclic F1 females. Thus, although hormone concentrations may differ with cycle length (for example the magnitude of the LH and FSH surge), not all differences we found between 4-day cyclic WU and 5-day cyclic F1 rats were related to cycle length. Together, these data suggest that, besides cycle length, strain differences also affect the regulation of the reproductive system.

In conclusion, the F1 strain appears to be reproductively aged to a further extent judging from the E_2, P, and PR data, while follicular depletion appears to be more advanced in the WU strain. Therefore, in some rat strains the capacity of the follicular pool may represent a serious limitation as found for certain mice strains (59). Although (strain)differences may be present in the sensitivity of different components of the reproductive system to aging, the primary limitation contributing to reproductive aging in rats appears to be at the hypothalamic level, i.e. the ability of steroid hormones to regulate the number of steroid receptors.

The data presented in this thesis suggest that the contribution and limitation of different components of the reproductive axis (hypothalamus, pituitary gland, and ovaries) may vary among individuals and (rat) strains.

7.3. Proposed mechanism of the hypothalamic-pituitary system and reproductive aging

In the present thesis we demonstrated that during early reproductive aging in the rat the number of hypothalamic cells containing ERα and PR falls dramatically. This led us to conclude that the sensitivity of hypothalamic steroid receptors to the feedback of E_2 and P changes with age, and that this alteration in the reproductive axis underlies the age-related attenuation of the preovulatory LH surge. But how are these changes in ERα and PR concentrations translated to the GnRH system, i.e. which neurotransmitter systems are involved?

Proposed mechanism for the induction of the LH surge

Previous research reported that ER- and PR-mRNA and/or protein is expressed in preoptic and hypothalamic neurons that have also been reported to express a whole array of neurotransmitters. These neurotransmitters include: nitric oxide (NO) (55,56), vasopressin (VP) (8), dopamine (DA) (105), somatostatin (55,56), galanin (272), γ-aminobutyric acid (GABA) (149,250), glutamate (250), NPY (271), substance P (55), enkephalin (234), dynorphin (234), and neurotensin (2,55,56). In addition, 2-4% of the β-endorphin (β-END)- and NPY-neurons in the ARC express the ERα (235), and 5-20% of the NPY-neurons binds E_2 (218), while gene expression of both β-END (198,280) and NPY (111) is influenced by E_2 and/or P. E_2 also affects glial cells, astrocytes, and vascular endothelial cells (33,126,127,202,204,301). In this way E_2 is able to influence glial ensheetment and synaptic morphology in the preoptic area (33) and the ARC/ME region (107,174,179), thereby regulating neuronal synaptic input and neurotransmitter release.

Hypothalamic PRs and the daily neural signal from the SCN

Recently it was demonstrated that the E_2-induced expression of PRs in the AvPv plays a crucial role in the regulation of the preovulatory LH surge (35). It was proposed that a daily neural signal from the SCN activates the PRs through a ligand-independent mechanism (i.e. indirectly via cAMP) and that these activated PRs regulate processes that culminate in the initiation of the GnRH surge (151). Based on the finding that P levels are elevated for one extra day in 5-day compared to 4-day cyclic females (265,this thesis), we expand this proposed

mechanism by stating that besides an increase in E_2, a decrease in P and hence a decreased down-regulation of its own receptor may also be important for the increase in PR expression.

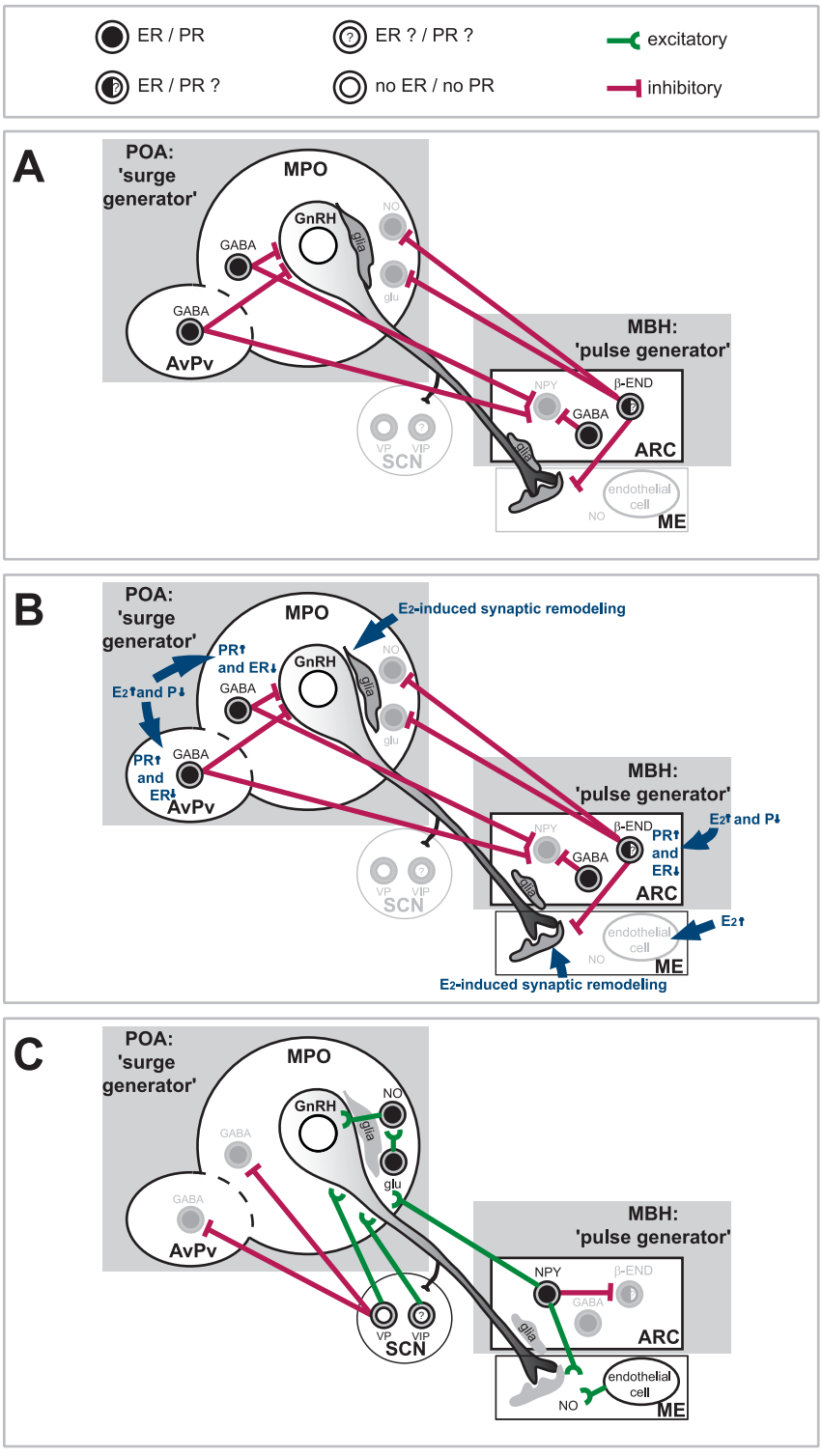
A number of studies suggest that VP is the SCN-derived daily signal that activates PRs in the POA. Not only is VP infusion in the POA capable to induce an LH surge in SCN-lesioned female rats (192), but also the circadian rhythm of GnRH and VP release are similar in co-cultures of the POA and SCN (274). Moreover, E_2 is able to increase VP receptor expression (V1a) in GABA-containing cells of the POA (78).

Besides VP, the SCN has direct sexually dimorphic projections to GnRH neurons that contain VIP (104,225,261) and VIP-innervated GnRH neurons are activated during the LH surge (260). Several studies indicate that these VIP connections are involved in the timing and magnitude of the preovulatory LH surge (259,278,279).

Thus, the SCN may stimulate GnRH release directly via VIP and indirectly via VP. With respect to VP we hypothesize that the E_2-induced increase in V1a receptors in GABA-containing neurons of the POA sensitizes these neurons to the daily increase in VP. The activation of V1a receptors by the increase in VP release on proestrus morning (112), may increase intracellular cAMP levels (125), resulting in the ligand-independent activation of the PRs in these GABA-containing neurons (34,35) (see also Figure 1A and 1B).

→

Figure 1. Schematic representation of the hypothalamic processes that result in the preovulatory GnRH surge. **A**, the normal negative feedback of low estradiol (E_2) levels on neurotransmitter release. **B**, the transition from negative to positive feedback on GnRH release by the increase in E_2 and the concurrent decrease in progesterone (P) levels. **C**, the positive feedback results in specific neurotransmitter release that stimulates GnRH release from the hypothalamus. For abbreviations see text.


ER / PR
ER ? / PR ?
excitatory
ER / PR ?
no ER / no PR
inhibitory
A
POA:
'surge
generator'
MPO
GnRH
NO
glia
GABA
glu
GABA
AvPv
MBH:
'pulse generator'
NPY
β-END
GABA
ARC
VP
VIP
SCN
glia
endothelial
cell
NO
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B
POA:
'surge
generator'
MPO
E2-induced synaptic remodeling
PR↑
and ER↓
GnRH
NO
glia
E2↑and P↓
GABA
glu
PR↑
and
ER↓
GABA
AvPv
MBH:
'pulse generator'
NPY
β-END
PR↑
and
ER↓
E2↑ and P↓
GABA
ARC
VP
VIP
SCN
glia
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The decrease in inhibitory GABA signaling and the activation of the NPY system

A role for GABA in the regulation of the female reproductive cycle appears very likely: GABA-ergic neurons innervate GnRH neuronal cell bodies, GABA inhibits GnRH release (44), GABA turnover shows a clear sex difference (223), and E_2 decreases GABA-ergic synaptic input to neuroendocrine ARC neurons (193). Therefore, VP release on proestrus morning (112) that appears to result in the activation of the PRs in GABA-containing neurons (34,35), may lead to the decrease in GABA release that is seen on proestrus afternoon (108).

The activation of hypothalamic PRs also leads to an increase in NPY release and NPY-receptor expression in the hypothalamus (297), suggesting that the E_2-induced PR expression not only decreases GABA levels and/or GABA-ergic input to the ARC, but also induces an increase in NPY release at the level of the ARC. The activation of the NPY system by the E_2-induced PR expression can be direct via PR activation of NPY neurons, or indirect via a decrease of the inhibitory GABA-ergic input to ARC-NPY neurons. An increase in NPY release stimulates GnRH release either via direct contacts with GnRH cell bodies (235,238) or by increasing the sensitivity of pituitary gonadotrophs to GnRH, both resulting in an increased LH release (111,296) (Figure 1C).

The decrease in negative feedback

The activation of the NPY system by PRs was proposed to lead to the inactivation of the β-END system (297). Indeed, NPY is a potent inhibitor of electrical activity in the ARC (135) and innervates β-END neurons in the ARC (111), suggesting that NPY inhibits the activity of β-END neurons. Furthermore, E_2 can inhibit and induce a diurnal rhythm in β-END mRNA expression (198,280), β-END neurons project to GnRH neurons in the POA and in the ME, and β-END inhibits GnRH release (111). At the POA, β-END also inhibits NO-containing cells directly (19) or via interneurons containing glutamate (16). Because NO stimulates GnRH release (129), the disinhibition of NO by a decrease in β-END on proestrus morning may result in an increase in GnRH release and subsequently the GnRH and LH surge. In summary, these data suggest that on proestrus morning the negative feedback on GnRH release (exerted by β-END and GABA) is abolished or diminished, thereby allowing the generation of the preovulatory GnRH surge (Figure 1C).

NO-induced synaptic remodeling at the ME

Besides its role at the preoptic area, NO appears to play an important role in the ARC/ME area. Endothelial cells in the ME release NO in an E_2-concentration dependent manner (126,129,203,204), whereafter NO enhances GnRH release by actions on GnRH nerve endings (203,204). Since NO is thought to induce an increase in cAMP formation in neuroendocrine nerve terminals in the ME, it was hypothesized that endothelial NO release underlies the synchronized, pulsatile and massive release of GnRH by facilitating GnRH release into the portal blood capillaries due to a cytomorphological reorganization in the ME (i.e. by modulating the extent of glial ensheetment and/or sprouting of GnRH nerve terminals) (203). Therefore, the E_2-induced increase in NO release on proestrus may enhance GnRH release by the hypothalamus.

The above represents a simplified model of the cascade of events at the level of the brain that regulate GnRH release during the normal estrous cycle (see also Figure 1 and 2).

In summary, during the estrous cycle the increase in plasma E_2 (growing follicles) in combination with a decrease in plasma P concentrations (inactivation of CL; thus an increased E_2/P ratio) leads to an increase in PR-containing cells and a decrease in ER-containing cells in several brain areas. The resulting sensitization of preoptic GABA neurons to the daily signal from the SCN (i.e. VP), leads to the timed inactivation of GABA-ergic neurons by VP. The decrease in GABA in concert with the increase in PRs activates the hypothalamic NPY system, which inhibits the activity of the hypothalamic β-END system. The decrease in inhibitory β-END and the increase in excitatory NPY both result in the activation of GnRH neurons, increased GnRH release, and an increased sensitivity of the pituitary gonadotrophs to GnRH, thereby inducing the preovulatory GnRH and LH surge that results in ovulation of mature follicles.

The proposed model is supported by the correlations we found between E_2 levels and ERα and PR concentrations in the brain. ERα concentrations in the ARC appear to be an indicator for the amount of negative feedback on GnRH release (i.e. β-END), while PR concentrations in the AvPv may be an indicator for the amount of positive feedback on GnRH release (i.e. VP-induced decrease in GABA release, stimulated NPY release).

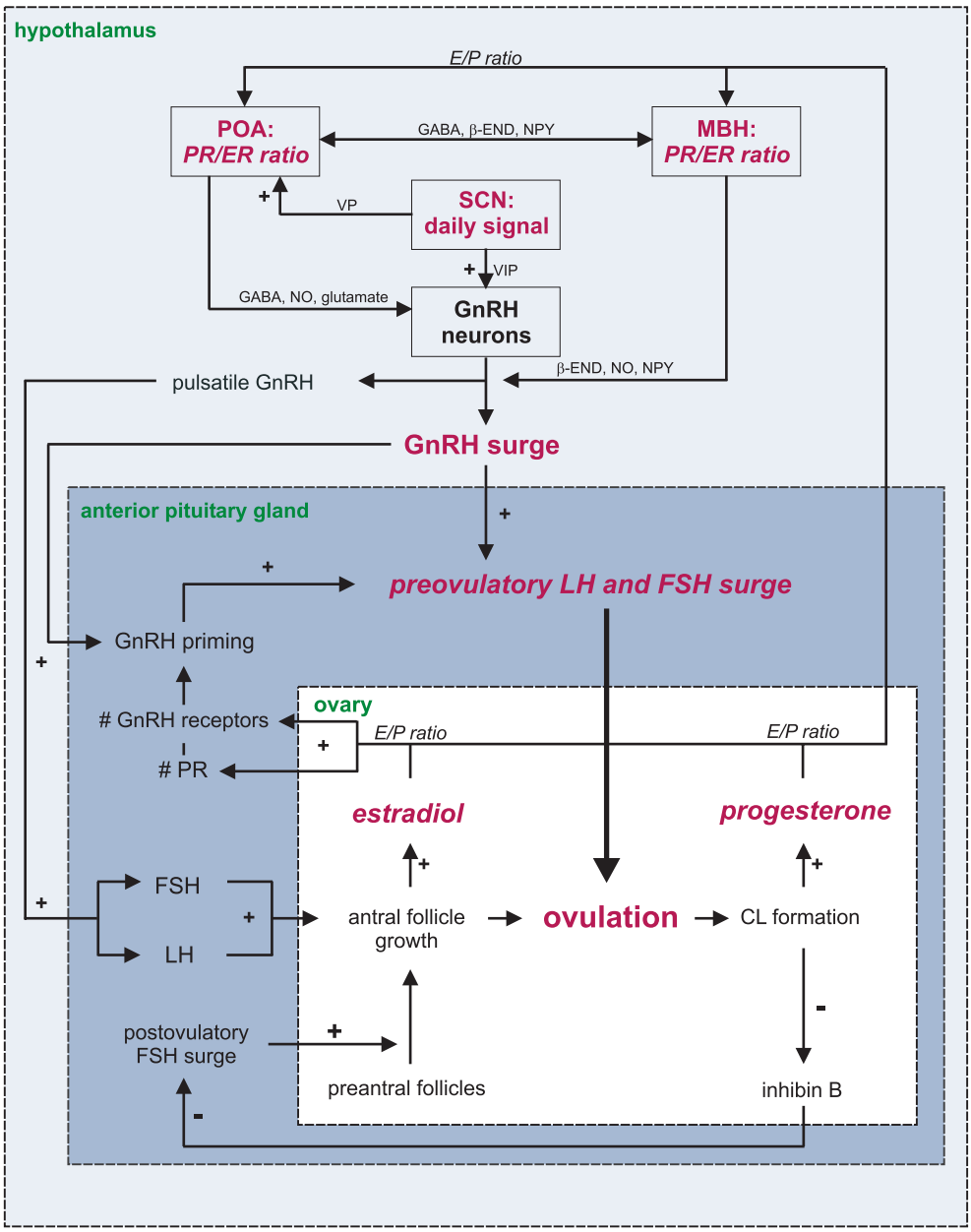

Figure 2. Schematical summary of the processes at the level of the hypothalamus, pituitary gland and ovary that were dealt with in the present thesis and that are involved in the regulation of the estrous cycle and ovulation in the rat. The components that were measured in this thesis are in italic. Arrows represent (hormonal) actions that can be stimulating (+) or inhibiting (-). For abbreviations see text.

Thus, the absence of a correlation between the PR in the AvPv and ERα in the ARC in middle-aged rats may suggest an impairment with age in the shift between negative and positive feedback on GnRH release.

In conclusion, the E_2-induced increase in PRs at the hypothalamic as well as pituitary level plays a crucial role in the cascade of events (i.e. changes in activity of the hypothalamic GABA-, NPY-, and β-END-systems and GnRH priming) that eventually result in the preovulatory GnRH and LH surge.

Proposed mechanism of reproductive aging

One of the first indications of female reproductive aging in rats, the attenuation of the LH surge, is associated with a decreased activation of GnRH cells (130,212) and neurons in the AvPv (144), as well as with a decrease in ER- and PR-containing cells in the AvPv, MPO, and ARC (**Chapter 3, 4, and 5**, see alse Figure 3). Since the amount of LH released per burst is decreased (117,161) at a relatively early age (9-11 months), while the total number of GnRH cells only decreases at a very progressed age (26 months) (76), these data suggest an early age-related change in the GnRH drive (i.e. a changed input to GnRH neurons) and/or in the responsivity to GnRH by the pituitary gland. Both options are supported by previous research and this thesis.

Firstly, the ability of E_2 to increase VP receptor mRNA in the POA appears to be lost with age (78), raising the possibility that the sensitivity of GABA-neurons to the daily signal from the SCN as well as to gonadal hormone feedback is impaired.

Secondly, previous research showed that the decrease in NPY levels in the ARC and the increase in NPY levels in the ME and POA, which results from the NPY release by the ARC to the ME and POA on proestrus, was absent in 7-10 months old Sprague-Dawley females (216), suggesting a decreased activity of the NPY system with age. Yet, the response of pituitary LH release to NPY appears unaffected by age (214).

Thirdly, the GnRH response to excitatory amino acids, like glutamate, dampens with age, possibly due to a decrease in *N*-methyl-D-aspartate (NMDA) receptors (4,85,86,300).

Fourthly, the ability of E_2 to both suppress and induce a diurnal rhythm in β-END mRNA levels in the ARC was abolished in 9-11 month-old Sprague-Dawley females (280) and E_2 exposure induces a selective loss of these β-END neurons

with age (20,47,48).

And finally, the number of VIP-innervated, Fos-expressing GnRH neurons is reduced in 10-12 month old Sprague-Dawley females (130) and the rhythm in VIP mRNA within the SCN is changed with age (131). Since the attenuated LH surge is also delayed in middle-aged Sprague-Dawley females (215,285), this may suggest that the functioning of the SCN is also impaired around the age of 10 months in this rat strain. Together, these data suggest that the activity of several systems that regulate GnRH release decreases with age and/or that the responsiveness of GnRH neurons to these signals may decrease with aging.

Although the above described changes were not all measured at a comparable age or within one rat strain, the available data support selective changes with age in E_2-sensitive neurotransmitter systems that may play a role in the regulation of GnRH and/or LH release. Based on our proposed mechanism for the induction of the LH surge and the finding that most of the age-related changes in neurotransmitter systems involve E_2, we speculate that the age-related changes as mentioned above are all secondary to a decrease in hypothalamic ER- and PR-containing cells (see also Figure 3). The absence of a correlation between PR concentrations in the AvPv and ERα concentrations in the ARC at the age of 9 months, but not at 4 months of age, in both WU and F1 females (this thesis) also supports the notion that steroid hormone effects on the brain may be impaired with age. ER and PR concentrations are regulated by E_2 and P, and E_2 and P were still unaffected by age in middle-aged WU females (**Chapter 3 and 4**).

We hypothesize that the age-related changes in gonadal hormone feedback on hypothalamic steroid receptors are fundamental to changes in relevant neurotransmitter systems and thus to the attenuation of the LH surge, to changes in steroid hormone concentrations as found in F1 females, and to the decrease in fertility and fecundity with age.

The gonadal hormone feedback on PRs is also involved in GnRH priming at the level of the pituitary gland (257,274) and in other hypothalamic systems, i.e. receptive and maternal behavior (15,41,159,189,196,200). Based on the findings that GnRH priming (117) and receptive behavior (148) change with age, we hypothesize that during reproductive aging, the E_2-induced induction of the PR may not only be impaired in the POA (**Chapter 3**) and in the ARC (**Chapter 5**), but may

also be altered in other systems in which the feedback of E_2 on the number of PR plays a role.

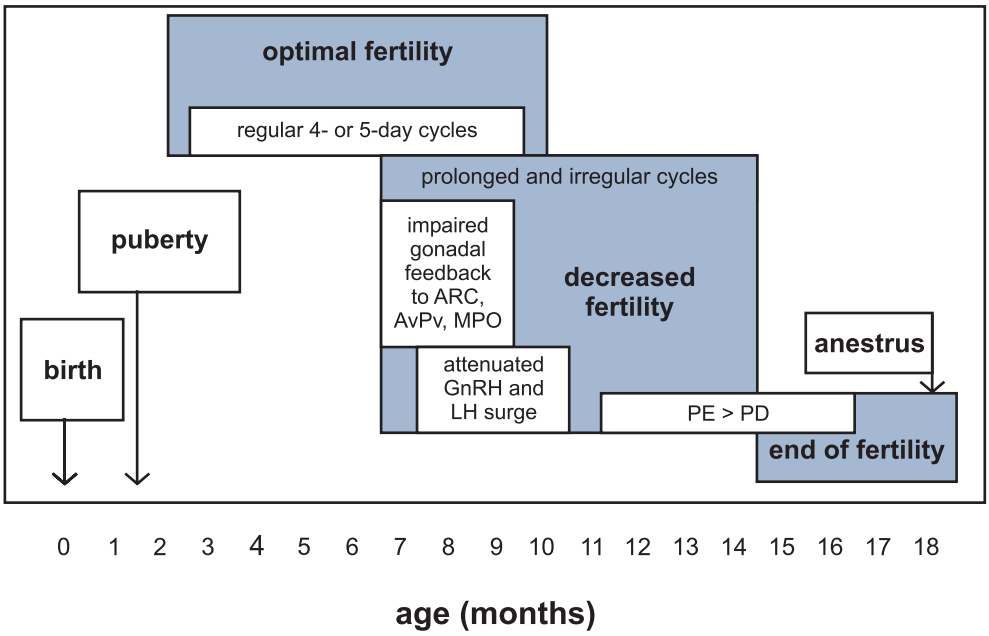

Figure 3. Simplified schematic representation of the processes during reproductive life in rats, including the associated stages of fertility.

7.4. Fertility, aging and the human reproductive system

This thesis presents age-related changes that may underlie the decline in fertility with age in rats, but what about aging and fertility in women? Data of hypothalamic connections and hypothalamic aging in the human are scarce, since the collection of brain material is very difficult. Therefore, fertility research in the past has mainly focussed on peripheral tissues, like the ovary and uterus, instead of the hypothalamus. Yet, most human studies that investigated the reproductive system during aging focussed on women without known fertility problems at a rather advanced age (40-45 years), on women that were undergoing assisted reproductive techniques like *in vitro* fertilization (IVF), or on women that were

postmenopausal. Therefore, little is known about possible age-related changes in the hypothalamus-pituitary-gonadal axis that occur before the age of 40 years and precede the decline in fertility besides the fact that the pregnancy chance declines in women from their late 20s onward (57).

Signs of early reproductive aging in women

The data in this thesis support the notion that in female rats, changes in GnRH secretion and GnRH priming ('hypothalamic aging') precede changes in inhibin or FSH concentrations ('ovarian aging') or in cycle length. In humans however, one of the first indications of reproductive aging appears to be a decrease in inhibin B levels, resulting in an increase in FSH levels and an acceleration in the rate of loss of primordial follicles around the age of 37-38 years (i.e. 'ovarian aging') (248).

The increase in FSH on day 3 of the menstrual cycle is commonly used in IVF procedures to detect women with a decreased ovarian reserve, a condition known to decrease their reproductive potential (71,121,190,224,247). There are, however, indications that the ovarian volume may be a better predictor for diminished ovarian reserve than day 3 FSH levels (226). Thus: are elevated basal FSH levels indeed the first sign of reproductive aging in women? Also, it is still the question if an elevated day 3 FSH level is the best tool for predicting IVF outcome (10,58,136,142). An increase in FSH is associated with a poor response to gonadotropin stimulation in IVF treatment (60,71,142,224), a decrease in inhibin B levels (224) and LH surge magnitude, a lower number of antral follicles (71), an increase in luteal P levels, and with a shorter cycle length (268). The shorter cycle length appears to result from an advanced follicular growth with age, leading to a shorter follicular phase (122,124,268). Indeed, the function of dominant follicles appears to be altered in 40-45 year old women (124).

Since an increase in FSH is associated with many other changes, these data underline that when FSH levels are elevated, it is likely that more than one component of the reproductive system is deteriorating, suggesting a rather advanced stage of reproductive aging.

Interestingly, regular cyclic infertile women that had unfertilized oocytes demonstrate a decrease in preovulatory LH, but not FSH levels compared to women with fertilized and cleaved oocytes (269). This corresponds to the results in our aging rats (**Chapter 2 and 6**) and raises the possibility that in a subset of women an improper functioning hypothalamus-pituitary system, as reflected by a

reduced LH surge (269) underlies infertility. In addition, IVF outcome appears to differ between white and black women (227), suggesting the presence of 'strain' differences in humans as well.

A preovulatory GnRH surge in women?

For ovulation in rats, hypothalamic GnRH release on proestrus plays a crucial role. There appear to be two subgroups of GnRH neurons in the rat brain: one subgroup located in the OVLT/POA that is associated with GABA-containing neurons and constitutes the "GnRH surge generator", and the other located in the MBH that is associated with opioid neurons and constitutes the "GnRH pulse generator" (82,119). In addition, rats display a clear GnRH surge that is thought to be associated with either an increase in GnRH pulsatility (102), or with an increase in GnRH pulse amplitude (150), but always with an increase in Fos-containing GnRH neurons in the POA (147). Thus, the POA and its GnRH neurons play a crucial role in the induction of the GnRH and LH surge in rats.

In humans, the distribution of GnRH neurons is different. In contrast to rats in which most GnRH neurons are located in the OVLT and POA, a large group of GnRH neurons is located in the MBH of humans as well as rhesus monkeys (52,53,150). Women appear to lack an increase in GnRH pulse frequency during the midcycle LH surge (1) and a normal LH surge can be generated in GnRH-deficient women without an increase in dose or frequency of exogenous GnRH (160). Since GnRH release may even be decreased during the LH surge (95), these data suggest that human females lack a robust preovulatory GnRH surge.

Yet, in a species that is more related to humans than rats, i.e. the rhesus monkey, an E_2-induced GnRH surge has been demonstrated (295), although a concomitant increase in Fos-containing GnRH neurons was absent (294). Since lesions of the POA do not abolish gonadotropin surges in female rhesus monkeys (128), we hypothesize that the contribution of the POA, or the so-called "GnRH surge generator", to the gonadotropin surges is limited in monkeys and in humans. In monkeys, E_2 is able to increase the PR expression in the MBH, but not (157) or only slightly (14) in the POA, while in rats PRs in the POA play a vital role in the induction of the GnRH surge (35). Hence, the contribution of the MBH, or the so-called "GnRH pulse generator", to the pulsatile GnRH and subsequent gonadotropin release may be more important in humans and monkeys than in rats. Unlike in rats, GnRH in humans may have a permissive role in the generation of

the LH surge and ovulation, while the role of pituitary GnRH priming may be greater in humans than in rats (95,128).

Similarities in the hypothalamus of rats and women

It may appear as if the results obtained in this thesis in rats are difficult to extrapolate to reproductive aging in humans. Nevertheless, there are many similarities in the regulation of the reproductive system between rats and humans. Firstly, the LH surge in humans also appears to be 'timed': in most women the preovulatory LH surge starts between midnight and 8:00 h in the morning (28) while in rats the LH surge is limited to the end of the inactive period on proestrus (**Chapter 2**), indicating a clear role for the biological clock in both species. Secondly, GnRH pulse frequency is increased by E_2 and decreased by P in both rats and humans (21,128,167), which involves PRs (236) and β-END cells located in the ARC (128,206,236). Thirdly, the sparse data on hypothalamic neurotransmitter systems in humans appear to roughly correspond to those in rats. Indeed, human but not monkey (249) GnRH neurons have reported to be innervated by NPY neurons originating from the ARC (~64%) (53) and by catecholaminergic neurons from the ARC and brain stem (52). These findings suggest that the regulation of the GnRH pulse generator (i.e. the ARC) may be comparable between rats and humans. Based on the fact that in women a large group of GnRH neurons is located in the MBH, we hypothesize that the contribution of the ARC to GnRH release is larger in women than in rats.

The hypothalamus and aging in women

With respect to aging, many alterations at the hypothalamic level have been reported in rats (290,291) and humans (103). In humans, GnRH pulse frequency has been reported to decrease (162) or not (239) with age in premenopausal women. Also, the response of the pituitary gland to GnRH (75) and naloxone (an opioid inhibitor) (39) stimulation is attenuated in older compared to younger premenopausal women. These data suggest alterations in GnRH release (pulse frequency) as well as GnRH priming (pituitary response to GnRH) in premenopausal women, which is exactly what this thesis and previous literature conclude about the process of reproductive aging in rats. Therefore, the above mentioned findings suggest that like in rats, "hypothalamic aging" may play a role in humans as well.

The results in this thesis as well as in the literature indicate that the sensitivity of the hypothalamus to gonadal hormone feedback is altered with age in rats and that life-time exposure to high E_2 concentrations may influence the rate of reproductive aging in rats. This may also be true for humans.

Indeed, the exposure to high levels of E_2 may accelerate reproductive aging in humans as in rats (197,207,254), since the use of high-dose oral contraceptives (OC; containing high estrogen levels) appears to decrease the age at menopause (43). Alternatively, the use of high doses of OC in these women could be indicated by irregularity of their cycles, which itself is a sign of early ovarian aging.

In rats, exposure to elevated P levels appears to delay the reproductive aging process (156). Since in women a positive correlation was found between the number of pregnancies during life (i.e. exposure to elevated P levels) and the age when women enter menopause (43,114), this suggests that P exposure may delay reproductive aging in women as well. In addition, OC (which is nowadays most often a combination of E_2 and P) suppress hypothalamic and pituitary activity, thereby inhibiting follicular growth and the development of a mature follicle. The response to OC has been reported to increase with age (64), suggesting that the sensitivity to gonadal hormones indeed also changes with age in humans.

Together, the findings suggest that also in women the sensitivity of the hypothalamus-pituitary system to gonadal hormone effects may change with age, resulting in alterations of the hypothalamus-pituitary system that could lead to “hypothalamic aging”.

In conclusion, there are species-, strain-, and individual- differences present in the contribution of the different levels of the HPG-axis to the regulation of the female reproductive system. We think that the same holds for reproductive aging: the alterations that occur during aging are similar across species and it depends on the individual, which of these changes occurs first and eventually limits fertility and fecundity.

7.5. Conclusions

(1) Individual and strain differences in the regulation of the reproductive cycle may reflect differences in contribution of processes to the regulation of the reproductive system

(2) Despite existing strain differences in rats, the first change in the reproductive axis that may underlie the age-related decline in fertility and fecundity appears to be at the level of the hypothalamus, i.e. an impaired E_2-induced increase in PRs primarily in the ARC, but also in the AvPv and MPO.

(3) The decrease in proestrous hypothalamic PR concentrations may lead to a decrease in the negative feedback (GABA, β-END) and a decrease in the positive feedback (i.e. NO, glutamate, NPY), resulting in an attenuation of the GnRH and LH surge.

(4) In women, the contribution of the POA or 'GnRH surge generator' to GnRH release appears to be limited, while the contribution of the ARC or 'GnRH pulse generator' may be comparable to or even higher than that in rats.

(5) We hypothesize that hypothalamic aging also occurs in women, but in most women its contribution to the decline in fertility may be less crucial than is the case in rats.

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Nederlandse samenvatting

In dit proefschrift draait alles om vruchtbaarheid, veroudering en de hersenen.

Vruchtbaarheid

Vrouwelijke zoogdieren zijn over het algemeen de 'beperkende factor' wat betreft vruchtbaarheid, want het zijn met name de vrouwelijke dieren die het tijdstip en het aantal nakomelingen bepalen. Dit komt doordat zij tijdens elke voortplantingscyclus maar gedurende een korte periode vruchtbaar zijn. Bovendien worden vrouwelijke dieren met een beperkt aantal *eicellen* in hun eierstokken (de *ovaria*) geboren, terwijl mannelijke dieren gedurende hun hele volwassen leven nieuwe zaadcellen produceren.

Veroudering

Omdat er elke dag eicellen beginnen met groeien vanuit de 'ovariële reserve' terwijl het aantal eicellen beperkt is, zal het aantal eicellen dat nog beschikbaar is in de loop der tijd afnemen. Ook de vrouwelijke vruchtbaarheid neemt af met leeftijd, en stopt met het einde van de cycliciteit. Bij de mens wordt de laatste menstruele cyclus, die zo rond het 50ste levensjaar plaatsvindt, de *menopauze* genoemd. De vruchtbaarheid begint echter al veel eerder, ongeveer vanaf het dertigste levensjaar, af te nemen. Omdat steeds meer vrouwen in de Westerse wereld voor een carrière kiezen voordat ze een gezin willen stichten, zullen er ook steeds meer vrouwen moeite hebben met het 'krijgen' van kinderen. Door meer inzicht te krijgen in de veranderingen die met leeftijd in het voortplantingsstelsel optreden, hopen we oorzaken te vinden van de leeftijdsgebonden vermindering in vruchtbaarheid bij de vrouw, waardoor we vrouwen met vruchtbaarheidsproblemen in de toekomst misschien beter kunnen helpen.

De hersenen

Men denkt dat *'ovariële veroudering'*, dat wil zeggen de afname in het aantal eicellen met leeftijd, leidt tot bovengenoemde vermindering van de vruchtbaarheid. Er zijn echter ook aanwijzingen dat er factoren op andere niveaus van het regulatie mechanisme van de cyclus, zoals de hersenen of hypofyse, een rol spelen. Al voordat er een versnelde afname van het aantal eicellen te zien is (bij de vrouw

vanaf ongeveer 37 jaar) neemt de vruchtbaarheid namelijk af. Bovendien is het bekend dat er bij de rat nog een groot aantal eicellen 'over' is op het moment dat de cyclus stopt. Ook dat suggereert dat er nog andere dan alleen ovariële veranderingen met de leeftijd in het voortplantingsstelsel kunnen optreden. Veranderingen in de hersenen bijvoorbeeld, kunnen ook een rol spelen bij reproductieve veroudering en de bijdrage daarvan aan dit verouderingsproces is bij de rat waarschijnlijk groter dan bij de mens. Omdat het doel van dit proefschrift is inzicht te krijgen in de bijdrage van de hersenen aan het proces van reproductieve veroudering, hebben we er bij al onze studies voor gekozen om de rat als diermodel te gebruiken.

De hypothalamus-hypofyse-ovaria as en de cyclus

Om veranderingen die bij veroudering in het voortplantingsstelsel optreden te kunnen begrijpen, moeten we eerst het normale functioneren daarvan in kaart brengen. Daarom beginnen we hier met een uitleg over de regulatie van de normale cyclus.

Tijdens een cyclus begint er dagelijks wel een eicel met de omringende cellen (de *follikel*) te groeien, maar per cyclus bereikt maar één (bij de mens) of een aantal follikels (bij de rat) het volgroeide stadium. De volgroeide, rijpe follikels ovuleren (de eisprong of *ovulatie*), waarna de vrijgekomen eicel bevrucht kan worden.

De groei van eicellen en follikels en de uiteindelijke ovulatie worden geregeld door goed gecoördineerde processen in het voortplantingssysteem dat bestaat uit de hypothalamus, de hypofyse en de ovaria. De hypothalamus is een gebied in de hersenen dat de afgifte van hypofysehormonen reguleert door middel van afgifte van gonadotropine-releasing hormoon (GnRH). De hypofyse is een orgaan dat vlak onder de hersenen ligt en dat hormonen aan het bloed afgeeft die onder andere de activiteit van de ovaria reguleren. De twee belangrijkste hypofysehormonen in dit verband zijn: follikel stimulerend hormoon (FSH) en luteïnizerend hormoon (LH). Onder invloed van deze twee hypofysehormonen groeien en rijpen er follikels in de ovaria en gaan deze follikels hormonen produceren. Die hormonen, oestradiol (E_2) en progesteron (P), kunnen via terugkoppeling (*feedback*) op de hypothalamus en de hypofyse de activiteit van de ovaria, en dus hun eigen afgifte, reguleren. Het complete systeem van elkaar beïnvloedende hormonen dat de voortplanting reguleert wordt de hypothalamus-hypofyse-ovarium as genoemd.

Bij de rat speelt de licht-donker cyclus een grote rol bij de regulatie van de hypothalamus-hypofyse-ovarium as. Zo treedt de LH piek die de ovulatie teweeg brengt altijd tegen de donkerperiode (de actieve periode, want ratten zijn nachtdieren) tijdens de proestrus op, terwijl de ovulatie ongeveer 13 uur later in de vroege ochtend van de estrus plaatsvindt. Bovendien vertoont de vrouwelijke rat in de periode rondom de LH piek en de ovulatie typisch bronstgedrag (estrus = bronst) en dat is een teken voor het mannetje is dat ze vruchtbaar is. In tegenstelling tot de ~28-dagen durende cyclus van de mens, duurt de cyclus van de rat maar 4 of 5 dagen. Daardoor verlopen er bij de rat processen tegelijkertijd die bij de mens achtereenvolgens plaatsvinden. Zo is er bij de rat nauwelijks een activatie van het CL (bij de mens ~14 dagen), terwijl de follikelgroei net als bij de mens ~14 dagen (dus 3 tot 4 cycli) duurt. Daardoor vindt alleen de laatste groeispurt met de bijbehorende toename van de E_2 concentratie plaats tijdens de cyclus waarin de follikels ovuleren.

Bij de rat duurt de totale cyclus dus maar 4 of 5 dagen en bestaat uit de volgende fasen: estrus, metestrus, diestrus en proestrus. Tijdens de estrus vindt er een selectie plaats van groeiende follikels die een bepaald stadium bereikt hebben: een aantal follikels (~10) groeit door terwijl de rest in atresie gaat (‘wordt afgebroken’). Onder invloed van FSH groeit zo’n follikel in een aantal cycli verder uit tot een Graafse follikel met een rijpe eicel. Doordat een groeiende follikel uit steeds meer hormoonproducerende cellen gaat bestaan, zal die follikel meer E_2 gaan produceren waardoor de E_2 concentratie in het bloed toeneemt. Via een *negatieve feedback* op de hypothalamus en de hypofyse zorgt deze verhoogde E_2 concentratie ervoor dat de afgifte van GnRH en LH geremd wordt. Op deze manier kan de follikel door middel van E_2 aan het hypothalamus-hypofyse systeem ‘doorgeven’ hoe het met zijn groei en rijping staat. Op het moment dat de follikel volgroeid is, is de concentratie E_2 in het bloed zeer sterk verhoogd en dat leidt juist tot een sterke toename in afgifte van GnRH en LH, de *positieve feedback*.

Deze omslag in de feedback (van negatief naar positief) wordt veroorzaakt door inwerking van E_2 (en P!) op de hypothalamus en hypofyse. De feedback werkt via receptoren (R) die E_2 (de ‘ER’) en P (de ‘PR’) kunnen binden, waardoor processen in de zenuwcel worden geactiveerd. Deze processen leiden tot een veranderde afgifte van signaalstofjes, de *neurotransmitters*, door deze cel. Omdat GnRH cellen deze receptoren niet of nauwelijks hebben, werkt de feedback van E_2 en P in de hypothalamus voornamelijk via andere zenuwcellen die wel ER en PR bevatten en

contact maken met GnRH cellen. Dus, de terugkoppeling van E_2 en P via de ER en PR leidt tot afgifte van bepaalde neurotransmitters in de hypothalamus, die zowel een remmend als een stimulerend effect kunnen hebben. Een verandering in het aantal receptoren zal dus een verandering in de balans tussen remmende en stimulerende neurotransmitters teweegbrengen, en zo tot een veranderde activiteit van GnRH cellen leiden. De positieve feedback die optreedt wanneer de follikels volgroeid zijn en de E_2 concentratie hoog is, ontstaat doordat het aantal PR onder invloed van E_2 toeneemt en processen geactiveerd kunnen worden die uiteindelijk leiden tot een stimulatie van GnRH cellen en een verhoging van de afgifte van GnRH, en dus ook van LH en FSH. Deze zogenaamde *preovulatoire LH piek* zorgt er voor dat de volgroeide follikels gaan ovuleren en luteïnizeren.

Door de luteïnizatie van de follikelcellen vormt zich vervolgens een corpus luteum (CL) dat actief P produceert. Doordat hoge concentraties P, in tegenstelling tot $E_{2,}$ tot een afname van het aantal PR leiden, heeft dit een remming van de GnRH afgifte en de follikelgroei tot gevolg. Bovendien zorgt P ervoor dat de baarmoeder klaargemaakt wordt voor de eventuele implantatie van een bevruchte eicel. Op het moment dat er geen bevruchting heeft plaatsgevonden, stopt het CL met de P productie en gaat het in regressie: er kan dan weer een nieuwe set follikels uitgroeien tot Graafse follikels.

De hypothalamus-hypofyse-ovaria as en veroudering

Omdat één van de eerste kenmerken van reproductieve veroudering in de rat een verlaging van de preovulatoire LH piek is die al optreedt nog vóórdat er een verlenging van de cyclus meetbaar is, hebben wij in dit proefschrift getracht te achterhalen wat de oorzaak van deze verlaagde LH piek is. We hebben er daarom voor gekozen om in al onze studies ratten met een regelmatige cyclus van 4.5 maand (volwassen) en van 9 maanden oud (vroege middelbare leeftijd) met elkaar te vergelijken. Verder hebben we ratten van twee stammen vergeleken, de Wistar (WU) en de F1 (UxRP) stam, waarvan bekend is dat ze respectievelijk een 4-daagse en 5-daagse cyclus vertonen. Bovendien zijn er aanwijzingen dat de vruchtbaarheid van F1 ratten eerder afneemt dan in Wistar ratten. Door deze twee rattenstammen op deze leeftijden te onderzoeken, hoopten we meer inzicht te krijgen in wat er verandert met de leeftijd én wat de volgorde van de veranderingen is.

Als eerste hebben we de LH piek bij deze ratten gemeten (**Hoofdstuk 2**) door

bloedmonsters te nemen tijdens de proestrus. Vervolgens hebben we ook onderzocht of de verlaging van de LH piek veroorzaakt wordt door een vermindering van de gevoeligheid van de hypofyse voor GnRH met de leeftijd. Hiervoor hebben we op de volgende proestrus vóór het begin van de LH piek een synthetisch GnRH analoog toegediend en daarna weer bloedmonsters afgenomen. Bovendien hebben we P concentraties in het bloed ook gemeten, want P kan de LH afgifte op proestrus sterk stimuleren. Doordat LH de luteïnizatie van de follikels stimuleert, wat resulteert in een verhoogde P afgifte, stijgt de P concentratie op proestrus. De resultaten lieten duidelijk zien dat de LH piek bij de 9 maanden oude ratten van beide rattenstammen verlaagd is, terwijl we geen verschillen vonden in de gevoeligheid van de hypofyse voor GnRH of in de hoogte van de P waarden met de leeftijd. Dit suggereert dat de hypofyse en de ovaria nog normaal reageren op respectievelijk GnRH en LH, waardoor de verlaging van de LH piek dus waarschijnlijk het directe gevolg is van een verminderde GnRH afgifte tijdens de proestrus.

Vervolgens hebben we onderzocht of de indirecte terugkoppeling van ovariële hormonen op de GnRH afgifte uit de hersenen met de leeftijd verandert. Hierbij spelen de cellen uit twee hersengebieden een belangrijke rol. Ten eerste is dat het *preoptisch gebied*, dat bestaat uit de 'anteroventral periventricular area' (AvPv) en 'medial preoptic area' (MPO) en dat betrokken is bij de generatie van de GnRH piek (positieve feedback; **Hoofdstuk 3 en 4**), en ten tweede is dat de *nucleus arcuatus* (ARC) die betrokken is bij de basale, pulsatiele afgifte van GnRH en die het ontstaan van een GnRH piek onderdrukt (negatieve feedback; **Hoofdstuk 5**). Voor deze experimenten hebben we normaal cyclische ratten gebruikt en ratten waarvan de ovaria verwijderd waren. In de cyclische ratten werden bloedmonsters genomen tijdens deze cyclus om E_2 en P te meten, waarna de hersenen op verschillende tijdstippen van de cyclus werden verzameld (metestrus, diestrus, proestrus). De hersenen werden vervolgens in heel dunne plakjes gesneden en die werden immunocytochemisch gekleurd voor ER of PR. Het aantal cellen in de AvPv, MPO en ARC dat een hoge concentratie ER of PR in de celkern bevatte, werd geteld door microscopische plaatjes van deze gebieden te analyseren met behulp van een beeldanalyse computerprogramma. Bij de ratten waarvan de ovaria verwijderd waren, hebben we het effect onderzocht van eenzelfde concentratie E_2 op het aantal ER en PR, om te zien of dat zou verschillen tussen ratten van jonge en middelbare leeftijd. Hiervoor werd twee weken na het

verwijderen van de ovaria een onderhuidse injectie met oestradiol benzoaat (een E_2-achtige stof) gegeven waarna de ratten 2, 8, 26 of 32 uur later gedood werden om de hersenen te verzamelen en te kleuren voor de ER en PR.

Onze resultaten lieten duidelijk zien dat de hormoonconcentraties in cyclische ratten tijdens de cyclus fluctueren en verder dat ze met de leeftijd toenemen bij F1 maar niet bij Wistar ratten (**Hoofdstuk 3 en 4**). Deze toename bij F1 ratten zou het gevolg kunnen zijn van veranderingen in follikelgroei of activiteit van het CL. Ook vonden we een fluctuatie tijdens de cyclus van het aantal cellen met hoge concentraties ER en PR in de AvPv, MPO en ARC. Het meest opvallende was echter dat in beide stammen het aantal cellen met ER en PR in de AvPv, MPO en ARC drastisch was afgenomen met leeftijd ondanks de onveranderde of zelfs gestegen hormoon concentraties (**Hoofdstuk 3, 4 en 5**)! Deze afname was, zij het in mindere mate, ook in de geovariëctomeerde dieren te zien. Samenvattend wijzen deze resultaten er sterk op dat de terugkoppeling van ovariële hormonen op de hersenen sterk veranderd is in deze 9 maanden oude, cyclische ratten. De veranderingen in steroid receptor concentraties in de AvPv and MPO suggereren een minder sterke positieve feedback en dus een afname in de hoogte van de GnRH piek. De veranderingen in de ARC suggereren veranderingen in de negatieve feedback, hetgeen gevolgen kan hebben voor de onderdrukking van de GnRH piek, en/of de basale, pulsatiele GnRH afgifte.

Bij de mens lijkt één van de eerste veranderingen bij veroudering niet een verlaging van de LH piek te zijn, maar juist een verhoging van de FSH concentraties. Bovendien lijkt bij de mens het aantal follikels op een bepaalde leeftijd de ‘beperkende factor’ voor vruchtbaarheid te zijn. Beide suggereren een belangrijke bijdrage van ‘ovariële veroudering’. Om te onderzoeken of deze factoren ook al veranderd zijn bij ratten met een verlaagde LH piek, hebben we de FSH concentratie in het bloed tijdens de proestrus gemeten bij dezelfde dieren waarbij we de LH piek bepaald hadden. Bovendien hebben we bij die dieren de ‘ovariële reserve’ (het aantal follikels wat nog niet aan het groeien is) geteld (**Hoofstuk 6**). Zoals verwacht was de ovariële reserve gedaald met de leeftijd. We vonden echter geen leeftijdsverschiilen in de FSH concentratie tijdens proestrus. Deze resultaten geven duidelijk aan dat bij 9 maanden oude, cyclische ratten de gevoeligheid van de hersenen voor de ovariële hormonen E_2 en P al wel veranderd is, wat waarschijnlijk de oorzaak is van de verlaging van de LH piek op deze

leeftijd, maar dat er niet, zoals bij de mens, een verhoging van FSH te zien is. Omdat de verhoging van FSH bij de mens veroorzaakt wordt door een verlaging van het door de ovaria geproduceerde inhibine, suggereren deze gegevens ook dat de inhibine concentratie nog normaal is, en dus dat de bijdrage van 'ovariële veroudering' bij de rat kleiner is dan die van 'hersenveroudering'.

Behalve de effecten van leeftijd hebben wij stamverschillen en een effect van cycluslengte gevonden in veel van de parameters die we hebben onderzocht. De resultaten suggereren dat de bijdrage aan reproductieve veroudering van elk van de factoren die tezamen het voortplantingssysteem vormen per stam, en waarschijnlijk zelfs per individu, kan verschillen. Bovendien vonden we in de F1 ratten meer en grotere veranderingen met de leeftijd en dat bevestigt het idee dat F1 ratten wat het voortplantingssysteem betreft sneller verouderen dan Wistar ratten.

Ten slotte hebben we de resultaten samengevat en bediscussieërd in **Hoofdstuk 7** en doen we een voorstel over welke processen en neurotransmitters er bij de normale regulatie van de cyclus betrokken zijn op het niveau van de AvPv, MPO en ARC. Wat reproductieve veroudering betreft doen we een aantal suggesties voor de mogelijke aard van de leeftijd-gerelateerde veranderingen in neurotransmittersystemen en hersengebieden. Als laatste vergelijken we onze resultaten bij ratten met wat er bekend is bij de mens en bekijken we wat onze resultaten ons kunnen leren over de vermindering van de vruchtbaarheid met leeftijd bij de mens.

Kort samengevat suggereren onze resultaten sterk dat één van de eerste veranderingen met leeftijd bij de rat een verandering in het effect van ovariële hormonen E_2 en P op de hersenen. Dat leidt mogelijk tot veranderingen in neurotransmitter-afgifte in de hersenen, waardoor de GnRH afgifte door de hersenen vermindert. Dat resulteert vervolgens in de verlaging van de LH piek die we op middelbare leeftijd zien. Veroudering van de hersenen lijkt bij de rat dus de beperkende factor te zijn voor de vruchtbaarheid.

Bij de mens zijn er aanwijzingen dat het voortplantingssysteem op hersenniveau globaal op dezelfde wijze gereguleerd wordt als bij de rat. Er lijkt echter een verschil te zijn met de rat in het belang van de GnRH piek (preoptisch gebied) en de pulsatiele, basale GnRH afgifte (nucleus arcuatus) voor reproductieve veroudering. Bovendien lijkt er een verschil te zijn tussen de rat en

de mens in het belang van de ovaria en de hersenen in dit opzicht. Hoewel ovariële veroudering bij de vrouw de dominante beperkende factor is, verwachten wij niettemin dat de oorzaak van reproductieve veroudering bij sommige vrouwen gezocht moet worden in de hersenen.

Curriculum Vitae

Antje Nicoline (Annelieke) Franke werd geboren op 24 oktober 1975 te Rhenen. Zij groeide op in Nuenen en behaalde in juni 1994 het VWO diploma aan het Strabrechtcollege in Geldrop. In september van dat zelfde jaar startte zij met de studie Biologie aan de Universiteit Utrecht.

Tijdens deze studie groeide bij haar de fascinatie voor de hersenen, wat terug te zien is in de keuze van de twee stages en de scriptie tijdens de specialisatiefase. Haar eerste stage betrof onderzoek naar de ontwikkeling van het zenuwstelsel (met name de oogzenuw) in zebravis-embryo's. Dit onderzoek werd uitgevoerd bij het Hubrecht Laboratorium, Nederlands Instituut voor Ontwikkelings Biologie (NIOB) te Utrecht onder begeleiding van dr. A. van der Sar en dr. J. den Hartog. Haar tweede stage betrof neurofysiologisch onderzoek naar de rol van neurotransmitter vasopressine, afkomstig uit de biologisch klok in de hersenen, in de regulatie van schildklierhormoon afgifte in de rat. Dit werd uitgevoerd bij het Nederlands Instituut voor Hersenonderzoek (NIH) te Amsterdam onder begeleiding van dr. A. Kalsbeek en prof. dr. R.M. Buijs. Doordat tijdens haar laatste stage duidelijk werd dat ze na haar studie in Wageningen zou gaan werken, heeft ze haar scriptie gewijd aan haar nieuwe interesse: de hypothalame bijdrage in reproductieve veroudering in vrouwelijke zoogdieren, onder begeleiding van dr. E.M. van der Beek en prof. dr. D. van der Heide. Annelieke studeerde af op 30 november 1998. De scriptie bleek een goede voorbereiding te zijn voor haar baan als Onderzoeker in Opleiding op een door NWO gesubsidieerd project bij dezelfde vakgroep aan de Wageningen Universiteit in de periode van 16 oktober 1998 tot 16 april 2003. Dit onderzoek werd verricht onder begeleiding van dr. E.M. van der Beek en prof. dr. V.M. Wiegant en de resultaten hiervan staan beschreven in dit proefschrift. Sinds 1 augustus 2003 is Annelieke werkzaam als histologisch analiste bij de vakgroep Anatomie van het Radboud Ziekenhuis, Katholieke Universiteit Nijmegen.

List of Publications

Abstracts

A.N. Franke, H.H. van Vugt, J.J.M. Swarts, J. Kastelijn, V.M. Wiegant, E.M. van der Beek 2000 Gonadal steroids and steroid receptors: the role of the hypothalamus in reproductive aging. 4th Endo-Neuro Meeting, Doorwerth, The Netherlands, p 100

A.N. Franke, J.J.M. Swarts, J. Kastelijn, V.M. Wiegant, E.M. van der Beek 2000 Gonadal steroids and steroid receptors: the role of the hypothalamus in reproductive aging. 30th Annual Meeting of the Society for Neuroscience, New Orleans, USA, 542.8, p 1450

A.N. Franke, A. Oosting, J.J.M. Swarts, J. Kastelijn, E.M. van der Beek 2001 Early changes in luteinizing hormone release in female rats with age. 5th Endo-Neuro Meeting, Doorwerth, The Netherlands, p 102

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Full papers

A. Kalsbeek, E. Fliers, A.N. Franke, J. Wortel, and R.M. Buijs 2000 Functional connections between the suprachiasmatic nucleus and the thyroid gland as revealed by lesioning and viral tracing techniques in the rat. Endocrinology 141(10): 3832-3841

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