GROWTH HORMONE AND REPRODUCTION IN THE FEMALE RAT: A CENTRAL ROLE FOR SOMATOSTATIN

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GROWTH HORMONE AND REPRODUCTION IN THE FEMALE RAT: A CENTRAL ROLE FOR SOMATOSTATIN

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It is well known that the somatotropic and gonadotropic axes are closely related: Both underproduction and overproduction of growth hormone (GH) affect reproduction. During aging, the decline in GH release and reproductive function appear to occur concurrently. Data from the literature suggest that somatostatin (SOM), the inhibiting factor in the bimodal hypothalamic regulation of GH release, is not only crucial for GH release patterns, but may also affect reproductive function. The aim of this thesis was to test the hypothesis that SOM neurons in the periventricular nucleus (PeVN) may play a central role in the interaction between both axes in the female rat.

GH release patterns are sexually dimorphic, which has been suggested to result mainly from differences in SOM release from the median eminence (ME). Our data support the notion that the hypothalamic SOM system differs between the sexes. The PeVN in female rats likely possesses more SOM cells, but synthesis and, possibly, storage of SOM peptide may be higher in males. Also, the acute GH feedback on SOM cells, as monitored by the presence of a cFos-positive nucleus, is different between the sexes and, moreover, occurs in different subregions of the PeVN in male and female rats. Gonadal steroids may be involved in the regulation of hypothalamic GH feedback in the female, as we found that estrogen decreases the sensitivity of SOM cells to GH. Our findings suggest that the organization of SOM cells in the PeVN differs between the sexes, which may direct the proposed different SOM release patterns from the ME.

In this thesis we show that SOM decreases the estrogen-induced luteinizing hormone (LH) surge in the female rat by, at least in part, decreasing hypothalamic GnRH cell activation. These data suggest that SOM may indeed directly affect reproductive function at the level of the hypothalamus and, possibly, the pituitary. Also, our data indicate that SOM neurons in the PeVN are regulated by both estrogen and progesterone, of which plasma levels increase during the day of proestrus. Hence, our data, combined with data from the literature, suggest that hypothalamic SOM neurons may be involved in the regulation of the proestrous LH surge in the female rat. Based on our results, we suggest that in the normal female cycle, SOM may be involved in the descending phase of the LH surge.

Interestingly, in female rats of 9 months old that already showed an attenuated proestrous LH surge, we found changes in SOM peptide concentrations within the PeVN compared to young animals. The decline in GH release with age is suggested to result from increased SOM secretion from the ME. Our findings suggest that early aging of the somatotropic axis may be correlated with changes in the hypothalamic SOM system. Thus, early reproductive aging and age-related changes in the somatotropic axis occur simultaneously in the female rat, suggesting that SOM may also be involved in the age-related changes in reproductive function.

In conclusion, we show that changes in GH release patterns (e.g. between the sexes or with age) are associated with altered activity of the PeVN SOM cells. These findings support our notion of the proposed central role of the hypothalamic SOM system in the interaction of the somatotropic axis with the reproductive system. Therefore, PeVN SOM neurons may indeed provide the “cross-link” between the functionality of the somatotropic and gonadotropic axis.

CONTENTS

CHAPTER 1

General introduction

*Underproduction of **growth hormone** (GH) leads to short-stature in children, while overproduction causes gigantism or, when GH is secreted excessively in the adult, acromegaly. Strikingly, both GH-deficiency and -overexpression negatively affect **reproductive function**.*

1.1. THE SOMATOTROPIC AXIS

Growth hormone

As its name implies, growth hormone (GH) promotes growth and development of the body. GH is a single-chain peptide that consists of 191 amino-acids and is secreted by somatotroph cells in the anterior pituitary gland. GH directly affects muscle and bone growth, lipid metabolism, milk production, and the release of several hormones. Moreover, GH also affects several processes in the body indirectly by stimulating the production and release of insulin-like growth factor (IGF-I) from the liver (see Figure 1). Besides the direct effects on growth and differentiation, GH also affects the regulation of the reproductive system: it is, amongst others, involved in pubertal maturation, in the ovarian cycle, and has additional roles in pregnancy and lactation [1]. This thesis will focus on the interaction between the GH and the reproductive system in the adult female rat.

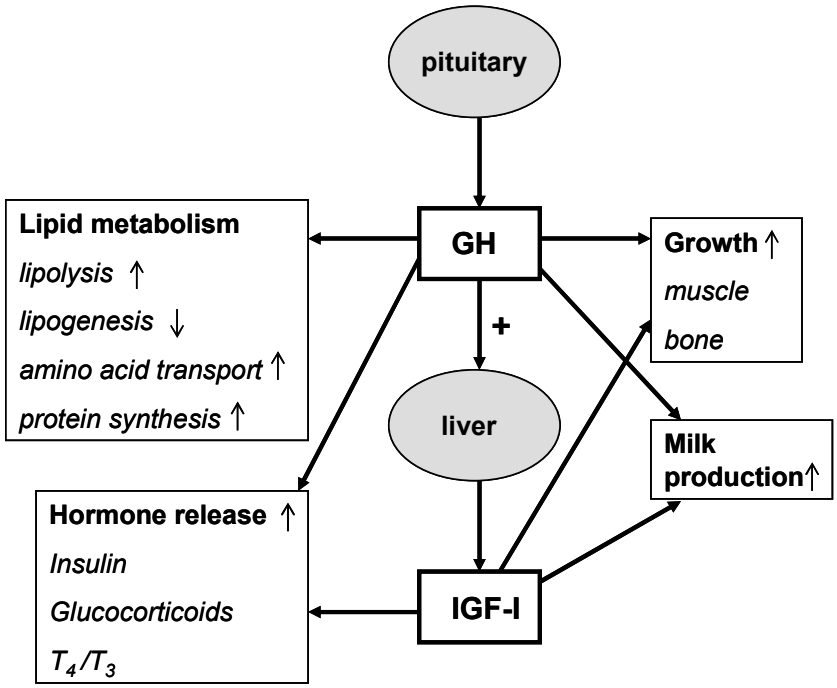

Figure 1. Schematic representation of the actions of growth hormone (GH). IGF-I: insulin-like growth factor-I; T_4: thyroxine; T_3: triiodothyronine. (Adapted from [2, 3]).

Growth hormone secretion patterns

GH release is pulsatile, which is essential for normal growth, and differs between the sexes in many mammalian species, including humans and rats [4]. In humans, mean plasma GH concentrations and GH pulse amplitude are greater in adult females than in males. In contrast, mean GH concentrations and GH pulse amplitude are greater in adult male rats compared to female rats [4]. GH pulse frequency, however, is higher in females than in males in both humans and rats [5]. In adult male rats, GH release patterns are characterized by regular high amplitude GH pulses occurring at approximately 3 h intervals, and low baseline levels. Female rats exhibit lower amplitude GH pulses with a higher but irregular frequency, and higher baseline levels compared to males [4, 6, 7].

In humans, GH release shows a diurnal rhythm: mean plasma GH levels and pulse frequency increase during sleep [8]. Although in male rats GH secretion patterns are clearly independent of the time of day, GH release in female rats seems to follow a diurnal rhythm: pulse frequency and amplitude were found to be increased during the dark period [5, 6].

Effects of gonadal steroids on growth hormone secretion

The gonadal steroid milieu during neonatal development is very important to establish the sexual dimorphism in GH secretion during adulthood [9]. Testosterone (T) is essential for the generation of low GH baseline levels and determines GH pulse height and duration, but not pulse frequency [10-12]. On the other hand, estradiol (E_2) reduces GH peak amplitude and increases GH baseline levels [11, 12].

Although the neonatal gonadal steroid milieu is crucial in determining GH release patterns, the continuous presence of T and E_2 is required to maintain the typical male- and female-like GH release patterns during adulthood [10-12]. In castrated (CX) adult male rats, GH peak amplitude is decreased and GH pulses are irregular [13]. Treatment with T restores the typical male-like GH release pattern in CX animals [10, 11]. Ovariectomized (OVX) female rats show increased GH peak amplitude and decreased GH baseline levels [14]. Following E_2 treatment, GH baseline levels increase and GH peak amplitude decreases in these animals (Van Vugt *et al.*, unpublished data). Interestingly, in CX or intact male rats treated with E_2, GH baseline levels and GH pulse frequency increase [13], whereas intact or OVX females treated with T show a male-like GH secretion pattern [14-

16]. Thus, it is clear that in adult rats circulating gonadal steroids dictate the feminine or masculine GH secretion pattern (see also Figure 2).

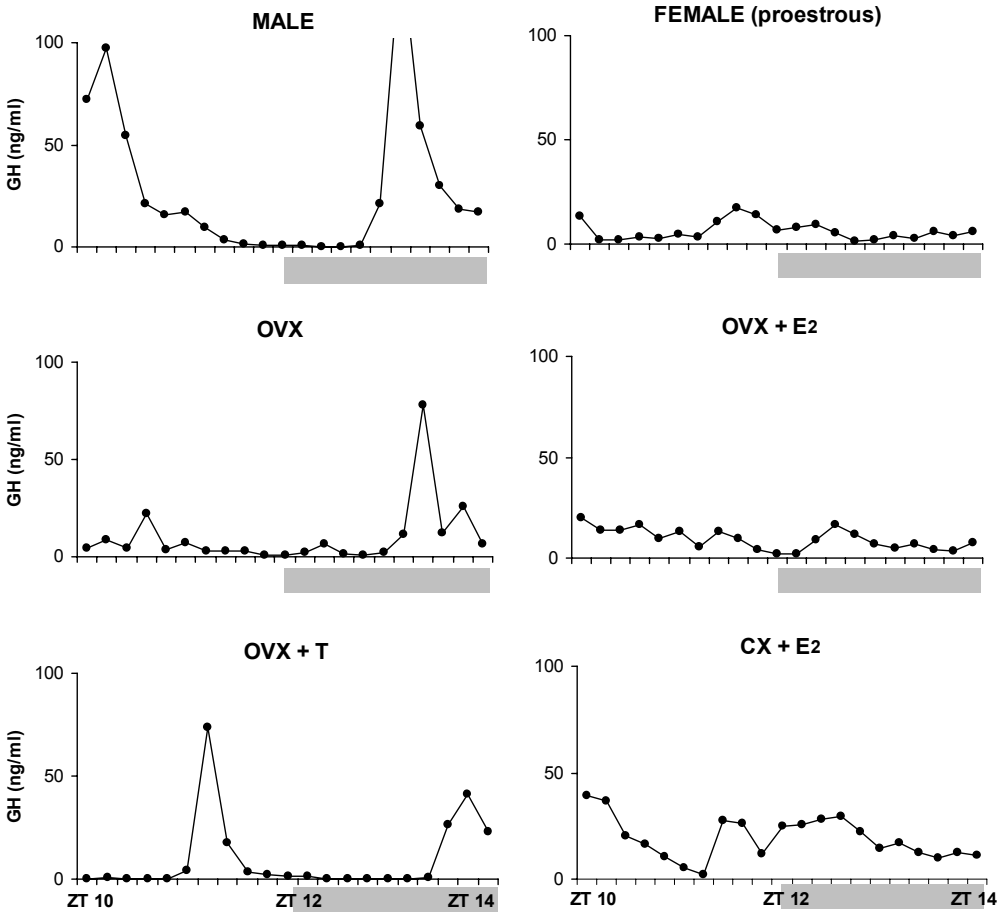

Figure 2. Representative examples of growth hormone (GH) secretion patterns in adult male and female rats with different gonadal steroid status. Rats were kept under light/dark conditions of 12/12 h; lights on at zeitgeber time (ZT) 0. All animals received an indwelling jugular vein catheter directed towards the right atrium. On day 12 and 13 after ovariectomy (OVX) or castration (CX) rats were treated with estradiol (E_2), testosterone (T), or vehicle. On day 14 after OVX or CX blood samples were taken. Intact male rats were sampled randomly, and regularly 4-day cycling female rats were sampled on the day of proestrus. Blood samples were taken every 12 minutes, 2 h before until 2 h after onset of the dark period (ZT 12) (Van Vugt *et al.*, unpublished data). Gray bars represent dark period.

Hypothalamic regulation of growth hormone secretion

GH release from the pituitary is mainly regulated by the interplay of the two neurohormones GH-releasing hormone (GHRH) and somatostatin (SOM). GHRH is synthesized in neurons located in the arcuate nucleus of the hypothalamus (ARC) that project to the median eminence (ME). SOM-producing neurons are widely distributed

throughout the brain [17], but the SOM cells located in the periventricular nucleus of the hypothalamus (PeVN) project to the ME and are responsible for the regulation of pituitary GH release [18, 19]. GHRH and SOM released from the ME are subsequently transported to the anterior pituitary gland via the hypothalamo-hypophyseal portal blood vessels to, respectively, stimulate and inhibit GH release. GHRH stimulates both the synthesis and secretion of GH and influences somatotroph cell number, whereas SOM inhibits GH secretion but not synthesis [19].

Besides projecting directly to the ME, SOM- and GHRH-producing neurons can influence each other via intrahypothalamic connections. Also other neuropeptides synthesized by neurons in the ARC, like neuropeptide-Y (NPY), are able to modulate the synthesis and release of GHRH and SOM [18]. Moreover, SOM regulates its own release by “short-loop” negative feedback actions within the PeVN [20].

GH is able to regulate its own secretion by feedback actions at the level of the pituitary and hypothalamus. As GH-receptors (GH-Rs) were found on SOM cells in the male PeVN [21, 22], it is likely that GH stimulates SOM release by a direct feedback action on these neurons. GH feedback on GHRH neurons in the ARC, however, has been suggested to be indirect. GH likely affects SOM- and/or NPY-containing neurons with GH-Rs that in turn send signals to the GHRH neurons resulting in a decrease in GHRH release. In addition, IGF-I may also feed back at the level of the pituitary, and possibly the hypothalamus, to inhibit GH release from the pituitary (reviewed in [23]) (see Figure 3).

Sexual dimorphism in GHRH and SOM release

The typical masculine and feminine GH release patterns are the direct result of differences in GHRH and SOM release patterns between the sexes. In male rats, both GHRH and SOM are released in regular pulses, however exactly out of phase with each other: at the time of a GH peak, SOM levels are low whereas GHRH levels are high and vice versa during GH trough levels [25]. In the female rat, SOM release is suggested to be more continuous at a level in-between the maximal and minimal concentrations found in males, whereas GHRH is released in irregular pulses. Whenever GHRH is “high enough” to exceed inhibitory SOM concentrations, a GH peak will occur [26] (see Figure 4).

With respect to the bimodal hypothalamic regulation of GH release, differences in SOM release patterns between the sexes are most striking. Sex differences in GH release patterns have therefore been suggested to depend mainly on SOM rather than GHRH

release in the rat [19, 23, 25]. Consequently, SOM synthesis and release may, like GH release, be influenced by gonadal steroids as well. Indeed, male rats that are castrated shortly after birth show decreased SOM mRNA concentrations in the PeVN on postnatal day 5 compared to intact males of the same age [27]. Interestingly, treatment with T in these animals does not affect SOM mRNA, whereas treatment with E_2 results in SOM mRNA levels similar to that in intact males, indicating that the organizational effects of T require aromatization of T to E_2.

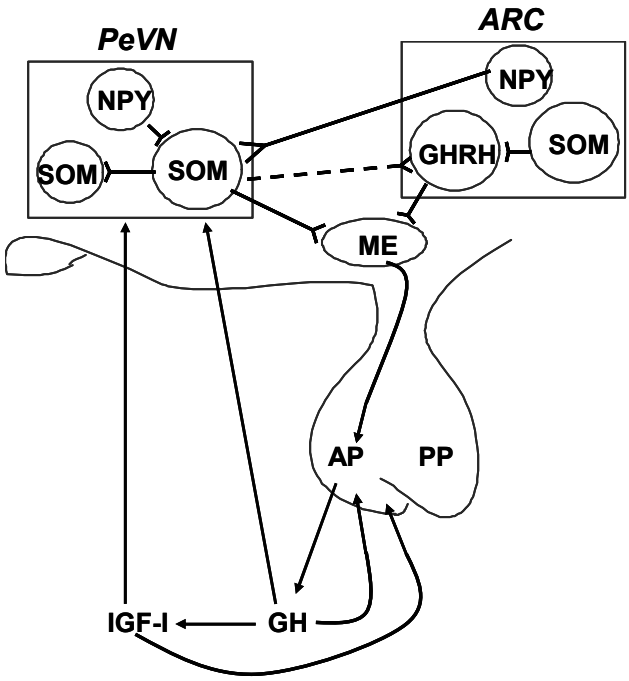

Figure 3. Schematic representation of somatostatin (SOM) and growth hormone (GH)-releasing hormone (GHRH) projections within the hypothalamus, regulating GH release. PeVN: periventricular nucleus; ARC: arcuate nucleus; NPY: neuropeptide-Y; ME: median eminence; AP: anterior pituitary; PP: posterior pituitary; IGF-I: insulin-like growth factor-I. (Adapted from [18, 24]).

In adult rats, gonadal steroids affect hypothalamic SOM mRNA, peptide and release [14, 23, 28-32]. CX decreases SOM mRNA and peptide levels in the PeVN of male rats, which can be reversed by treatment with T [28, 29, 32]. Also in the female rat, OVX results in decreased levels of SOM mRNA and peptide in the PeVN. Treatment with E_2 may restore hypothalamic SOM concentrations, although the precise role of E_2 in the regulation of hypothalamic SOM activity in the female remains unclear, as contradictory results have been reported [28, 29, 32, 33]. Hypothalamic GHRH mRNA concentrations, however, are

affected by neither gonadectomy nor steroid treatment in both male and female rats [14, 34].

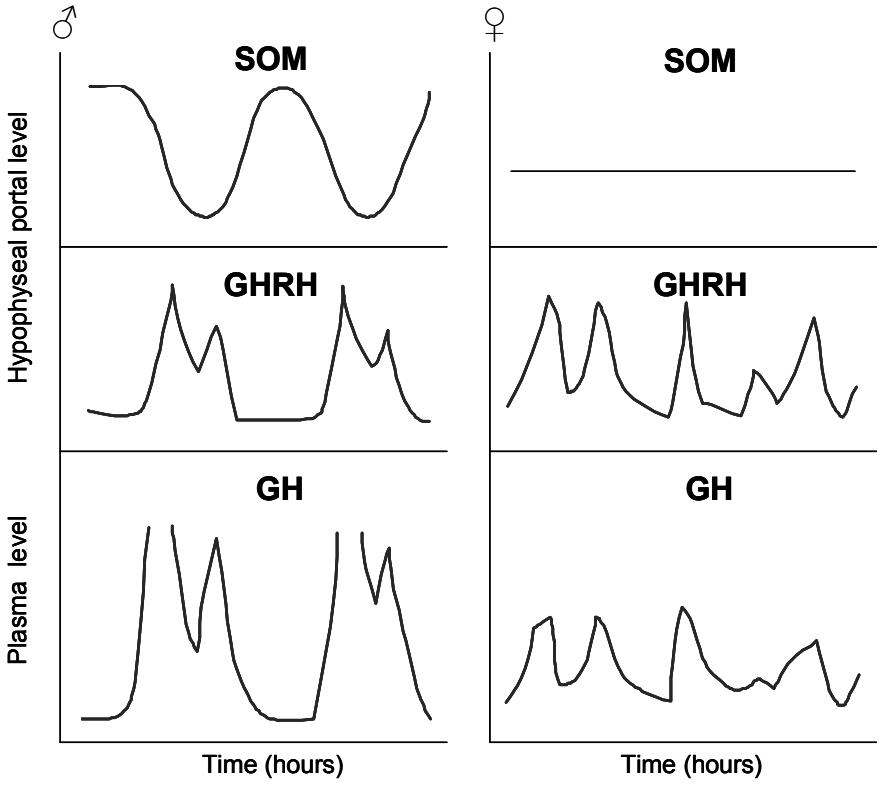

Figure 4. Schematic representation of somatostatin (SOM) and growth hormone (GH)-releasing hormone (GHRH) release patterns in the male (left) and female (right) rat, which are suggested to result in the typical masculine and feminine GH release patterns (adapted from [25, 35]).

Ghrelin

Besides GHRH and SOM, ghrelin, a recently discovered peptide, is likely involved in the regulation of GH release as well. Ghrelin, a 28 amino-acid peptide, was originally isolated from the stomach, but was also found to be present in small amounts in the intestine and hypothalamus. This peptide is the endogenous ligand for the GH secretagogue receptor, which is expressed in the hypothalamus and pituitary. Although ghrelin is able to stimulate GH release, its exact role in the regulation of GH secretion remains uncertain. Data from studies on the possible role of ghrelin in physiological GH regulation suggest a facilitating, rather than a regulatory effect. It was suggested that the role of ghrelin may become more prominent during a state of negative energy balance (reviewed in [36]).

Growth hormone release during life

Plasma GH levels are low at birth, but increase shortly thereafter [37]. In 22 days old rats, GH secretion patterns are already pulsatile, although peaks are still lower compared to those found in adult animals. At this age, the GH secretory pattern does not yet differ between the sexes. During early puberty, GH secretion patterns become different between females and males, but it is not until early adulthood that the typical feminine and masculine GH secretory patterns are fully established [7, 38].

During adulthood, GH secretion gradually declines with age in both humans and rats, which is thought to be responsible for the aging-related metabolic and physiological changes (reviewed in [39]). In female rats, mean plasma GH concentrations and peak amplitude are already significantly decreased at 11 months of age and are even more decreased at the age of 25-29 months [40] (see Figure 5A for an overview). The decline in GH concentrations with age is concurrent with decreased GH mRNA concentrations [41, 42], and the number and size of pituitary GH cells [39], suggesting that the age-related changes in pituitary GH levels may occur at the transcription level of GH synthesis. However, somatotrophs can be restored to youthful secretory capacity when treated with GH-releasing peptides at any age [43]. Therefore, the decrease in GH release with age, and the subsequent decreased IGF-I levels, are more likely the result of changes at the level of the hypothalamus rather than the pituitary [39, 43].

GHRH and SOM during life

Although hypothalamic GHRH mRNA concentrations are increased and become different between the sexes after puberty [14], the amount of GHRH released from the hypothalamus remains constant during life and does not differ between male and female rats [44]. In male rats, SOM release is very low shortly after birth, but reaches secretion levels around puberty that are similar to those found in adulthood [44, 45]. In female rats, SOM release follows a pattern similar to males until adulthood but continues to increase thereafter [45]. Also hypothalamic SOM mRNA concentrations [14, 27] and SOM peptide content in the PeVN [45] start to increase immediately after birth and peak around puberty. Differences in SOM mRNA, SOM peptide in the PeVN and SOM release from the ME between the sexes become evident, respectively, on day 5, 10 and 40 [45].

It has been suggested that the gradual decrease in GH secretion with age may be due to increased SOM release [17]. SOM content in the ME is lower, whereas SOM immunoreactivity in the anterior pituitary is higher in old (20 months) compared to young male rats [46], suggesting that SOM release from the ME increases with age. Indeed, in 14 month old male and female rats, SOM release from the hypothalamus is increased compared to young animals [44]. Although increased secretion of SOM occurs with age, decreased hypothalamic gene expression and peptide was found in old rats, suggesting an increased efficiency of SOM translation with age [39]. On the other hand, GHRH release is found to be unchanged [44] or decreased (reviewed in [17, 39]) with age (see Figure 5B for an overview). Hence, changes in GH release patterns during aging are mainly the result of changes in SOM release, which further supports the notion of a prominent role for SOM to direct GH release patterns.

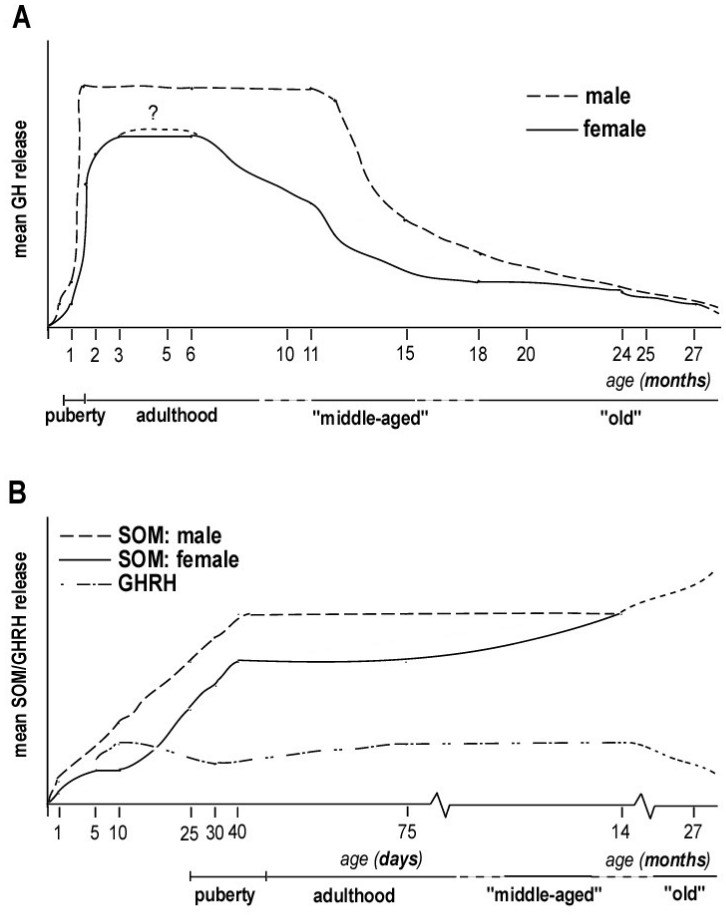

Figure 5. Proposed plasma GH levels (A, based on [40-42, 44, 47]), and SOM and GHRH release (B, based on [14, 27, 39, 44, 45]) during life in male and female rats.

1.2. THE GONADOTROPIC AXIS

Hypothalamus-pituitary-gonadal axis

In the adult female rat, the estrous cycle lasts for 4-5 days and the regulation of the cycle shows, despite differences in cycle length, many similarities with the menstrual cycle in humans [48].

Gonadotropin-releasing hormone (GnRH)-producing neurons that are located in the organum vasculosum of the lamina terminalis (OVLT) and in the preoptic area (POA) project to the ME, from where GnRH peptide is transported via the portal blood system to the anterior pituitary to stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH, in turn, affect the ovaries: LH stimulates ovarian androgen production, ovulation and corpus luteum (CL) formation, and FSH stimulates the development of ovarian follicles. In the follicles, the gonadal steroids are produced: the thecal cells synthesize T, which is aromatized to E_2 in the granulosa cells. P is synthesized in both granulosa cells and in the luteinized cells of the CL. E_2 and P are crucial for the induction of the preovulatory LH surge. Low concentrations of E_2 (during the first phase of the estrous cycle) inhibit the secretion of GnRH from the hypothalamus, but when adequate levels are present, E_2 is able to stimulate GnRH release, leading to the GnRH surge on the day of proestrus and, subsequently to an LH and FSH surge. P also plays a role in the timing and magnitude of the preovulatory LH surge [48] (see Figure 6).

E_2 and P feed back at the level of both the pituitary and hypothalamus. The feedback action at the level of the hypothalamus, however, has to be indirect, because GnRH cells only express very low concentrations of E_2-receptor β (ERβ), but in rats no ERα and PR [49]. Neurons in the POA that contain ERs and/or PRs, and project to GnRH cells, are therefore most likely to mediate the ovarian feedback on the hypothalamus.

Reproductive aging

Already at a relative early age, around 8-9 months of age (“middle-aged”), activation of GnRH cells on the day of proestrus is decreased in female rats compared to young females, despite a normal estrous cyclicity [51, 52]. This decreased activation may lead to a decline in GnRH synthesis and secretion and may be responsible for the attenuation of the preovulatory LH surge that is found in these middle-aged female rats [48, 50]. During reproductive aging, decreased GnRH cell activation and LH release is followed by

lengthening of the estrous cycle and/or irregular cycles, resulting in acyclicity (persistent estrous). Finally, the female rat becomes anovulatory and infertile (around 18 months of age, although this varies between strains). Interestingly, infertile females still have a considerable number of ovarian oocytes [48], indicating that ovarian failure is not the limiting factor in reproductive aging. Also, the early changes in estrous cyclicity occur despite the stimulating levels of E_2 and P, that are unchanged, or even elevated during the early phases of the reproductive aging process [50]. Thus, it seems that changes occurring at the level of the hypothalamus and pituitary are the first indications of reproductive aging, at least in the female rat.

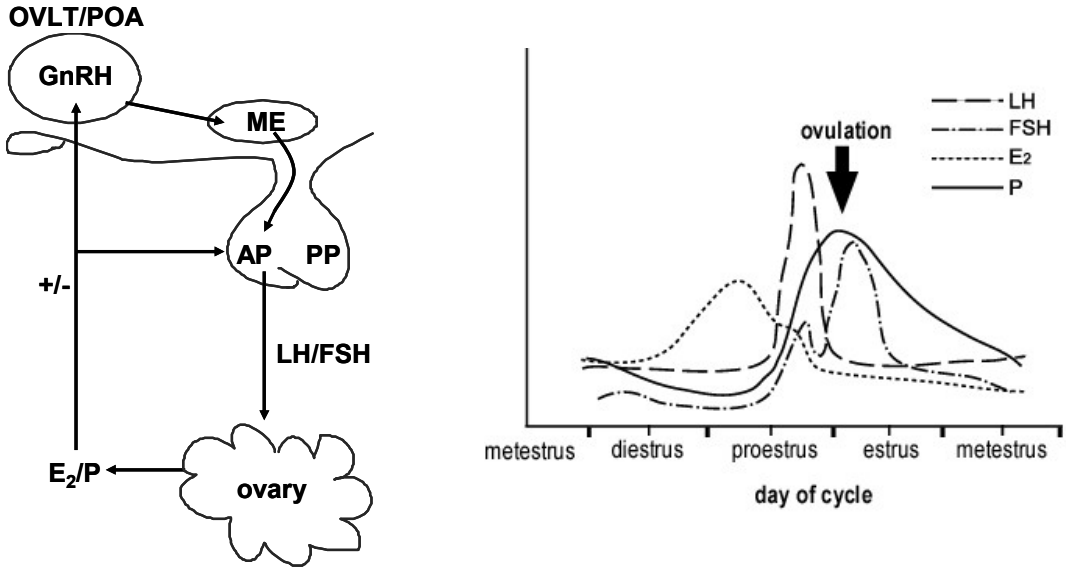

Figure 6. Schematic representation of the hypothalamus-pituitary-gonadal axis (left), and of plasma hormone concentrations during the estrous cycle (right; with permission from [50]) in the female rat. OVLT: organum vasculosum of the lamina terminalis; POA: preoptic area; ME: median eminence; AP: anterior pituitary; PP: posterior pituitary; LH: luteinizing hormone; FSH: follicle-stimulating hormone; E_2: estradiol; P: progesterone.

1.3. SOMATOTROPIC AND GONADOTROPIC INTERACTIONS

Changes in GH secretion during puberty, pregnancy and during the ovarian cycle indicate that GH is relevant to reproduction [38]. Also medical cases support this notion: although a proportion of the GH-deficient and GH-resistant women have normal menstrual cycles and are able to conceive, many of them require assisted reproductive technologies to conceive (reviewed in [1]). In rodents too, an altered GH status affects reproduction: GH-deficient animals are hypogonadal and completely infertile [53, 54], whereas animals

overexpressing GH show reduced fertility [55, 56]. So, both reduced and elevated levels of plasma GH counteract normal reproductive function, suggesting that the activity of the somatotropic axis needs to be in balance for an optimal functioning of the reproductive axis. This paragraph will further illustrate how these two axes may interact.

Ovary

Ovarian dysfunction in women is often associated with an altered GH secretion [38], and animals with GH-R deficiency show distinct disturbances in ovarian development (reviewed in [57]). In women, GH release increases during the preovulatory phase of the ovarian cycle [8, 38], GH is locally produced in the ovaries, and GH-Rs are present on ovarian cells (reviewed in [1, 38, 57]), suggesting that GH directly affects ovarian function. Indeed, GH stimulates folliculogenesis, prevents atresia of follicles (reviewed in [1, 57, 58]), and has a facilitatory role in ovulation (reviewed in [1, 38]). Also, GH stimulates E_2 and P synthesis in the ovaries (reviewed in [1, 57]).

Many of the actions of GH on ovarian function may be induced by a local production of IGF-I: both IGF-I and IGF-I-Rs are present in ovarian tissues [1]. Almost all actions of FSH on the ovaries are amplified by IGF-I (reviewed in [59]). Moreover, recent experiments in GH-R-knock-out mice in our laboratory showed that IGF-I stimulates follicle recruitment (Slot *et al*., manuscript in preparation).

Pituitary

Circulating plasma GH levels correlate with plasma LH, FSH and E_2 concentrations during the ovarian cycle in humans [38]. The relevance of GH to normal sexual development is reflected by the fact that gonadotrophs are poorly, if at all, developed in rats lacking GH during the fetal stage [38]. GH-deficient animals show decreased plasma gonadotropin levels [53] and a reduced GnRH-induced gonadotropin release [60]. GH-overexpression results in a decreased proestrous LH surge [53] and in decreased GnRH-induced gonadotropin release [54, 61], which is probably caused by reduced LH and FSH mRNA levels in the pituitary [38]. In the adult female rat, 40% of the GH cells co-express LH mRNA and 60% co-express FSH mRNA. The percentage of somatotroph and gonadotroph co-expression peaks on the day of proestrus. Moreover, 30-40% of the GH cells bind GnRH [58], implying a very close interaction between the two axes at the level of the pituitary.

IGF-I also plays a role at the level of the pituitary in the somatotropic and gonadotropic interaction. Long-term treatment with IGF-I increases GnRH-stimulated LH secretion [62], and incubation with IGF-I increases the release of both LH and FSH from cultured anterior pituitary cells [63].

Hypothalamus

Possible interactions between the GH and reproductive axis at the level of the hypothalamus have been rarely studied. Yet, the hypothalamic changes found in animals with an altered GH status may provide information on the possible mechanisms affecting reproductive function. In GH-deficient animals, NPY mRNA levels and the number of NPY-containing neurons in the ARC are reduced [64], indicating possible changes at the regulatory level of GH release. The distribution of GnRH neurons in several hypothalamic areas is changed in animals overexpressing GH [65], and *in vitro* GnRH release is decreased in transgenic males [53].

In GH-deficient animals, the amount of SOM mRNA in the PeVN is decreased [66], whereas in animals overexpressing GH, the amount of SOM mRNA and the number of SOM neurons in the PeVN is increased. The number of SOM and GHRH neurons in the ARC of GH overexpressing mice, however, is decreased [65, 66]. An altered GH status hence results in changes at the hypothalamic level of both the somatotropic and gonadotropic axis.

In the POA, the majority of GnRH cell bodies show IGF-I immunoreactivity and in the ME GnRH neuroterminals are located close to IGF-I-immunopositive cells. IGF-I is able to regulate GnRH gene expression [67] and may affect GnRH release from the ME [68]. Thus, also the hypothalamic regulation of GH and LH and FSH release may be functionally related. In addition, one study showed that also GnRH may have the properties of a GH-releasing factor [69], while on the other hand, SOM may directly inhibit LH release and affect both gonadotropic cell numbers and morphology [70, 71]. Figure 7 shows an overview of the possible interactions between the gonadotropic and somatotropic axis.

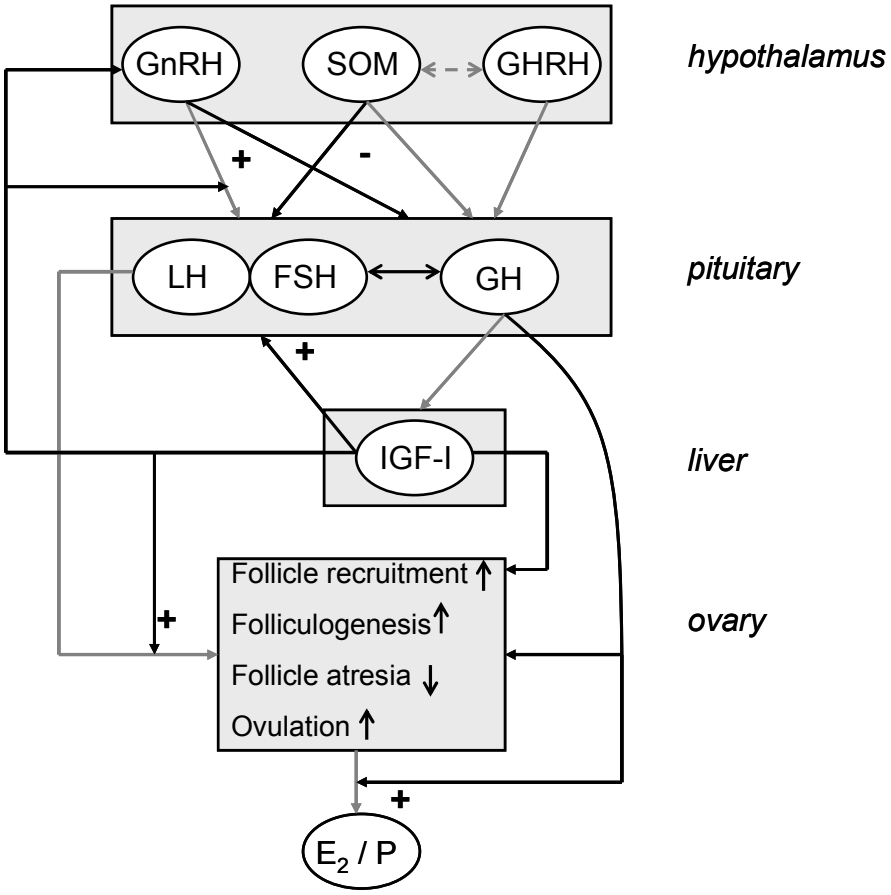

Figure 7. Schematic representation of the reported interactions between the somatotropic and gonadotropic axis (depicted by black arrows). Gray arrows represent normal regulation of both axes. For more information and references: see text. GnRH: gonadotropin-releasing hormone; SOM: somatostatin; GHRH: growth hormone (GH)-releasing hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone; IGF-I: insulin-like growth factor-I; E_2: estradiol; P: progesterone.

Somatotropic and gonadotropic interaction during life

During puberty, plasma GH levels are elevated in both humans and rats, which is the result of increased GH pulse amplitude. This increase in GH pulse amplitude is initiated by gonadal steroids and is crucial for the pubertal growth spurt and sexual maturation [38]. GH-deficiency delays puberty onset in both male [72, 73] and female [74, 75] rats, and in women (reviewed in [1]). On the other hand, GH overexpression advances puberty in female mice [61], indicating that “normal” plasma GH concentrations are needed for a normal sexual maturation.

Transgenic mice that overexpress GH exhibit various symptoms of early aging including reduced reproductive function [61]. On the other hand, GH treatment in aged (18

months old) female rats restores plasma LH and E_2 levels to those found in young females. Moreover, in 60% of these animals, estrous cyclicity could be restored following long-term GH treatment [76].

The effects of GH on puberty and reproductive aging may, at least in part, be mediated by changes in plasma IGF-I concentrations: During puberty, IGF-I mRNA levels in the POA and ME increase, whereas they decrease during reproductive aging [68]. In reproductively aged women, plasma GH and IGF-I levels decline despite the stimulatory effects of E_2, which may even be elevated in these women [77].

Hence, during all phases of life, the function of the somatotropic and gonadotropic axis is closely related. Clearly, an altered GH status leads to changes in reproductive function at all levels of the gonadotropic axis. With respect to the regulation of GH release, the hypothalamic SOM system may be vital. Moreover, GH-deficiency as well as GH-overexpression, both correlated with a decreased reproductive function, result in altered hypothalamic SOM levels. Changes in reproductive function following changes in GH status may therefore be dictated, at least in part, by hypothalamic SOM actions.

1.4. SOMATOSTATIN

Structure

SOM is synthesized as a prohormone, preproSOM, consisting of 92 amino-acids. PreproSOM undergoes tissue-specific processing to produce either a 14 amino acid peptide (SOM-14) or a 28 amino acid, N-terminally extended form (SOM-28), which are present in the hypothalamus in the ratio of 4:1 (reviewed in [19, 78]). Both are biologically active forms with similar potency with respect to GH secretion, and bind to all 5 known SOM-receptors with high affinity. The biological significance of the two forms in relation to neuroendocrine function, however, remains to be determined [19]. SOM secretion from the PeVN may be regulated by pathways originating outside the hypothalamus (cholinergic and adrenergic pathways), as well as by intrahypothalamic pathways (gamma-aminobutyric acid (GABA), glutamate, and possibly dopamine) [19].

Distribution

SOM has a widespread distribution in the CNS: high levels can be found in the amygdala and brainstem, and lower levels in the limbic system and neocortex [17], as well

as in the periphery [19]. Within the hypothalamus, SOM cells are located, besides in the PeVN, in the paraventricular nucleus (PVN, which regulates the neuroendocrine stress response), the ARC (also containing GHRH neurons that stimulate GH release), the suprachiasmatic nucleus (SCN, which plays an important role in the regulation of seasonal and circadian hormonal rhythms), and in the ventromedial nucleus (VMN, involved in the regulation of reproductive functions). These neurons do not project to the ME, in contrast to SOM neurons located in the PeVN, indicating that SOM neurons have several different functions within the hypothalamus [19].

Besides its involvement in the regulation of GH release, periventricular SOM also inhibits the release of thyroid stimulating hormone (TSH) and possibly also adrenocorticotropic hormone (ACTH), prolactin [17] and LH, as already mentioned in paragraph 1.3 [70].

SOM receptors

The transmembrane G-protein-coupled SOM receptor (SSTR) now comprises five subtypes ($SSTR_{1-5}$). All five receptor subtypes bind SOM-14 and SOM-28 with equal affinity [78]. SSTR subtypes are very specifically distributed within the hypothalamus and pituitary. For instance, in the PeVN and ARC, as well as the anterior hypothalamus, containing most GnRH neurons, $SSTR_1$ and $SSTR_2$ are expressed in much higher numbers than $SSTR_{3-5}$ [79-81]. Also, somatotrophs in the pituitary express mainly $SSTR_4$ and $SSTR_5$, whereas gonadotrophs mainly express $SSTR_2$ [82]. Specific SSTR subtypes may hence be involved in specific mechanisms with respect to the regulation of somatotropin and gonadotropin release. Moreover, the fact that SSTRs are present in hypothalamic areas involved in the regulation of gonadotropin release, and in gonadotrophs themselves, supports the hypothesis that hypothalamic SOM may be involved in the somatotropic and gonadotropic interaction.

1.5. SUMMARY AND SCOPE OF THE THESIS

Both GH secretion and reproductive function can be restored in aged animals, indicating that neither pituitary capacity, nor ovarian function is crucially changed during the early stages of reproductive aging. This strongly suggests that aging of neuroendocrine function may start at the level of the hypothalamus. Hence, as the somatotropic and gonadotropic axes are closely related, their interaction may also take

place, at least in part, at the level of the hypothalamus. With respect to the hypothalamic regulation of GH release, SOM plays a crucial role in determining and maintaining the sexual differences. In aged animals and in animals with an altered GH status, hypothalamic SOM concentrations and release are significantly changed. Also, SOM has been shown to affect LH release and gonadotropic cell numbers and morphology directly. Moreover, SSTRs are present in the hypothalamic area containing GnRH neurons, and on gonadotrophs in the pituitary, indeed indicating direct effects of SOM on the hypothalamic-pituitary regulation of female reproduction. Based on the above, we hypothesize that SOM, originating from the PeVN, may play a central role in the interaction between the somatotropic and gonadotropic axis.

To test our hypothesis, adult female rats were studied. In addition, we used male rats to compare our data with previous studies from the literature, as most research on the hypothalamic regulation of GH release to date has been performed in males. To test the hypothesis that periventricular SOM neurons may play a central role in the interaction between the GH and reproductive axis in the female, we first studied the hypothalamic SOM system of the female rat in more detail. Literature on the specific anatomical definition of the PeVN area containing the SOM cells that project to the ME is scarce. Therefore, the PeVN area was strictly defined with respect to anatomical boundaries and the SOM cell location within the PeVN was determined in more detail in both adult male and female rats (*chapter 2*).

Next, we aimed to study the hypothalamic regulatory mechanism of GH secretion in the adult female rat. To this end, the acute feedback effect of GH on (the activation of) SOM cells in the PeVN was studied in intact cycling female (on the day of proestrus), ovariectomized (OVX) rats, OVX rats treated with estradiol (E_2), and in addition, to compare with previous data from the literature, adult male rats (*chapter 3*).

Data from literature indicate that E_2 is likely to affect SOM cell activity. The reported data on the precise role of E_2 on SOM cells in the PeVN, however, are somewhat contradictory. Our data from *chapter 3* suggested that not only E_2 but also other ovarian factors, e.g. progesterone (P), may affect the feminine hypothalamic SOM system. Therefore, the effects of gonadal steroids (E_2 and/or P) on the number of SOM peptide-containing cells in the PeVN were studied in adult OVX female rats, and compared to intact male rats (*chapter 4*).

It may be clear from the literature that interactions between the somatotropic and gonadotropic axes seem to influence neuroendocrine output. We tested the hypothesis that SOM, originating from the PeVN, not only regulates GH release from the pituitary, but may also be the central factor in the interaction between the two axes. To this end, the effect of a single central injection with a SOM analog, just prior to surge onset, on the E_2-induced LH surge and on the activation of hypothalamic GnRH cells was studied in adult OVX female rats (*chapter 5*).

During aging, there appears to be a concurrent decline in the activity of the somatotropic and gonadotropic axis. A previous study in our laboratory showed that already in 9 months old female rats the LH surge was decreased, which appeared to be primarily correlated to hypothalamic changes. These changes occurred despite the fact that stimulatory levels of E_2 were still present [50]. Moreover, evidence exists that exposure to high levels of E_2 during life may in fact accelerate reproductive aging. Thus, we studied the effect of acute E_2 feedback on SOM cells in the PeVN of young adult (4 months old) and middle-aged (9 months old) OVX female rats (*chapter 6*). To this end, rats from two sub-strains were used: the Wistar, displaying predominantly 4-day estrous cycles, and a hybrid of two Wistar strains, the (UxRP)F1, displaying predominantly 5-day estrous cycles due to diestrous lengthening. The (UxRP)F1 females are hence exposed to high plasma E_2 levels for one extra day each ovarian cycle. Moreover, from literature it is known that female (UxRP)F1 rats become reproductively aged earlier in life than Wistar rats.

Finally, in *chapter 7* the main conclusions of this thesis are presented and discussed. In addition, we propose a hypothesis for the possible mechanism(s) via which SOM may act on both the somatotropic and gonadotropic axis. The possible consequences of an altered GH status, in particular during aging, on hypothalamic SOM and consequently on reproductive function are discussed.

CHAPTER 2

Distribution pattern of somatostatin cells within the rat periventricular nucleus: effect of sex and steroidal environment

Harmke H. Van Vugt, Bert J.M. Van de Heijning, and Eline M. Van der Beek

ABSTRACT

To determine the distribution of somatostatin (SOM) cells within the periventricular nucleus of the hypothalamus (PeVN) in the adult rat, we studied the number of SOM cells in a strictly defined series of PeVN sections of male and ovariectomized (OVX) female rats, following sham or gonadal steroid treatment.

The distribution of SOM cells within the PeVN showed a very characteristic pattern in both male and female rats. Both the rostro-caudal distribution and the total number of SOM cells in each PeVN section was different between the sexes. Moreover, our data suggest that gonadal steroids may affect the number of SOM cells in specific subregions of the PeVN in female rats.

Based on these observations and data from the literature, we divided the PeVN into three subareas. We hypothesize that within the PeVN specific SOM cell sub-populations may exist.

INTRODUCTION

In the adult rat, the growth hormone (GH) secretory pattern is sexually dimorphic. In males, GH secretion is characterized by regular high amplitude GH pulses occurring at approximately 3-h intervals, and low baseline levels. Females exhibit irregular low amplitude GH levels and higher baseline levels than males [4, 6, 7]. GH release from the pituitary is mainly regulated by the interplay between the stimulating effects of GH-releasing hormone (GHRH), synthesized in the arcuate nucleus of the hypothalamus (ARC), and the inhibiting effects of somatostatin (SOM), synthesized in the periventricular nucleus of the hypothalamus (PeVN) [19, 83-85].

The sexual dimorphism in GH secretion has been suggested to be predominantly the result of differences in SOM release patterns from the median eminence (ME) between the sexes [19, 23, 25]. In males, SOM is released in regular pulses with maximal levels during a GH trough and minimal levels during a GH peak [25, 83, 84, 86, 87]. In female rats SOM release is thought to be more continuous at a level in-between the maximum and minimum levels that are found in males [26, 35], although relatively small SOM peaks have been reported in female rats [33]. Thus, SOM release patterns from the ME may indeed contribute considerably to the sexual differences in GH release from the pituitary. Consequently, SOM synthesis and release may be influenced by gonadal steroids. Indeed,

in both male [23, 28-31] and female rats [28, 29, 31, 33, 88, 89] gonadal steroids have been shown to affect hypothalamic SOM mRNA and peptide concentrations, although the exact role of estradiol (E_2) in the female remains unclear.

It is well known that periventricular SOM cells located in the anterior part of the hypothalamus are the neurons that project to the ME to regulate GH release from the pituitary [90-94]. However, this periventricular part of the hypothalamus is still extensive and not very well defined. Previous studies [20, 24, 95, 96] and personal observations suggested that the anterior periventricular area is not homogeneous with respect to SOM cell function and distribution. Therefore, we studied the distribution of SOM cells within the PeVN in more detail. We compared SOM cell distribution between the sexes and studied the possible effects of gonadal steroids on the distribution of SOM cells in adult female rats.

MATERIALS AND METHODS

Immunocytochemistry

For animal treatment and brain collection, see *chapter 4* of this thesis.

One third of the 40 µm coronal brain sections was stained for SOM peptide using free-floating immunocytochemical techniques [97]. Sections were pretreated with 3% H_2O_2 (Merck) in 0.1 M Tris buffer (TBS; pH 7.4) for 30 min, followed by extensive washing with TBS. Subsequently, sections were incubated with the primary polyclonal rabbit antibody raised against SOM peptide (Somaar 080289, NIH, Amsterdam, The Netherlands; final dilution 1:30,000) [98], diluted in TBS containing 0.5% Triton-X-100 and 0.25% gelatin ("Supermix") for 1 night at 4ºC. This was followed by incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories; 1:500 in Supermix) for 1.5 h at room temperature, and Avidin-Biotin Complex-elite (Vector Laboratories; 1:1500 in Supermix) for 1.5 h at room temperature. Between incubation steps, sections were thoroughly washed with TBS. SOM immunoreactivity was visualized by incubation with 0.05% 3,3'-diaminobenzidine (Sigma Chemical Company) in TBS containing 0.1% imidazole and 0.03% H_2O_2 for 14.5 min. Stained sections were mounted on albumin coated slides, dried, dehydrated and coverslipped in DEPEX (BDH Laboratory Supplies, Poole, England). SOM-immunoreactive (SOM-ir) neurons in the PeVN were counted using a computer assisted image analysis system (SION Image software program) following background correction.

Data analysis

The first section of the PeVN was determined to be the section in which the suprachiasmatic nucleus (SCN) first appeared (Plate 22 according to Paxinos and Watson [99]). The last section of the PeVN was determined to be the section just before the ventral medial nucleus (VMN) first appeared (Plate 26 [99]). The total PeVN, defined as described above, consisted of nine 40 µm sections with 80 µm intervals and included all periventricular SOM cells that have been shown to project to the ME [90, 94].

Images of only the left side of the brain sections containing the PeVN were captured using a microscope (10 x 5 magnification) with a digital black-and-white CCD camera (Sony, XC-77CE) connected to a computer, as no differences in SOM peptide content were anticipated between the left and right side of the hypothalamus (personal observations). Next, 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 [50]. By capturing each image at the same gray level (i.e. the average gray level of the entire image), a background correction was made. Subsequently, a threshold was determined by background measurements (i.e. the gray level in an area that contained SOM-ir fibers, but no SOM-ir cell bodies) in a representative selection of the images. Using this threshold, SOM-ir neurons (i.e. cell bodies, without fibers, that consisted of a minimal size of 5 pixels) were counted automatically (for more details: [50]).

Data were analyzed using the SPSS statistical analysis system. To compare the number of SOM-ir cells between the groups in each PeVN section, a Oneway ANOVA was used and in addition separate Bonferroni tests were used as a post-hoc test. Differences were considered to be significant when $p<0.05$.

For the schematic camera lucida drawings of the PeVN, images were imported into Adobe Illustrator (version 8.0), a drawing program in which schematic drawings can be made by placing an empty layer over the image file. In this layer, immunoreactive neurons were marked and saved.

RESULTS AND DISCUSSION

SOM-ir cell distribution within the PeVN showed a very characteristic pattern with respect to the location of the SOM cells in relation to the third ventricle and to total cell numbers. In the anterior part of the PeVN a small, dense population of SOM-ir cells was located just above the SCN, lined along the ventricle wall. Moving more caudally towards the medial part of the PeVN, a dense population of SOM-ir cells covered the whole length of the third ventricle side area, with maximum numbers of SOM-ir cells at the mid-level (from ventral to dorsal) of the ventricle. Finally, in the posterior part of the PeVN, small populations of SOM-ir cells were mainly located along the mid- and dorsal part of the ventricle side (Figure 1 and 2).

The total SOM-ir cell number was higher in male compared to ovariectomized (OVX) female rats in each PeVN section (Figure 3). The rostro-caudal distribution within the PeVN appeared to be slightly different between the sexes, i.e. maximal SOM-ir cell numbers were found in section 7 in males, but consistently in section 6 in the OVX females, irrespective of gonadal steroid treatment (Figure 3). Although the general rostro-caudal distribution of SOM-ir cells was largely unaffected by steroid treatment, the number of SOM-ir cells seemed to be different between the treatments in specific PeVN sections. Estradiol (E_2) or progesterone (P) treatment alone appeared to decrease the number of SOM-ir cells mainly in the anterior and medial parts of the PeVN compared to the untreated OVX females, whereas combined treatment with E_2 and P seemed to increase the number of SOM-ir cells in the medial and posterior parts of the PeVN. These data suggest that in the female rat gonadal steroids may affect the number of SOM peptide-containing cells in specific parts of the PeVN.

Based on the observations described above, we divided the PeVN into three subregions: the anterior (containing sections 1-3), medial (sections 4-6), and posterior (sections 7-9) PeVN. In line with others who also proposed a possible subdivision of the periventricular SOM cells [20, 24, 95], we hypothesize that within the PeVN specific SOM cell sub-populations may exist with specific functions with regard to for instance the regulation of GH secretion.

In the next two chapters, we will study the possible functions of the presumed different SOM cell subpopulations within the PeVN.

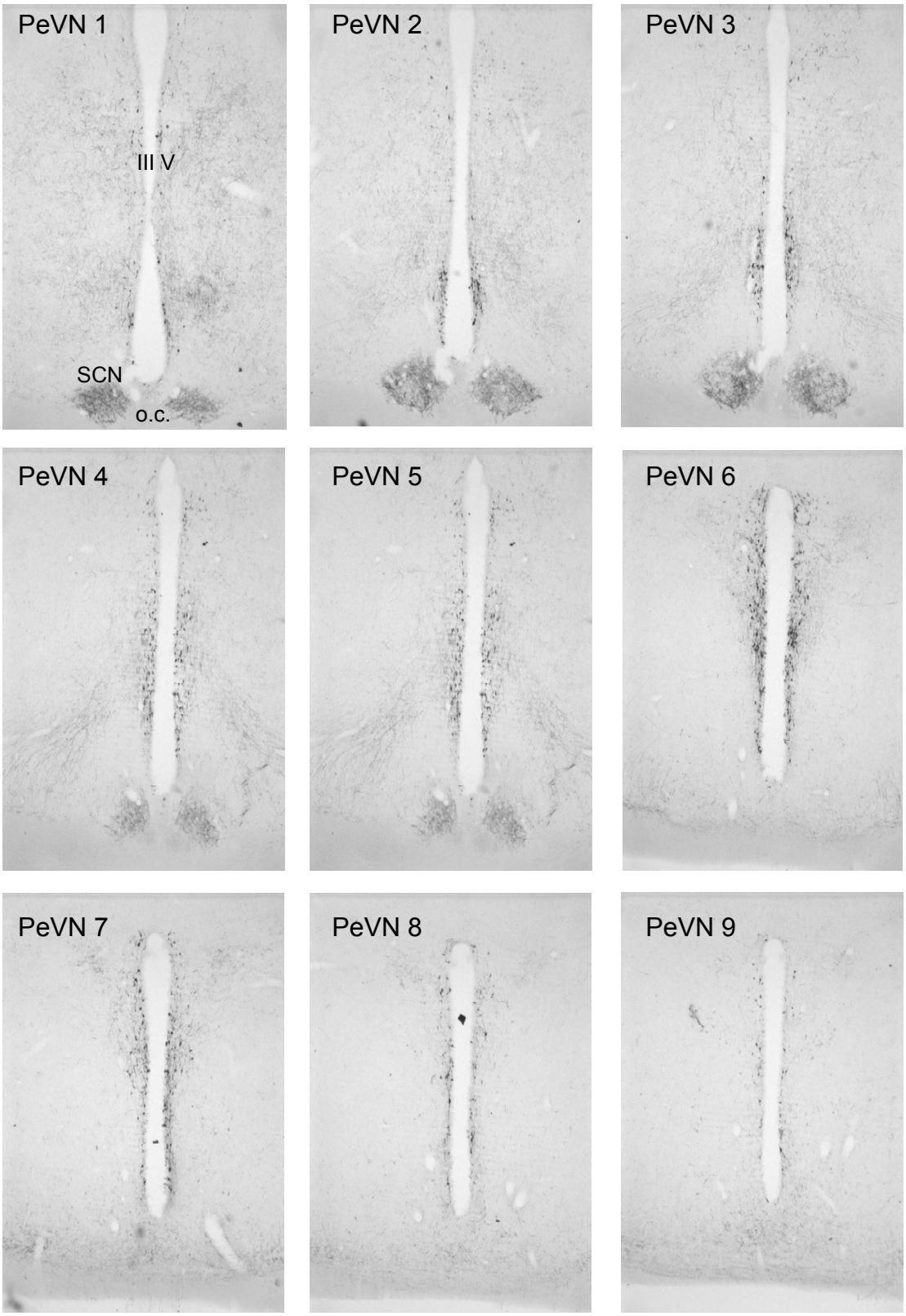

Figure 1A. Pictures of 9 successive PeVN sections at a 10 x 3.75 magnification of one ovariectomized E_2-treated female rat. Each PeVN section is 40 µm thick and the distance between the sections is 80 µm. III V: third ventricle; o.c.: optic chiasm; SCN: suprachiasmatic nucleus.

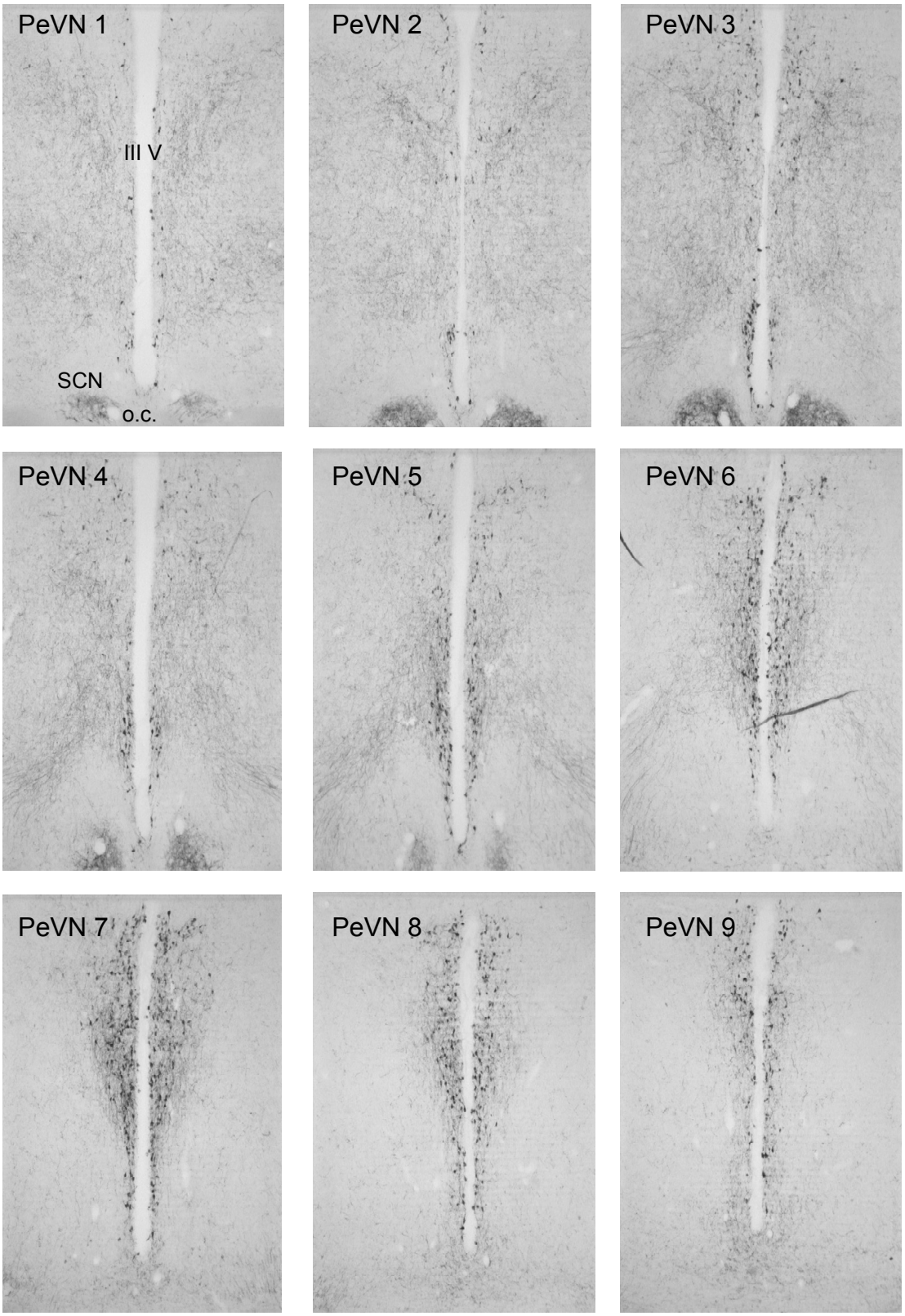

Figure 1B. Pictures of 9 successive PeVN sections at a 10 x 3.75 magnification of one male rat. Each PeVN section is 40 µm thick and the distance between the sections is 80 µm. III V: third ventricle; o.c.: optic chiasm; SCN: suprachiasmatic nucleus.

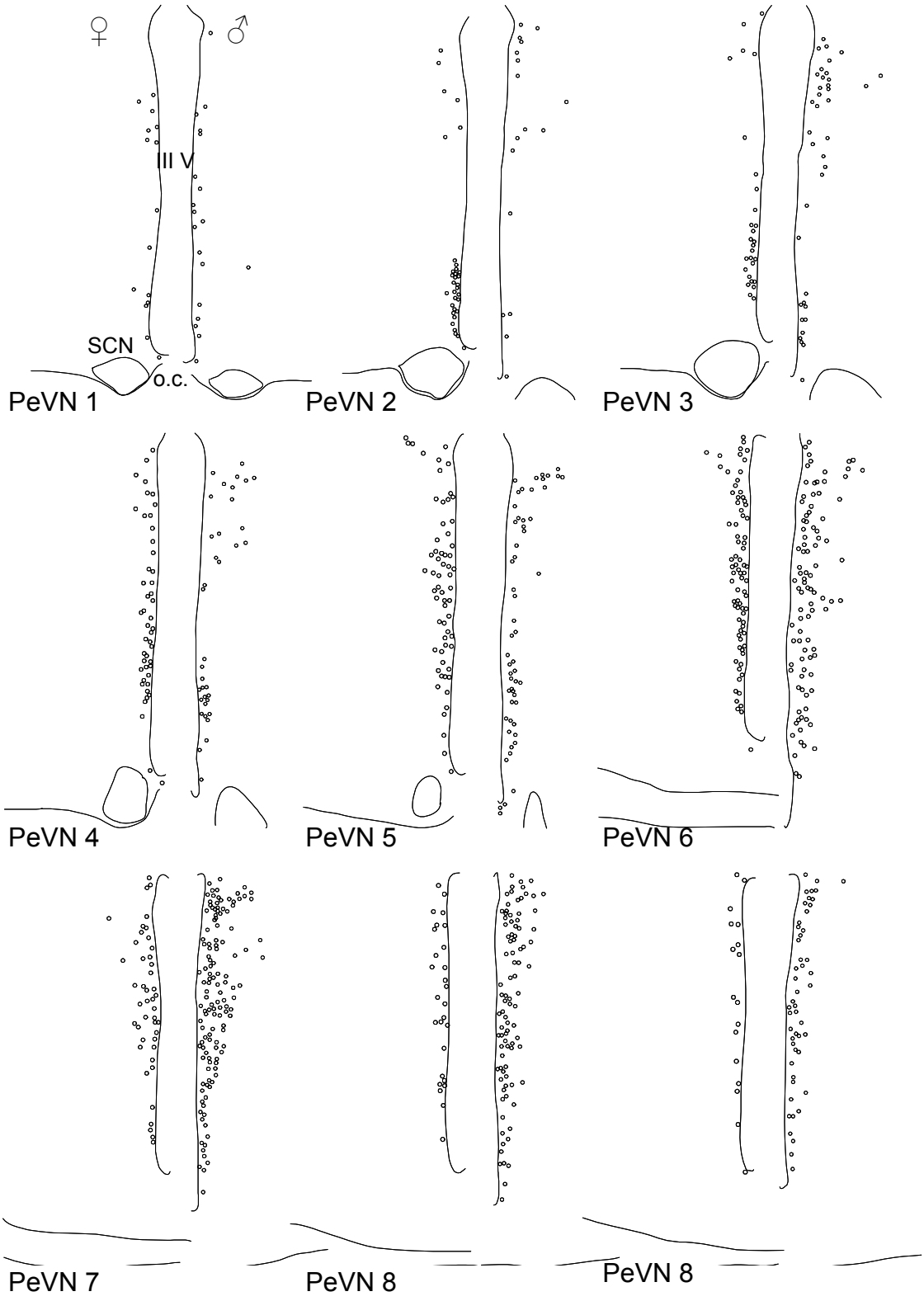

Figure 2. Camera lucida drawings of somatostatin cells throughout the PeVN of the adult rat. Immunoreactive cell bodies at the left (female) and right (male) side of the third ventricle are represented with open circles. These are representative drawings of the 9 successive PeVN sections of the OVX+E_2 female and the male rat as shown in figure 1A and 1B, respectively. III V: third ventricle; o.c.: optic chiasm; SCN: suprachiasmatic nucleus.

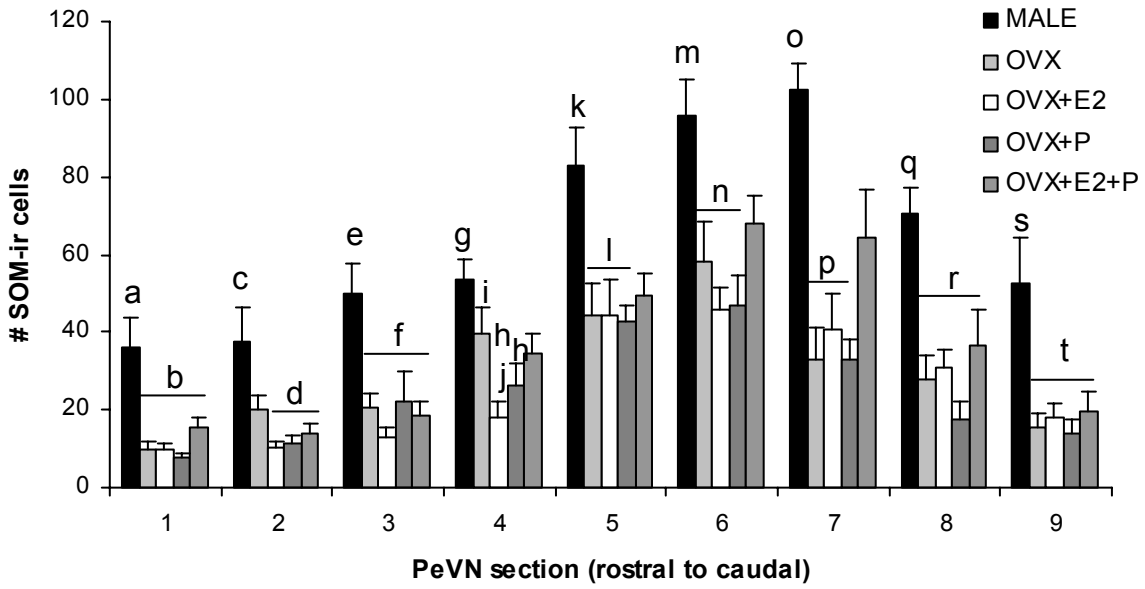

Figure 3. Number of SOM-ir cells (means ± SEM) in the 9 successive PeVN sections of male (black bars), OVX (light gray bars), OVX+E_2 (white bars), OVX+P (dark gray bars), and OVX+E_2+P (gray bars) rats (n=8 for all groups).

CHAPTER 3

Activation of somatostatin cells in the periventricular nucleus after a single, intravenous growth hormone injection: effect of steroidal environment

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ABSTRACT

In the adult rat, the sexual dimorphism in growth hormone (GH) secretion has been suggested to predominantly result from differences in somatostatin (SOM) release patterns from the median eminence (ME). Both GH secretion and the hypothalamic SOM system are influenced by gonadal steroids. GH regulates its own secretion mainly by feeding back at the level of the hypothalamus. In males, the effects of GH on SOM in the periventricular nucleus (PeVN) are likely to be direct, whereas in the female little is known about possible direct feedback effects of GH on SOM cells.

We studied the acute effects of a single GH stimulus on PeVN SOM cells and SOM mRNA in both sexes, and we examined the possible role of estradiol on the hypothalamic GH feedback in the female rat.

Intact adult male and female (proestrous) rats, and ovariectomized (OVX) female rats, treated with either estradiol benzoate (OVX+EB) or vehicle, were given a single i.v. injection with GH or saline and perfused 90 min later. To study the time course effect of GH feedback, OVX+EB females were given a single i.v. injection with GH and perfused after 15, 30, 60, 120, or 240 min. Brain sections were subsequently double stained for SOM and cFos peptide, a nuclear marker for cell activation, or for SOM mRNA.

We found a significant higher number of SOM cells in the PeVN of proestrous female rats compared to males and both OVX groups, but a significant higher number of SOM mRNA-containing cells in the males compared to all females, irrespective of GH treatment. A single injection with GH resulted in changes in SOM cell numbers in different PeVN subregions of male and proestrous female rats. In all female experimental groups, GH treatment increased SOM cell activation in the PeVN, albeit only significantly in OVX females, but not in male rats. The number of SOM mRNA-containing cells was not significantly affected by GH treatment.

In conclusion, our study confirms previous findings showing that the hypothalamic SOM system differs between the sexes. Moreover, we are the first to demonstrate that GH acutely affects SOM cell activity in the PeVN of female rats. Our data suggest that gonadal steroids may modulate the acute GH feedback on SOM cells in female rats.

INTRODUCTION

In the rat, the growth hormone (GH) secretory pattern is sexually dimorphic. In males, GH secretion is characterized by regular high amplitude GH pulses occurring at approximately 3-h intervals, and low baseline levels. Females exhibit irregular low amplitude GH levels and higher baseline levels than males [4, 6, 7, 26].

Gonadal steroids play an important role in the generation of the specific male- and female-like GH release patterns. In female rats, ovariectomy (OVX) increased GH peaks and lowered GH baseline levels [11] and treatment with testosterone (T) induced a male-like GH secretory pattern [6, 14, 16]. Estradiol (E_2) treatment in OVX rats increased GH baseline levels and decreased GH peak amplitude (Van Vugt *et al*., unpublished data). In castrated (CX) male rats, GH peak amplitude was reduced. Exposure of these animals to E_2 increased basal GH levels and increased GH pulse frequency [13], whereas T treatment restored the typical masculine GH secretory pattern [10].

GH release from the pituitary is mainly regulated by the interplay between the stimulating effects of GH-releasing hormone (GHRH), synthesized in the arcuate nucleus of the hypothalamus (ARC), and the inhibiting effects of somatostatin (SOM), synthesized in the periventricular nucleus of the hypothalamus (PeVN) [19, 83-85, 91, 92, 100, 101].

The sexual dimorphism in GH secretion has been suggested to be predominantly the result of the differences in SOM release patterns from the median eminence (ME) between the sexes [19, 23, 25]. In males, SOM is released in regular pulses with maximal levels during a GH trough and minimal levels during a GH peak [83-87, 102], whereas in female rats SOM release is thought to be more continuous [26, 35]. One study reported small SOM peaks in female rats [33], suggesting that SOM release in females may be pulsatile. However, these pulses are irregular and relatively small, and presumably with mean levels in-between the maximum and minimum levels that were documented in males [26, 35]. GHRH release from the ME is released in pulses in both sexes, albeit regular in male rats, with peak levels occurring during the SOM troughs, but irregular in females [25, 26, 35, 83, 84, 86]. Considering the above, SOM release patterns from the ME may thus contribute to a high extent to the sexual differences in GH release from the pituitary, suggesting that also SOM synthesis and release may be influenced by gonadal steroids.

Indeed, in CX male rats both hypothalamic SOM mRNA and peptide content was decreased, which could be reversed by treatment with T [28-30, 32]. In female rats E_2 seems to affect both hypothalamic SOM peptide and mRNA, although conflicting results

have been reported [14, 28, 29, 31-33]. In cycling female rats, hypothalamic SOM concentrations fluctuate: SOM mRNA in the PeVN was found to be minimal on the day of diestrus and maximal on the day of proestrus [88], when SOM peptide levels in the preoptic area, posterior hypothalamus and ME were demonstrated to be decreased [89]. Yet, SOM release from the ME was found to be highest on the day of proestrus [33], concurrent with high plasma E_2 levels [103]. Hence, in the female rat E_2 may influence hypothalamic SOM synthesis and release, probably inducing the typical feminine GH secretory pattern. However, the exact role of E_2 on the hypothalamic SOM system in female rats remains to be further elucidated.

Regulation of GH release from the pituitary by SOM and GHRH involves direct feedback actions of GH itself at the level of the hypothalamus [104-107]. In male rats, the effects of GH on periventricular SOM neurons are most likely to be direct. Prolonged as well as acute administration of GH resulted in increased SOM levels in portal blood [108], in increased SOM mRNA levels in the PeVN [109], and in increased cFos gene expression in SOM cells in the PeVN of male rats [110-112]. Moreover, in male rats SOM cells in the PeVN have been demonstrated to express GH-receptors (GH-R) [21, 22, 113]. Feedback of GH at the level of GHRH neurons in the ARC seems to be mainly indirect and may involve neuropeptide-Y (NPY) neurons in the ARC, or local SOM neurons projecting to the GHRH cells, or both, leading to a decreased GHRH release from the ME [24, 86, 110, 112, 114-117].

Although different from males, also in female rats evidence exists for a direct negative feedback loop of GH on its own secretion [107, 118, 119]. Little is known, however, about a possible involvement of periventricular SOM cells in GH feedback in the female. To our knowledge, no data exist on GH-R colocalization on specific hypothalamic SOM cell populations in the female, although GH-R expression has been demonstrated in the ARC of female rats [120, 121].

The data described above suggest that, at least in male rats, GH regulates its own release by directly activating the hypothalamic SOM system. Based on the proposed differences in SOM release that may underlie the sexual dimorphic pattern of GH release, we hypothesize that also the GH feedback on SOM neurons may be different between male and female rats.

In the present study we compared the acute feedback effects of a single injection with GH on the activation of SOM cells and the SOM mRNA content in the PeVN between

the sexes, and we examined the role of estradiol in SOM cell activation following acute GH feedback in female rats.

MATERIALS AND METHODS

Acute GH feedback on hypothalamic SOM cells: role of gonadal steroids

Twelve male and 32 female Wistar rats (Harlan, Horst, The Netherlands), 10 weeks of age on arrival, were individually housed in a room with controlled lighting (LD 12:12, lights on at 04.00 h), temperature (21°C) and humidity (70%). In the same room, 2 naïve male rats, 4 months of age on arrival, were housed together in a cage. These males were used for the monitoring of receptive behavior. Standard pelleted food and water were available *ad libitum*. All experiments were approved by the Wageningen University Animal Ethics Committee.

Estrous cyclicity was monitored by daily vaginal lavages in all female rats. In addition, receptive behavior was monitored daily by introducing a naïve male rat to each female rat just prior to dark onset. Twelve female rats that showed regular estrous cycles (i.e. at least 3 successive regular cycles) were selected. The remaining twenty female rats were bilaterally ovariectomized (OVX) under gas anesthesia with a mixture of Isoflurane (Isoflo; Abbott Laboratories Ltd, England) and oxygen (3:1 ratio). After a recovery period of 10 days the OVX rats received either a subcutaneous (s.c.) injection with estradiol benzoate (EB; 12.5 µg/0.1 ml oil; n=12) or with oil (n=12) at 9:00 h on two consecutive days before the day of perfusion. On the day of perfusion, all rats were given either an intravenous (i.v.) injection with recombinant human growth hormone (rhGH; Zomacton, Ferring, Hoofddorp, The Netherlands; 0.2 mg/0.2 ml, dissolved in saline) (n=6 for each experimental group) or saline (0.2 ml; n=6 for each experimental group). The rhGH or saline injection was given via the tail vein under a short gas anesthesia with a mixture of Isoflurane and oxygen (5:1 ratio) 90 min before perfusion (i.e. just before onset of the dark phase: between 15:30 and 16:00 h). Cycling female rats were perfused at the same time on the afternoon of proestrus. Prior to perfusion animals were given an overdose of Nembutal® (CEVA sante animale B.V., Maassluis, The Netherlands) anesthesia intraperitoneally (i.p.; 1.5 ml/kg body weight). Female rats were perfused transcardially with 200 ml saline followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate buffer (4% PFA; pH 7.4), and male rats with 300 ml saline followed by 400 ml 4% PFA. Brains

were removed from the skull immediately after perfusion and postfixed in the same fixative for 25 h at 4ºC. Prior to sectioning, tissue blocks containing the hypothalamus were embedded in 20% gelatin and fixed in 4% PFA for 4 h at 4ºC [122]. Coronal sections of 40 µm were sliced using a Vibratome (Vibratome Company; series 1500) and were stored in 0.1 M Tris buffer (TBS; pH 7.4) containing 1% polyvinylpyrrolidone-40 (PVP-40; Sigma), 30% ethylene glycol (Sigma) and 30% sucrose (Sigma) ("cryprotectant" [123]) at 4ºC until further use.

Time course of GH feedback on hypothalamic SOM cells in female rats

Twenty female Wistar rats (Harlan, Horst, The Netherlands), 10-12 weeks of age on arrival, were individually housed in a room with controlled lighting (LD 12:12, lights on at 04.00 h), temperature (21°C) and humidity (70%). Standard pelleted food and tap water were available *ad libitum*. All experiments were approved by the Wageningen University Animal Ethics Committee.

All animals were bilaterally ovariectomized under gas anesthesia with a mixture of Isoflurane and oxygen (3:1 ratio). After a recovery period of 10 days all rats received an s.c. injection with EB (12.5 µg/0.1 ml oil) at 9:00 h on two consecutive days. On the day following the EB injections, the rats were given an i.v. injection with rhGH (0.2 mg/0.2 ml; dissolved in saline) at 15, 30, 60, 120 or 240 min (n=4 for each time point) before perfusion. The rhGH injection was given via the tail vein under a brief anesthesia with a mixture of Isoflurane and oxygen (5:1 ratio). All animals were perfused just before onset of the dark phase, i.e. between 15:30 and 16:00 h, as described above.

Immunocytochemistry

One third of the brain sections was double stained for SOM and cFos peptide using free-floating immunocytochemistry techniques [97]. Sections were pretreated with 3% H_2O_2 in TBS for 30 min, followed by extensive washing with TBS.

For the cFos staining, sections were incubated with the primary polyclonal rabbit antibody raised against cFos peptide (SC-052, Santa Cruz, #C076; final dilution 1:10,000) diluted in TBS containing 0.5% Triton-X-100 and 0.25% gelatin ("supermix") for 1 h at room temperature followed by 4 nights at 4ºC. This was followed by incubation with biotinylated goat anti-rabbit IgG (GaR-bio; Vector Laboratories; 1:400 in supermix) for 1.5 h at room temperature, and Avidin-Biotin Complex-elite (ABC; Vector Laboratories; final

dilution 1:1200 in supermix) for 1.5 h at room temperature. Between incubation steps, sections were thoroughly washed with TBS. cFos Immunoreactivity was visualized by incubation with 0.05% 3,3'-diaminobenzidine (DAB; Sigma Chemical Company) in TBS containing 0.2% nickelammoniumsulphate and 0.03% H_2O_2 for 15-20 min.

For the double staining, cFos-stained sections were washed in graded series of methanol, including 100% methanol with 0.3% H_2O_2 (9 short wash steps of 5 min each). After intense washing with TBS, the sections were incubated with the primary polyclonal rabbit antibody raised against SOM peptide (Somaar 080289, NIH, Amsterdam, The Netherlands; final dilution 1:30,000) [98] for 1 night at 4ºC. This was followed by incubation with GaR-bio (Vector Laboratories; 1:500 in supermix) for 1.5 h at room temperature, and ABC (Vector Laboratories; final dilution 1:1500 in supermix) for 1.5 h at room temperature. Between incubation steps, sections were thoroughly washed with TBS. SOM immunoreactivity was visualized by incubation with 0.05% DAB in TBS with 0.1% imidazole and 0.03% H_2O_2 for 12-13 min.

Stained sections were mounted on albumin coated slides, dried, dehydrated and coverslipped in DEPEX (BDH Laboratory Supplies, Poole, England).

In Situ Hybridization

Riboprobes complementary to the coding region of the preproSOM mRNA, were generated by *in vitro* transcription of the 395-bp cDNA fragment of rat preproSOM cloned into pSP65 vector [124, 125]. The DIG-labeled antisense cRNA probes were generated on linearized plasmids using SP6 RNA polymerase [126] (kindly provided by dr. M. Fodor, VUMC, Amsterdam, The Netherlands).

One third of the brain sections of the gonadal steroid experiment was used for the non-radioactive *in situ* hybridization (ISH), using free-floating ISH techniques based on techniques developed by dr. J. Veening (UMC St. Radboud, Nijmegen, The Netherlands) [127]. Sections were thoroughly washed with milliQ at 4ºC to remove all cryoprotectant. Next, sections were treated with 0.1 M triethanolamine (TEA), containing 3% 5 M NaCl for 10 min at 4ºC, and TEA containing 0.25% Acetic Anhydride for 10 min at 4ºC, followed by 4 x SSC containing 50% formamide for 1 h at 4ºC. 2.5 µl DIG-labeled cRNA (360 ng/µl) was added to 0.8 ml TE buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8) and 0.2 ml tRNA and heated for 3 min at 80ºC to degrade the probe, and immediately thereafter cooled on ice. Hybridization-mix was then prepared by adding this mixture with the probe to 3 ml

hybridization buffer (2.5 ml deionized formamide, 1 ml 50% dextransulphate, 300 µl 5 M NaCl, 40 µl 1 M Tris-HCl, 8 µl 0.5 M EDTA, 100 µl 50 x Denhardts, 12 µl milliQ, 40 µl 1 M dithiothreitol (DTT)). Subsequently, sections were hybridized overnight at 54ºC.

Sections were washed with 4 x SSC for 30 min and thereafter incubated with RNAse buffer (250 ml milliQ, 50 ml 5 M NaCl, 5 ml 1 M Tris-HCl, and 250 µl 0.5 M EDTA, pH 8) containing RNAse (Roche; 10 mg/ml) for 30 min at 37ºC, followed by incubation with RNAse buffer without RNAse for 30 min at 37ºC. Sections were subsequently washed with 2 x SSC for 30 min, 0.1 x SSC for 30 min at 54ºC, and 0.1 x SSC for 5 min, and thereafter blocked by incubation with buffer-1 (150 mM NaCl, 0.1 M Tris-HCl, pH 7.5), containing 2 g blocking reagent and 600 µl Triton-X (in 200 ml buffer-1). Next, sections were washed with buffer-1 for 10 min and incubated with anti-DIG AP antibody (diluted 1:1000 in buffer-1) overnight at 4ºC.

On day three, sections were washed with buffer-1 for 30 min, followed by washing with buffer-2 (0.1M Tris-HCl, 0.1 M NaCl, and 50mM $MgCl_2$, pH 9.5) for 10 min. To visualize the reaction product, sections were incubated with the freshly prepared staining solution (800 µl NBT/BCIP (2%) stock solution (Roche) and 4 ml Levamisole (48 mg/ml) in 35.2 ml buffer-2) for 2 h. The color reaction was performed in the dark and was stopped by adding TE buffer to the sections. Thereafter, sections were stored in 0.1 M phosphate buffer comtaining 0.01% sodium azide. Stained sections were mounted on superfrost slides, air dried and coverslipped in water-based mounting medium (Kaiser's gelatin).

Data analysis

The PeVN was defined as described in detail in *chapter 2*, and consisted of nine sections with 80 µm intervals that included the entire part of the periventricular SOM cells that has been shown to project to the ME [94].

SOM-immunoreactive (-ir) cells (brown) and SOM-ir cells containing a cFos-ir nucleus (blue-black) ("activated SOM-ir cells") in the PeVN were counted consistently at the left side of the brain under a light microscope (10 x 10 magnification), assuming that SOM peptide content is equal in the left and right side of the hypothalamus (personal observations). The total number of SOM-ir cells in the PeVN was determined to be the sum of activated and non-activated SOM-ir cells in the left side of the PeVN. Also, the percentage of activation (i.e. of the total number of SOM cells) was determined in the left part of the PeVN. A SOM-ir cell was defined as a cell body (that could be round, oval or

irregular shaped) from which at least one dendrite was clearly emerging. cFos positive nuclei appeared from very faint (light gray) to intensely stained (blue-black).

In addition to analyzing total numbers of SOM-ir cells and activated SOM-ir cells in the complete PeVN, the nucleus was divided into three subregions: the anterior (containing PeVN sections 1-3), medial (sections 4-6), and posterior (sections 7-9) PeVN (for more details: see *chapter 2* of this thesis).

For SOM mRNA analysis, images of the left side of the brain containing the PeVN were captured using a microscope (10 x 5 magnification) with a digital black-and-white CCD camera (Sony, XC-77CE) connected to a computer. 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 [50]. By capturing each image at the same gray level, i.e. the average gray level of the entire image, a background correction was made. Subsequently, the gray level threshold was determined by background measurements (i.e. the gray level in an area without SOM mRNA-containing cells) in a representative selection of the images. Using this threshold, SOM mRNA-containing neurons (i.e. the cell bodies that had a minimal size of 5 pixels) were counted automatically (for more details: [50]). Due to chemicals used, not all 9 PeVN sections were still present after the ISH in some animals, therefore the average number of SOM mRNA-containing cells in the PeVN was used for further analysis.

Data were analyzed using the SPSS statistical analysis software program. To compare the total number of SOM-ir cells and the percentage of activated SOM-ir cells in the PeVN at different time points before perfusion (time course experiment), a Dunnett T3 test and a Bonferroni test were used, respectively. To compare the total number of SOM-ir cells in the PeVN between groups and between treatments within the groups (gonadal steroid experiment), a Bonferroni test and a MANOVA were used, respectively. The effect of GH treatment on the percentage of activated SOM cells within groups was tested with separate T-tests. The effect of GH treatment on the total number of SOM-ir cells and the percentage of activated SOM cells in the PeVN subregions were analyzed using MANOVAs. The average number of SOM mRNA-containing cells in the PeVN was compared between treatments and between groups using Oneway ANOVAs and Bonferroni tests, respectively. Differences were considered to be significant when $p<0.05$.

RESULTS

Effect of a single i.v. injection with rhGH on SOM cell activation and on the number of SOM mRNA cells in the PeVN of male and female rats: role of gonadal steroids

As previously described in *chapter 2*, SOM-ir cell distribution within the PeVN showed a very characteristic pattern, with highest numbers of SOM-ir cells appearing in the medial PeVN. The distribution of SOM-ir cells in the PeVN was similar in all different experimental groups, although the absolute number of SOM-ir cells varied between sex and treatment.

The total number of SOM-ir cells was higher in proestrous female rats compared to all other groups (Figure 1). In contrast, the average number of SOM mRNA-containing cells was higher in males compared to all female groups (Figure 2). In general, a single i.v. injection with rhGH did not affect the total number of SOM-ir cells or the average number of SOM mRNA-containing cells in the PeVN in any of the groups. In male rats however, the number of SOM-ir cells specifically in the medial portion of the PeVN was significantly increased after GH treatment (Figure 3A). In proestrous females, the number of SOM-ir cells was significantly decreased in the posterior PeVN following GH treatment (Figure 3B). In both OVX rats and OVX rats treated with EB, no differences were found in the number of SOM-ir cells in the different PeVN subregions following GH treatment (data not shown).

SOM-ir cell activation (i.e. SOM-ir cells that contained a cFos-positive nucleus) in the PeVN was characterized by high individual variation in almost all groups. Steroid status alone induced small differences in basal SOM-ir cell activation, i.e. following treatment with saline only, albeit not statistically significant (Figure 4). A single i.v. injection with GH increased SOM-ir cell activation in the PeVN in all groups, but only significantly in OVX rats (Figure 4). Increased SOM-ir cell activation was most abundant in the posterior PeVN in male rats (n.s.; data not shown), whereas in all female groups the increased activation of SOM-ir cells was consistent between the PeVN subregions, but reached significance in the anterior PeVN of OVX rats only (values were 1.69 ± 0.65 in saline-treated and 7.64 ± 2.51 in GH-treated OVX females; $p=0.045$; data not shown).

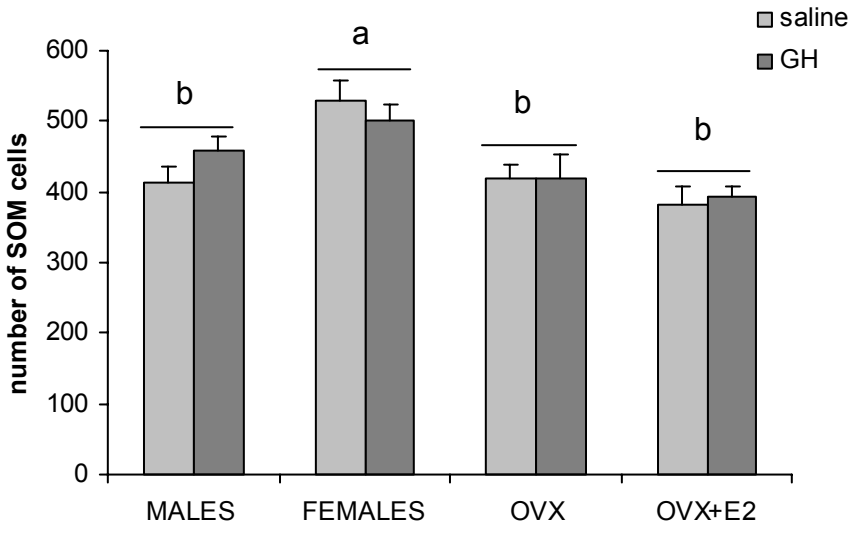

Figure 1. Total number of SOM-ir cells (bars represent mean ± SEM) in the PeVN 90 min after a single i.v. injection with either rhGH or saline (n=6 for each group). a: significantly different from b ($p \leq 0.01$).

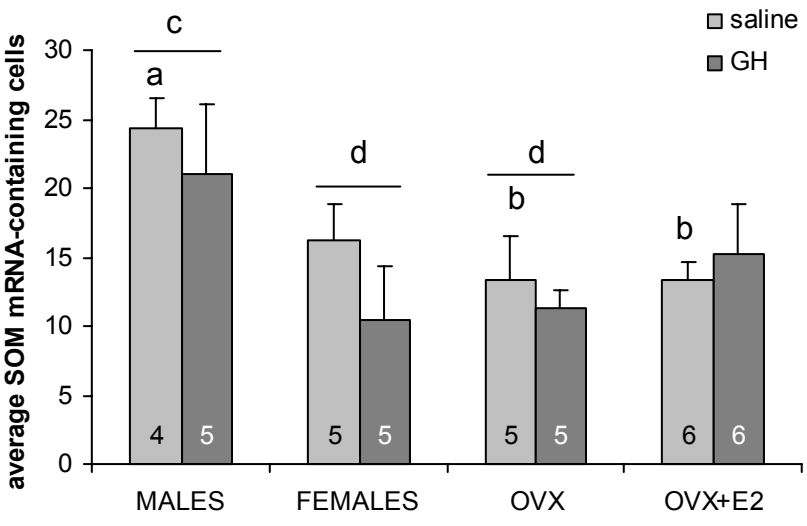

Figure 2. Average number of SOM mRNA-containing cells (bars represent mean ± SEM) after a single i.v. injection with either rhGH or saline. a: saline-treated males significantly different from saline-treated OVX+E_2 ($p=0.036$) and saline-treated OVX ($p=0.046$) females. c: males significantly different from proestrous females ($p=0.040$) and OVX females ($p=0.019$). Numbers within bars indicate the number of animals.

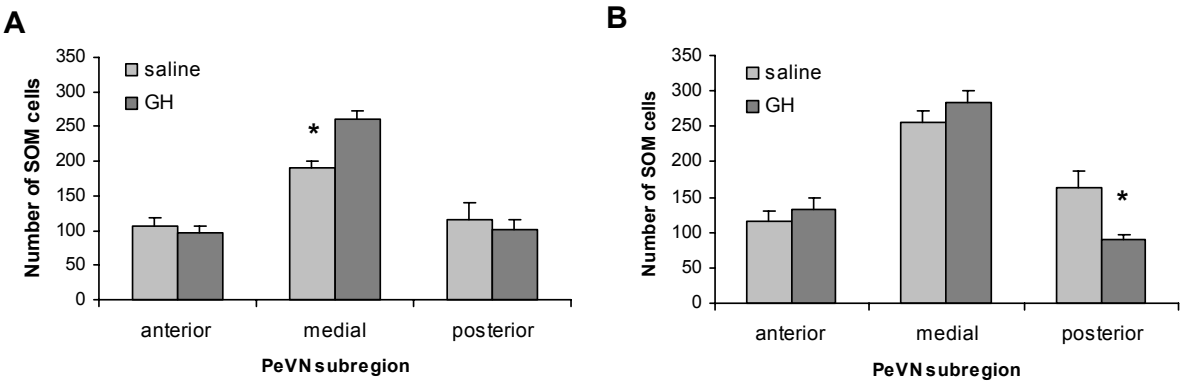

Figure 3. Total number of SOM-ir cells (bars represent mean ± SEM) in the three subregions of the PeVN 90 min after a single i.v. injection with either rhGH or saline in male (A; n=6 for each group) and proestrous female (B; n=6 for each group) rats. *: Number of SOM-ir cells in the medial PeVN significantly different in GH-treated compared to saline-treated males (figure A; p=0.001), or number of SOM-ir cells in the posterior PeVN significantly different in GH-treated compared to saline-treated proestrous females (figure B; p=0.02).

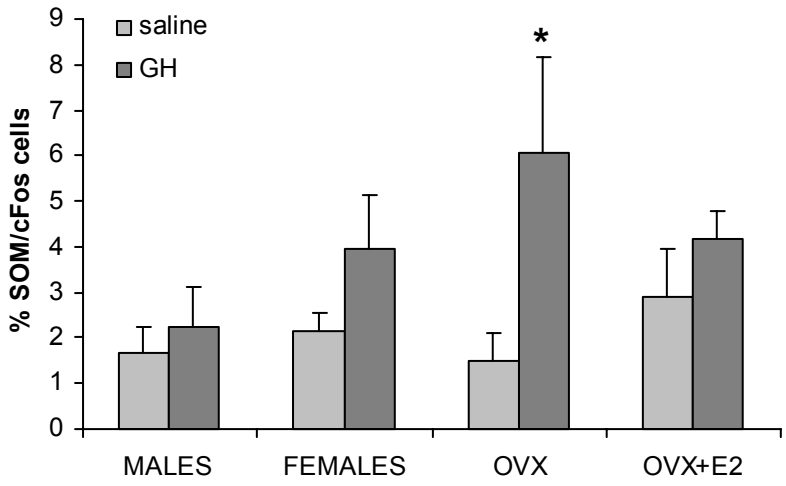

Figure 4. Percentage of SOM-ir cells containing a cFos-positive nucleus (bars represent mean ± SEM) in the PeVN 90 min after a single i.v. injection with either rhGH or saline (n=6 for each group).
*: Percentage of activated SOM-ir cells in GH-treated OVX rats significantly different from saline-treated OVX rats (p=0.047).

Time course of SOM cell activation in the PeVN after a single i.v. injection with rhGH

The total number of SOM-ir cells in the PeVN of OVX+EB rats was comparable at all time points after GH injection. Only at 90 min the total number of SOM-ir cells was slightly, but significantly decreased compared to 30 min and 60 min after GH injection (values were 529.5 ± 24.6 at 30 min and 499.8 ± 6.3 at 60 min, compared to 392.2 ± 15.9 at 90 min after GH injection, $p<0.04$; data not shown).

SOM-ir cell activation in the PeVN of OVX+EB rats following a single i.v. rhGH injection was significantly increased after 60 and 90 min, and returned back to normal levels after 120 min (Figure 5; analysis of the combined data of the gonadal steroid and time course experiment).

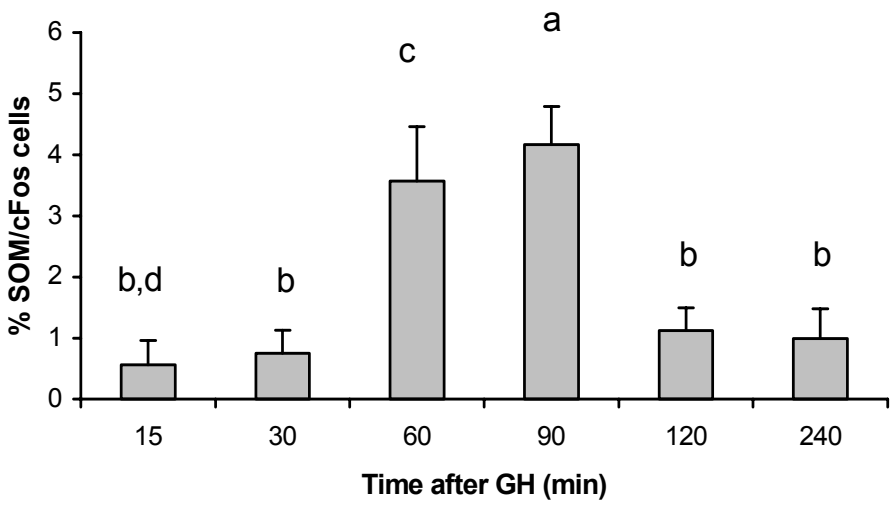

Figure 5. Percentage of SOM-ir cells containing a cFos-positive nucleus (bars represent mean ± SEM) in the PeVN of OVX+E_2 rats (n=4 for each group, except for T90: n=6) at different time points after a single i.v. injection with rhGH. a: significantly different from b ($p \leq 0.01$); c: significantly different from d ($p=0.03$).

DISCUSSION

To our knowledge, the present study is the first to demonstrate that a single i.v. GH injection induced activation in a small population of SOM cells in the PeVN of female rats. Our results also suggest that estrogen may play a role in the acute feedback effects of GH at the level of the hypothalamic SOM cells in the female. Moreover, we showed that the

number of SOM peptide-containing cells in the PeVN is higher in proestrous females compared to males, whereas the number of SOM mRNA-containing cells in the PeVN is higher in male compared to female rats. These data suggest a higher SOM peptide turnover in male compared to female rats.

We found a consistent increase in SOM-ir cell activation (i.e. cFos-containing SOM-ir cells) 90 min after a single i.v. GH injection in all female rats, but only significant in OVX female rats (Figure 4). These findings are in agreement with previous studies in male rats, in which cFos gene expression was found to be induced in periventricular SOM cells after repeated GH treatment [110], and a maximum of cFos mRNA levels was found 60 min after both repeated and single GH treatment in male rats [110-112]. We showed that also in female rats the hypothalamic SOM system is able to respond acutely to a single GH injection, suggesting that GH feedback on hypothalamic SOM cells is direct. Moreover, these findings suggest that in females SOM cells in the PeVN may also contain GH-Rs.

GH feedback on periventricular SOM cells in male rats is thought to be direct, as GH has been shown to affect SOM cell activation in the PeVN [110-112] and GH-Rs were demonstrated on SOM cells [21, 22, 113]. Therefore, we also expected to find an increased SOM-ir cell activation after a single GH injection in our male rats. However, in these animals SOM-ir cell activation was hardly increased after GH treatment (Figure 4). In previous studies injections of GH were given repeatedly to animals that were hypophysectomized, whereas we administered GH just once to intact rats, suggesting that one single GH injection may not be sufficient to stimulate the hypothalamic SOM system in males. The endogenous SOM release in the male is pulsatile and exactly out of phase with GH pulses: during a GH peak SOM release is very low and during a GH trough SOM release is high [19, 25, 35, 83, 86, 105]. Most researchers reported a synchronization of GH peaks with the light-dark cycle in male rats [6, 83, 86, 128, 129], suggesting the occurrence of a peak just prior to dark onset [6, 83, 129]. We perfused our rats just prior to the onset of the dark period, i.e. during an assumed GH peak when a low activity of the endogenous SOM system is anticipated [19, 25, 35, 83, 86, 105]. The persisting low SOM-ir cell activation level after a single GH injection may be a consequence of the already increased plasma GH levels at the time of perfusion. Consequently, an additional single injection with rhGH is likely inadequate to activate the SOM-ir cells in the PeVN. Alternatively, acute hypothalamic GH feedback in males could have acted at the level of the ARC and not the PeVN. Minami *et al*. [111] found an increase in cFos expression in

the PeVN only after repeated GH injections, whereas cFos expression in the ARC was already present 20 min after initial GH treatment. Possibly, in male rats acute GH feedback at the level of the hypothalamus involves, besides the proposed direct effect on SOM cells in the PeVN, an increased activity of local SOM cells projecting to arcuate GHRH cells [25, 114, 130, 131].

The total number of SOM-ir cells following a single GH injection in the OVX+EB females was comparable at all time points except for 90 min on which the total number of SOM-ir cells was slightly, but significantly decreased compared to the other time points. Although this may suggest that in females GH feedback may lead to reduced SOM synthesis and/or increased SOM release, the total number of SOM-ir cells in the PeVN was not different between GH and saline treatment (Figure 1). This may indicate that not GH, but the i.v. injection itself resulted in the small change in SOM-ir cell number in the OVX+EB females, which is indeed supported by a study showing that acute stress decreases the number of SOM mRNA containing cells in the PeVN in male rats [132].

The single GH injection appeared to affect SOM-ir cells in different subregions of the PeVN in male and female rats: In male rats the number of SOM-ir cells was increased only in the medial PeVN, whereas in proestrous female rats a decreased number of SOM-ir cells was found only in the posterior PeVN following the single GH injection. The increased number of SOM-ir cells as found in male rats is consistent with previous studies, reporting an increase in PeVN SOM mRNA levels following bGH treatment, albeit that prolonged elevated GH levels were used in this study [109]. To our knowledge, we are the first to report that GH given as a single i.v. injection acutely affects the number of SOM-ir cells in the PeVN in both male and female rats. Moreover, this effect was PeVN subregion- as well as sex-specific, suggesting a sexual dimorphism in the response of the PeVN SOM system to acute GH feedback.

We found a higher total number of SOM-ir cells in the PeVN of female rats on the day of proestrus compared to both intact males and the OVX groups, irrespective of the treatment (Figure 1). Interestingly, the average number of SOM mRNA-containing cells in the PeVN was significantly higher in male rats compared to all female groups (Figure 2). This difference between the sexes is in line with previous findings reported in the literature, in which both SOM mRNA [23, 30] and SOM peptide [31, 45] content in the PeVN was higher in male than in proestrous female rats. In contrast, both stimulated and basal SOM release from the PeVN and/or ME have been shown to be higher in female than in male

rats [31, 45]. Our data, combined with data from literature, suggest that the female rat may possess more SOM cells, but that these cells synthesize less SOM peptide compared to the male.

Following ovariectomy, we found a decreased number of SOM-ir cells compared to intact females, which could not be restored by treatment with EB (Figure 1). This is in line with a study that reported a decrease in total hypothalamic SOM peptide content after OVX, which was not affected by E_2 treatment [33]. The number of SOM mRNA-containing cells in the PeVN was not significantly affected by OVX or EB treatment in our females (Figure 2). In contrast, several studies reported a decrease in SOM mRNA concentrations in the PeVN after OVX, which was found to be reversed after E_2 treatment [28, 29, 32]. This discrepancy between our data and data from literature may be attributed to differences in period of OVX and/or in length and dose of E_2 treatment. The specific effects of E_2 on hypothalamic SOM levels are thus somewhat controversial. However, the fluctuating SOM release levels, concurrent with plasma E_2 levels during the estrous cycle [33, 88, 89], do suggest that estrogen may play an important role in the synthesis and release of SOM peptide in the female. Interestingly, the effect of GH on SOM-ir cell activation was only significant in OVX females that were not treated with EB (Figure 4). Our data suggest that “basal” SOM-ir activation is higher, but GH-induced SOM-ir cell activation is lower in both proestrous and OVX+EB female rats compared to OVX females. Based on these data, we hypothesize that estrogen may attenuate the sensitivity of SOM neurons in the PeVN to acute GH feedback effects.

Moreover, based on the fact that EB has no effects on the number of SOM-ir and SOM mRNA-containing cells in the PeVN of female rats (Figure 1 and 2), but appears to contribute to the female-like response of SOM cells to an acute GH feedback (Figure 4), we hypothesize that estrogen alone may not be sufficient to regulate the activity of hypothalamic SOM neurons in the female rat and that other ovarian hormones, e.g. progesterone may also play a role in this.

In conclusion, in the present study we clearly demonstrate that GH acutely affects periventricular SOM cells in female rats. The effect of GH on SOM cells was PeVN subregion- as well as sex-specific, suggesting a sexual dimorphism in the response of the PeVN SOM system to acute GH feedback. Gonadal steroids play an important role in this acute hypothalamic feedback in the female, although the exact mechanisms need further investigation.

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CHAPTER 4

Gonadal steroids regulate somatostatin peptide content in the periventricular nucleus of adult female rats

Harmke H. Van Vugt, Mireille Baart, Bert J.M. Van de Heijning, and Eline M. Van der Beek

ABSTRACT

In the rat, growth hormone (GH) release patterns are sexually dimorphic, which is suggested to be the result of differences in somatostatin (SOM) release patterns from the median eminence (ME). SOM synthesis and release may therefore be influenced by gonadal steroids. Indeed, estradiol (E_2) seems to affect hypothalamic SOM content, but no data exist on the possible role of progesterone (P) in the female rat. In the present study we examined the effects of E_2, or P, or both on the number of SOM peptide-containing cells in the PeVN of female rats, and compared these data with male rats.

Adult female rats were treated with E_2, P, both E_2 and P, or vehicle 3 months (long-term), or with E_2 2 weeks (short-term) after ovariectomy (OVX). Perfusion-fixed brains were sliced and stained for SOM peptide. The number of SOM-immunoreactive (-ir) cells and total SOM-ir area (in μm^2) were analyzed using a computer assisted analysis system.

Both the total number of SOM-ir cells and SOM-ir area in the PeVN was significantly higher in males compared to all female groups. E_2 or P treatment alone did not affect either the number of SOM-ir cells or SOM-ir area in the PeVN, but treatment with the combination of E_2 and P significantly increased SOM-ir area compared to treatment with P alone. These effects appeared to result mainly from differences in the medial and posterior PeVN. The total number of SOM-ir cells in the PeVN was lower in long-term compared to short-term OVX after E_2-treatment, but only significantly in the anterior and medial PeVN.

The present study clearly shows that the amount of SOM peptide in the PeVN is higher in male compared to female rats. Moreover, we found that in the female rat E_2 and P may act synergistically to affect SOM cells in the PeVN, suggesting that not only E_2, but both gonadal steroids may play a role in the generation of the typical feminine SOM release patterns.

INTRODUCTION

Growth hormone (GH) release from the pituitary is mainly regulated by the interplay between the stimulating effects of GH-releasing hormone (GHRH), synthesized in the arcuate nucleus of the hypothalamus (ARC), and the inhibiting effects of somatostatin (SOM), synthesized in the periventricular nucleus of the hypothalamus (PeVN) [19, 25, 83-85, 91, 92, 100, 101].

In the rat, GH release patterns are sexually dimorphic [4, 6, 7], which has been suggested to result from differences in SOM release patterns from the median eminence (ME) [19, 23, 25]. In males, SOM is released in regular pulses with maximal levels during a GH trough and minimal levels during a GH peak [25, 83, 84, 86, 87, 102]. In females, SOM release is thought to be more constant at a level in-between the maximum and minimum levels that are observed in males [26, 35], although relatively small and irregular SOM peaks have been reported [33]. GHRH release from the ME on the other hand, appears to be pulsatile in both sexes, albeit regular in male rats, with peak levels occurring during the SOM troughs, but irregular in females [25, 26, 35, 83, 84, 86, 105]. The differences in SOM release from the ME between the sexes might thus contribute considerably to the sexual dimorphism in GH release from the pituitary, implicating that SOM synthesis and release itself may be affected by gonadal steroids.

Indeed, castration of male rats decreased both hypothalamic SOM mRNA and peptide content, which could be restored by treatment with testosterone (T) [28-32]. In female rats estradiol (E_2) also affects both hypothalamic SOM mRNA and peptide levels, although conflicting results have been reported [28, 29, 31-33] (*chapter 3*). Both the number of SOM cells in the PeVN (*chapter 3*) and total hypothalamic SOM content [33] were not affected by E_2 in ovariectomized (OVX) rats. However, the decrease in SOM mRNA levels in the PeVN following OVX was found to be reversed by treatment with E_2 [28, 29, 32]. In addition, in the cycling female rat hypothalamic SOM concentrations and SOM release from the ME seem to fluctuate during the estrous cycle concurrently with plasma E_2 levels [33, 88, 89, 103], suggesting that E_2 may indeed affect hypothalamic SOM synthesis and release. The exact role of E_2 on the hypothalamic SOM system in the female rat, however, remains to be further elucidated. In addition, data from our previous study (*chapter 3*) suggested that also progesterone (P) may play a role in the regulation of the typical feminine SOM release pattern.

In the present study we examined the role of E_2 or P, alone or combined, on the number of SOM peptide-containing cells in the PeVN of female rats, and compared these data with males.

MATERIALS AND METHODS

Animals

Eight adult male and 38 adult female Wistar rats (Harlan, Horst, The Netherlands) were housed two per cage in a room with controlled lighting (LD 12:12, lights on at 04.00 h), temperature (23°C) and humidity (70%), with standard pelleted food and tap water available *ad libitum*. Dim red light was left on constantly, including the dark period. Males were 4 months of age and females were 11-12 weeks (group 1, n=32) or 16 weeks (group 2, n=6) of age on arrival. All experiments were approved by the Wageningen University Animal Ethics Committee.

Experimental design

All female rats were bilaterally ovariectomized (OVX) at the age of 12 weeks (group 1) or at the age of 18 weeks (group 2) under gas anesthesia with a mixture of Isoflurane (Isoflo; Abbott Laboratories Ltd, England) and oxygen (3:1 ratio). Three months (3mo) after OVX, the females of group 1 received a subcutaneous (s.c.) injection with estradiol benzoate (EB; 12.5 µg/0.1 ml cottonseed oil; n=16) or with 0.1 ml cottonseed oil (n=16) between 8:30 and 9:00 h on two consecutive days (day 1 and 2). The day following the EB or control injections (day 3), rats received an s.c. injection with either progesterone (P; 0.25 mg/0.1 ml cottonseed oil) (OVX+P, n=8 and OVX+EB+P, n=8) or with 0.1 ml cottonseed oil (OVX, n=8 and OVX+EB, n=8) at 11:00 h. The female rats of group 2 received the two s.c. injections with EB and the s.c. injection with oil on the next day, 2 weeks after OVX. Group 2 was included in this experiment to compare the effects of short-term and long-term OVX on hypothalamic SOM peptide following EB treatment, because results obtained from previous studies in our laboratory suggested that the period of OVX might change the sensitivity of SOM cells to E_2 (Van Vugt *et al*., unpublished data).

On day 3, all female rats were perfused just prior to dark onset, i.e. between 15:30 and 16:00 h. Male rats were randomly perfused between 15:30 and 16:00 h. Prior to perfusion, animals were given an overdose of Nembutal® (CEVA sante animale B.V., Maassluis, The Netherlands) anesthesia intraperitoneally (i.p.; 1.5 ml/kg body weight). Rats were perfused transcardially with 200 ml (females) or 300 ml (males) saline followed by 300 ml (females) or 400 ml (males) Somogyi fixative (2% paraformaldehyde in 0.1 M

phosphate buffer pH 7.4 with 0.1% glutaraldehyde and 0.2% saturated picric acid) [133]. Brains were removed from the skull immediately after perfusion and postfixed in the same fixative for 25 h at 4ºC. The part of the brain that contains the hypothalamus was sliced into coronal sections of 40 µm using a Vibratome (Vibratome Company, St. Louis, USA; series 1500) after which the sections were stored in Tris-buffered saline (TBS, pH 7.4) at 4ºC until immunocytochemistry.

Immunocytochemistry

One third of the sections was stained for SOM peptide using free-floating immunocytochemistry [97]. Sections were pretreated with 3% H_2O_2 (Merck) in TBS for 30 min, followed by extensive washing with TBS. Subsequently, sections were incubated with the primary polyclonal rabbit antibody raised against SOM peptide (Somaar 080289, NIH, Amsterdam, The Netherlands; final dilution 1:30,000) [98], diluted in TBS containing 0.5% Triton-X-100 and 0.25% gelatin (“Supermix”) for 1 night at 4ºC. This was followed by incubation with biotinylated goat anti-rabbit IgG (Vector Laboratories; 1:500 in Supermix) for 1.5 h at room temperature, and Avidin-Biotin Complex-elite (Vector Laboratories; 1:1500 in Supermix) for 1.5 h at room temperature. Between incubation steps, sections were thoroughly washed with TBS. SOM immunoreactivity was visualized by incubation with 0.05% 3,3’-diaminobenzidine (Sigma Chemical Company) in TBS containing 0.1% imidazole and 0.03% H_2O_2 for 14.5 min. Stained sections were mounted on albumin coated slides, dried, dehydrated and coverslipped in DEPEX (BDH Laboratory Supplies, Poole, England). SOM-immunoreactive (SOM-ir) neurons in the PeVN were counted using computer assisted image analysis (Scion Image software program) using background correction.

Data analysis

Images of the left side of the brain only containing the PeVN, covering 9 consecutive sections taken at 80 µm intervals (for details: see *chapter 2*), were captured using a microscope (10 x 5 magnification) with a digital black-and-white CCD camera (Sony, XC-77CE) connected to a computer. No differences in SOM peptide content were found between the left and right side of the hypothalamus (personal observations). Thereafter, images were analyzed using an image analysis program (Scion Image Beta, version 4.02; Scion Corporation, Maryland, USA) applying gray level threshold

discrimination as previously described [50]. By capturing each image at the same gray level, i.e. the average gray level of the entire image, a background correction was made. Subsequently, the gray level threshold was determined by true background measurements (i.e. the gray level in an area that contained SOM-ir fibers, but no SOM-ir cell bodies) in a representative selection of the images. Using this threshold, SOM-ir neurons (i.e. the cell bodies without fibers that had a minimal size of 5 pixels) were counted automatically (for more details: [50]). In addition to cell counts, also the total area of SOM peptide, i.e. all gray levels that were above the theshold, was determined.

The total number of SOM-ir cells and total SOM-ir area, i.e. the sum of the 9 PeVN sections, was analyzed. In addition to this, we also analyzed the number of SOM-ir cells and SOM-ir area in the subregions of the PeVN, as previously described in *chapter 2*.

Data were analyzed using the SPSS statistical analysis system. To compare both the number of SOM-ir cells and SOM-ir area between the males and all female groups, and between the 4 different gonadal steroid treatments in OVX female rats (total PeVN as well as within the different PeVN subregions), Oneway ANOVAs were used. In addition, Bonferroni tests were used for post-hoc testing to compare both the number of SOM-ir cells and SOM-ir area between the 4 different gonadal steroid treatments in female rats. To compare the effect of short-term versus long-term ovariectomy on both the number of SOM-ir cells and SOM-ir area (total PeVN as well as within the different PeVN subregions), separate T-tests were used. Differences were considered to be significant when $p<0.05$.

RESULTS

The rostro-caudal distribution of SOM-ir cells in the PeVN of the male and OVX female rats was previously described in *chapter 2*. Maximal numbers of SOM-ir cells were found in PeVN section 7 (posterior PeVN) in male rats, whereas maximal numbers of SOM-ir cells in OVX females were found in the medial PeVN: PeVN section 5 in OVX(2wk)+E_2 and PeVN section 6 in all OVX(3mo) groups.

Both the total number of SOM-ir cells and the amount of SOM-ir peptide (as reflected by the SOM-ir area) in the PeVN was significantly higher in males compared to all different OVX female groups (Figure 1A and B).

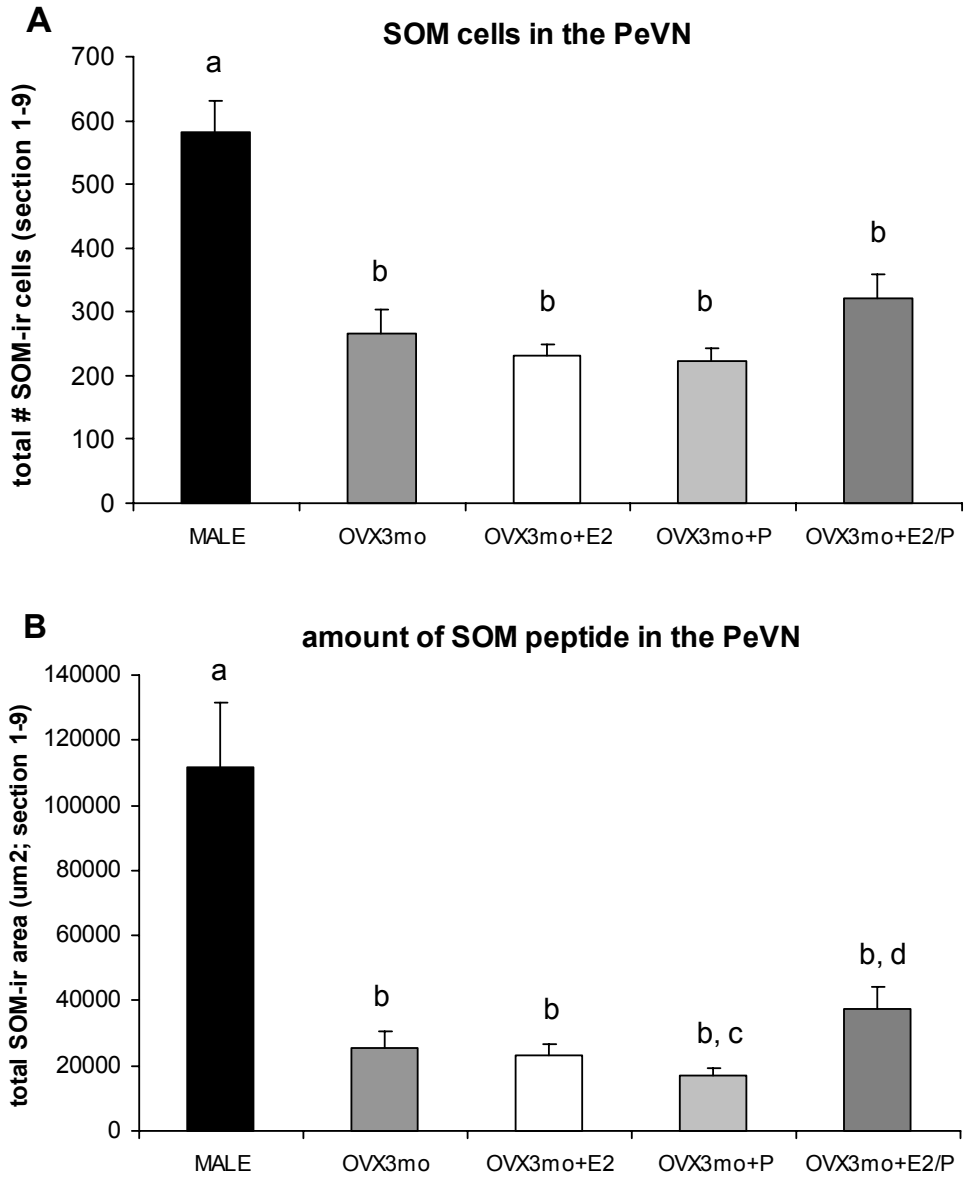
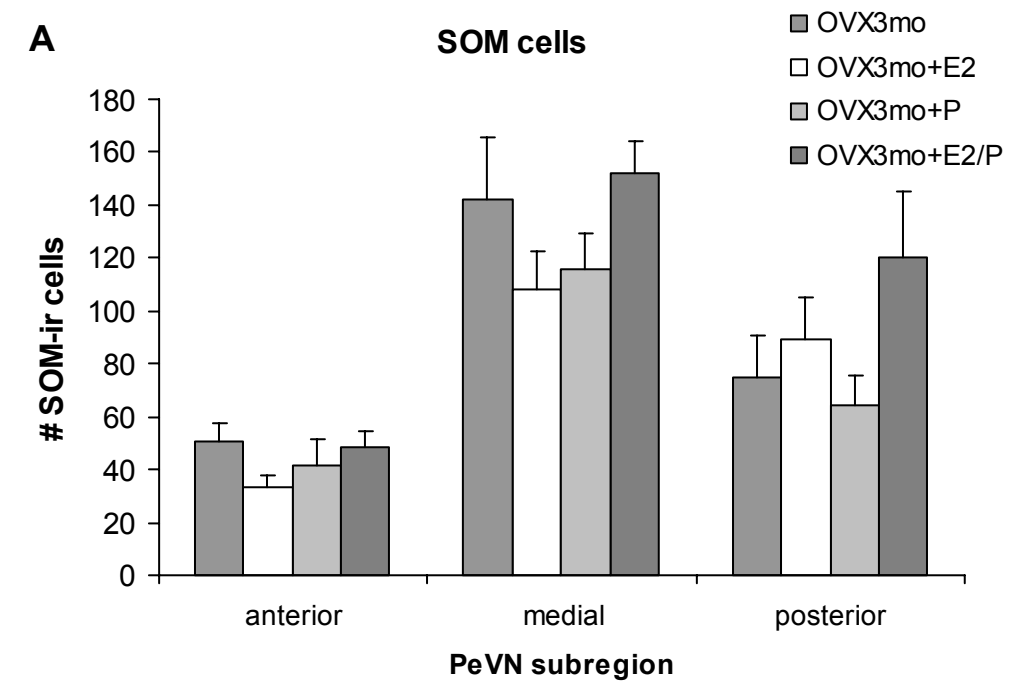
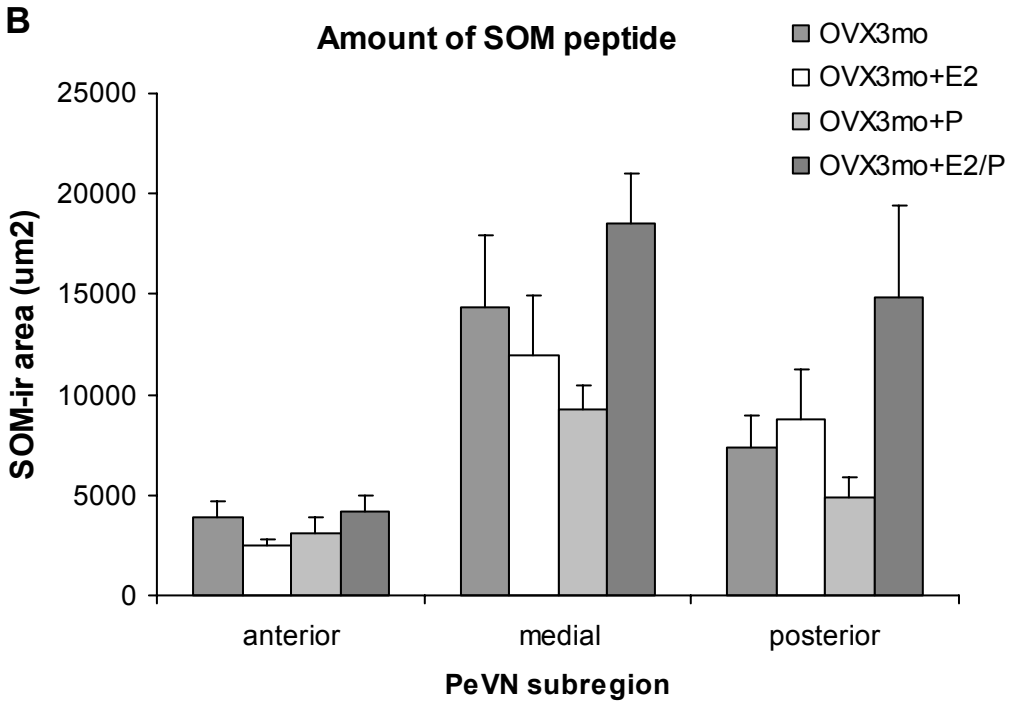


Figure 1. Total number of SOM-ir cells (A) and total amount of SOM-ir peptide, as reflected by SOM-ir area (B) (bars represent mean ± SEM) in the PeVN of males and 3-months ovariectomized (OVX3mo) females with different gonadal steroid treatments. a: significantly different from b (p=0.000; ANOVA); c: significantly different from d (p=0.033; Bonferroni).

E_2 or P treatment alone did not affect either the number of SOM-ir cells or the amount of SOM-ir peptide in the PeVN (Figure 1A and B). Treatment with both E_2 and P increased the amount of SOM-ir peptide, but only significantly compared to treatment with P alone (Figure 1B). The effect of combined E_2 and P was greatest on the number of

SOM-ir cells and was even more pronounced on the amount of SOM-ir peptide in the medial and posterior PeVN only, although these differences per subregion did not reach statistical significance (Figure 2A and B).





The total number of SOM-ir cells in the PeVN was significantly lower in long-term (3 months) compared to short-term (2 weeks) ovariectomized females after E_2-treatment (Figure 3, insert). The effect of E_2 on long-term OVX on the number of SOM-ir cells was only significant in the anterior and medial PeVN (Figure 3). Although the total amount of SOM-ir peptide in the PeVN was not significantly different between long-term and short-term OVX, the amount of SOM-ir peptide in the anterior PeVN was found to differ significantly between the two groups (data not shown; values were 5628 ± 810 μm^2 for short-term and 2467 ± 368 μm^2 for long-term OVX).

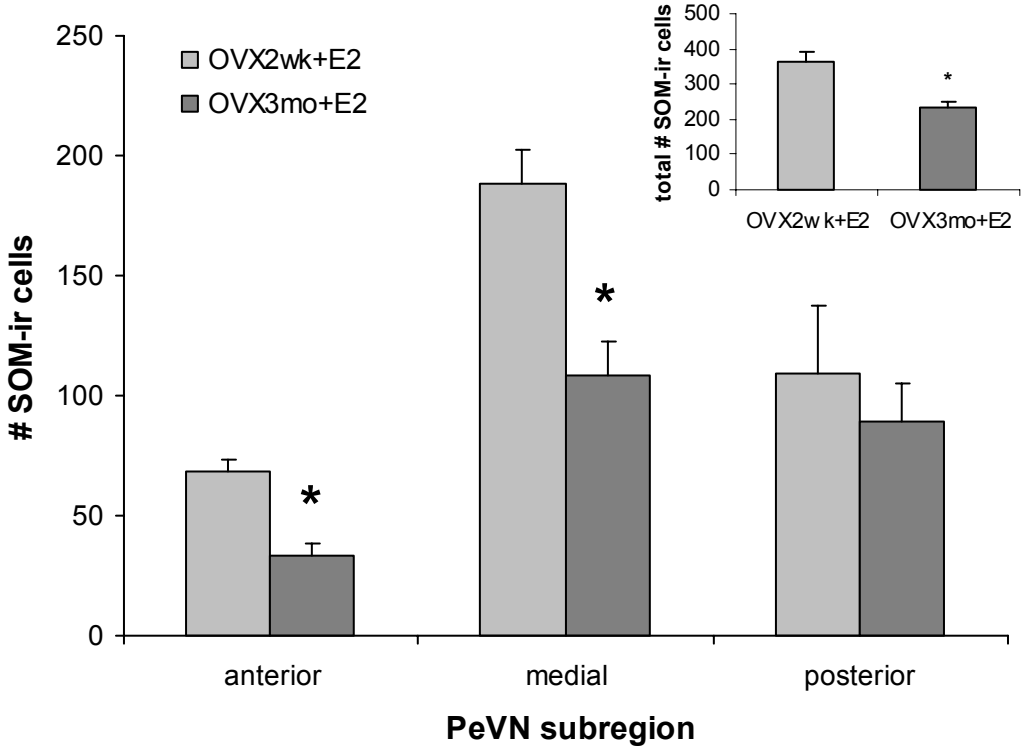


Figure 3. Effect of short-term (OVX2wk+E_2) versus long-term (OVX3mo+E_2) ovariectomy on the number of SOM-ir cells in the PeVN subregions and on the total number of SOM-ir cells (insert) (bars represent mean ± SEM). *: OVX2wk+E_2 significantly different from OVX3mo+E_2 ($p<0.003$; T-test).

←

Figure 2. Effects of gonadal steroids in 3-months ovariectomized (OVX3mo) female rats on the number of SOM-ir cells (A) and on the amount of SOM-ir peptide (B) (bars represent mean ± SEM) in the three different PeVN subregions.

DISCUSSION

In the present study, we clearly show that a sex difference exists in SOM peptide content of the PeVN: males showed significantly more SOM-ir cells and a higher amount of SOM-ir compared to OVX females. Also, in the female rat gonadal steroids slightly modulate the number of cells as well as the amount of SOM peptide stored in the PeVN (albeit not always significantly). Moreover, we found that the effect of E_2 treatment on both the number of SOM cells and the amount of SOM peptide in the PeVN is affected by the period of ovariectomy.

Males showed more SOM peptide-containing (SOM-ir) cells and a higher amount of SOM peptide (SOM-ir area) compared to female rats. These findings are supported by our previous study (*chapter 3*), in which we found that the number of SOM mRNA-containing cells in the PeVN was significantly higher in adult male rats compared to females, and by previous studies also showing higher SOM mRNA levels [23, 30] and a higher SOM peptide content [31, 45, 134] in the PeVN of male rats compared to female rats. In contrast, in the same study (*chapter 3*) we found that the total number of SOM cells in the PeVN of proestrus female rats was significantly higher compared to that in males. Interestingly, the discrepancy in SOM peptide-containing cell numbers between the present study and the data from *chapter 3* may very well result from the different methods that were used to quantify the cells. In our previous study, we double-stained the brain sections for SOM and cFos peptide and therefore had to count the SOM cells manually. Consequently, we were not able to distinguish between light and dark stained cells, but counted all cells that showed SOM immunoreactivity. In the present study however, we counted the SOM cells automatically using a threshold level and therefore included only cells that showed immunoreactivity above the threshold, i.e. the medium- to dark-stained SOM cells. Based on the results of these two studies, it appears that female rats may have more cells containing low levels of SOM peptide that are missed using an automated counting procedure. Indeed, based on personal observations, we found that male rats possess much more intensely stained SOM cells than female rats (compare Figure 1A and 1B in *chapter 2*). Thus, combining our own data with the data from the literature, we hypothesize that female rats may possess more SOM-producing cells than male rats, but that in males these cells may be able to synthesize and store more SOM peptide.

We found no effects of E_2- or P-treatment alone on either the total number of SOM-ir cells or on the amount of SOM-ir peptide in the PeVN of long-term OVX females. The

fact that E_2 did not have any effect on periventricular SOM peptide is in line with previous reports that also failed to observe effects of E_2 on total hypothalamic SOM peptide [33], or on the number of SOM cells in the PeVN (*chapter 3*). However several other studies showed that the effects of OVX on SOM mRNA levels in the PeVN could be reversed by E_2 alone [28, 29, 32]. These data may indicate that the possible effects of E_2 on hypothalamic SOM could be different concerning synthesis versus peptide storage and/or release.

To our knowledge, no data exist on possible effects of P on hypothalamic SOM mRNA or peptide in the female rat. P was found to have no effects on SOM-suppressed GH release from rat anterior pituitary cells [135], suggesting that P does not affect SOM receptor sensitivity or its expression on somatotrophs. Yet, studies in dogs showed that supraphysiological levels of plasma P resulted in increased plasma GH levels [136, 137], suggesting that P is somehow involved in the somatotropic axis. In the present study we found that treatment with the combination of E_2 and P increased both the number of SOM cells (n.s.) and the amount of SOM peptide in the PeVN compared to treatment with E_2 (n.s.) or P alone (Figure 1), indicating that gonadal steroids indeed affect hypothalamic SOM peptide content in the female rat.

Although the precise role of gonadal steroids on the periventricular SOM system remains unclear, SOM synthesis in the hypothalamus and release from the ME has been shown to fluctuate during the estrous cycle: SOM mRNA in the PeVN was found to be minimal on the day of diestrus and maximal on the day of proestrus [88], when SOM peptide levels in the preoptic area, posterior hypothalamus and ME were shown to be low [89]. Yet, SOM release from the ME was found to be highest on the day of proestrus [33], concurrent with high plasma E_2 and P levels [103]. These data suggest that E_2 or P, and more likely the combination of the two, affects SOM synthesis or release, or both, in the female rat. E_2 and P may hence act synergistically to affect SOM peptide in the PeVN and the effects of the combination of the two steroids may be essential for the generation of the typical feminine hypothalamic SOM release pattern.

The possible effects of E_2 and P on periventricular SOM cells are likely to be indirect. First, SOM neurons in the PeVN do not contain E_2-receptors α (ERα) [138]. Possibly, E_2 indirectly influences SOM neurons in the PeVN via SOM cells located in nearby hypothalamic areas that contain ERα [139], or via GABA-containing neurons that have been shown to be E_2-receptive [140] and were found to project to SOM cells in the

PeVN [141, 142]. Next, to our knowledge, no data exist on possible colocalization of the progesterone-receptor (PR) in SOM cells in the hypothalamus. Although studies in the guinea pig showed that SOM cells do contain PR [143, 144], no colocalization in the PeVN was found, suggesting that also the possible effect of P on SOM cells in the PeVN is likely to be indirect.

We found that the number of SOM cells in the PeVN was significantly lower in long-term (3 months) compared to short-term (2 weeks) OVX females that were treated with E_2, suggesting that the sensitivity of the SOM cells to E_2 treatment is dependent on OVX length. The effect of long-term OVX was only significant in the anterior and medial parts of the PeVN (Figure 3), whereas the effects of gonadal steroids were most pronounced in the posterior PeVN (Figure 2).

Interestingly, in our previous study (*chapter 3*) we found that the acute feedback effect of GH on the number of SOM cells was only significant in the posterior PeVN of proestrous female rats. These results suggest that both GH and gonadal steroids appear to predominantly affect SOM cells located in the posterior part of the PeVN in female rats.

In conclusion, the present study shows that the amount of SOM peptide in the PeVN is higher in male compared to female rats, although previous results from our laboratory suggest more SOM-producing neurons in proestrous females. We found that the sensitivity of hypothalamic SOM cells to steroid feedback is dependent on OVX length. Finally, our data suggest that in the female rat E_2 and P act synergistically to affect SOM cell activity in the PeVN of the female rat.

CHAPTER 5

Centrally applied somatostatin inhibits the estrogen-induced luteinizing hormone surge via hypothalamic gonadotropin-releasing hormone cell activation in female rats

Harmke H. Van Vugt, Hans J.M. Swarts, Bert J.M. Van de Heijning, and Eline M. Van der Beek

ABSTRACT

Overexpression of growth hormone (GH) as well as GH-deficiency dramatically impairs reproductive function. Decreased reproductive function as a result of altered GH release is, at least partially, due to changes at the hypothalamic-pituitary level. We hypothesize that hypothalamic somatostatin (SOM), the inhibiting factor of GH release from the pituitary, may play a central role in the "crosstalk" between the somatotropic and gonadotropic axis.

In the present study we investigated the possible effects of a centrally applied SOM analog on the luteinizing hormone (LH) surge and the concurrent activation of hypothalamic gonadotropin-reasing hormone (GnRH) neurons in female rats.

To this end, female rats were treated with estradiol 2 weeks after ovariectomy and were given a single central injection with either the SOM analog octreotide or saline just prior to surge onset, after which hourly blood samples were taken to measure LH. Two weeks later, the experimental setup was randomly repeated to collect brains during the anticipated ascending phase of the LH surge. Vibratome sections were subsequently double stained for GnRH and cFos peptide.

Following octreotide treatment, LH surges were significantly attenuated compared to those in saline-treated control females. Also, octreotide treatment significantly decreased the activation of hypothalamic GnRH neurons. These results clearly demonstrate that SOM is able to inhibit LH release, at least in part by decreasing the activation of GnRH neurons. Based on these results, we hypothesize that hypothalamic SOM may be critically involved in the physiological regulation of the proestrous LH surge.

INTRODUCTION

Growth hormone (GH) secretion from the pituitary is regulated by the inhibitory neurohormone somatostatin (SOM), released from neurons located in the periventricular nucleus of the hypothalamus (PeVN), and by the stimulating effects of GH-releasing hormone (GHRH), released from neurons located in the arcuate nucleus of the hypothalamus (ARC) [23, 25]. It is well known that the functionality of the somatotropic and gonadotropic axis are closely related. Overexpression of GH as well as GH-deficiency causes dramatic changes in reproductive function, i.e. a reduced fertility in animals that overexpress GH [54, 55, 61] and complete infertility in GH-deficient animals [53, 54]. At the

level of the ovaries, GH overexpression results in only primary, secondary and atretic follicles and no corpora lutea [65], whereas GH-deficient animals are hypogonadal [53].

Apart from effects on gonadal function, GH also appears to affect reproduction at the hypothalamic and pituitary level.

Steroid hormone (i.e. estradiol and progesterone) production in the gonads is regulated by the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are secreted from the pituitary, and in turn are stimulated by gonadotropin-releasing hormone (GnRH) released from the hypothalamus. Both LH and FSH stimulate several processes in the gonads [103]. In male Ames dwarf mice lacking GH, plasma gonadotropin levels are reduced [53] and the GnRH-stimulated LH synthesis and secretion *in vitro* is decreased [60]. On the other hand, also overexpression of GH in male transgenic mice results in decreased LH and FSH mRNA levels in the pituitary, in a decreased GnRH secretion from the hypothalamus *in vitro*, and in attenuated GnRH-induced LH and FSH responses *in vitro* [53, 145]. Also in male GH-receptor knockout mice, in which GH levels are consistently elevated, the LH response to a GnRH stimulus is attenuated [53, 61]. In female transgenic mice overexpressing GH, the proestrous LH surge is decreased [53] and the distribution of GnRH-containing neurons in the hypothalamus is altered [65]. These data show that decreased reproductive function as a result of altered GH release is, at least partially, characterized by changes at the hypothalamic-pituitary level.

PreproSOM mRNA levels in the PeVN are decreased in GH-deficient animals [66], whereas the number of SOM cells in the PeVN is elevated in animals overexpressing GH [65]. As SOM, originating from the PeVN, inhibits GH secretion from the pituitary, and as changes in the somatotropic axis clearly result in changes in the gonadotropic axis, at least partially evoked at the hypothalamic-pituitary level, we hypothesize that SOM may play a central role in the “crosstalk” between these two axes.

Indeed, SOM has been shown to affect the reproductive axis at the level of the pituitary: In male rats, SOM inhibits the GnRH-induced release of LH *in vitro* [70] and decreases plasma LH *in vivo* [146]. Moreover, multiple central injections with SOM or a SOM analog resulted in smaller and pycnotic gonadotropic cells and in decreased gonadotropic cell numbers in the pituitary of both male and female rats [71, 146-148].

These studies show that chronic high concentrations of SOM can inhibit LH release, probably by affecting the gonadotropic cells in the pituitary. However, based on the data

from animals that overexpress or lack GH, we hypothesize that SOM may also affect the reproductive axis within the hypothalamus.

In the present study, we aimed to obtain more insight in the possible direct effects of somatostatin at the level of the hypothalamus on the regulation of the LH surge in female rats. To this end, we studied the LH release from the pituitary as well as the activation of hypothalamic GnRH cells, following a single central injection with the SOM analog octreotide just prior to surge onset in ovariectomized estrogen-treated rats.

MATERIALS AND METHODS

Animals

Seventeen female Wistar rats (Harlan, Horst, The Netherlands), 10 weeks of age on arrival, were individually housed in a room with controlled lighting (LD 12:12, lights on at 02:00 h, Zeitgebertime (ZT) 0), temperature (21°C) and humidity (70%), with standard pelleted food and tap water available ad libitum. Dim red light was left on constantly, including the dark period. All experiments were approved by the Wageningen University Animal Ethics Committee.

Experimental design

Prior to surgery, rats were anaesthetized by an intraperitoneal (i.p.) injection with a mixture of Ketamine (Kombivet, Etten-Leur, The Netherlands) and Rompun (Bayer AG, Leverkusen, Germany) (respectively 16.7% and 60% diluted in saline; 1 ml/kg body weight). All animals were bilaterally ovariectomized (OVX; day 1) and received an indwelling jugular vein catheter directed towards the right atrium for the stress-free collection of blood samples. In addition, an intracerebroventricular (i.c.v.) guide cannula was placed in the right lateral ventricle of the brain.

On day 12 and 13 after OVX, all rats received a subcutaneous (s.c.) injection with estradiol benzoate (EB; 12.5 µg/0.1 ml oil) at 08:00 h (ZT 6) to induce an LH surge on day 14.

On day 14, rats received an i.c.v. injection with either the somatostatin analog octreotide (Sandostatine®, Novartis Pharma B.V., Arnhem, The Netherlands; 1.0 µg/2 µl; n=10) or saline (control; 2 µl; n=7) at 08:00 h (ZT 6). Octreotide is a synthetic octapeptide derivative of the natural somatostatin. The pharmaceutical effects of octreotide are similar

to those of somatostatin, but the effects last longer. Octreotide is used as treatment of patients with acromegaly and patients with symptoms associated with functional gastro-entero-pancreatic endocrine tumors, and clearly inhibits GH secretion [149]. Blood samples were collected every hour from 08:30 h (ZT 6.5) to 17:30 h (ZT 15.5) for determination of plasma LH concentrations. In addition, from 10:30 h (ZT 8.5) to 11:30 h (ZT 9.5) samples were drawn every 12 minutes for GH determination. Samples were centrifuged and the plasma was diluted in 0.02 M phosphate buffer (pH 7.4; 1:4 dilution for LH and 1:5 dilution for GH) and stored at –20ºC until assay.

After a recovery period of 3 weeks, all rats again received an s.c. injection with EB at 08:00 h (ZT 6) on two consecutive days. On the following day, the animals were randomly given an i.c.v. injection with either octreotide (n=8) or saline (n=9) at 08:00 h (ZT 6), after which all animals were perfused between 13:30 and 14:00 h (ZT 11.5 and 12), just prior to the onset of the dark period.

Prior to perfusion, animals were given an overdose of Nembutal® (CEVA sante animale B.V., Maassluis, The Netherlands) anesthesia (1.5 ml/kg body weight; i.p.), after which a blood sample was taken directly from the heart for plasma LH determination. Subsequently, each rat was perfused transcardially with 200 ml saline followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate buffer (4% PFA; pH 7.4). Brains were removed from the skull immediately after perfusion and postfixed in the same fixative for 25 h at 4ºC. Prior to sectioning, tissue blocks containing the hypothalamus were embedded in 20% gelatin and fixed with 4% PFA for 4 h at 4ºC [122]. Sections of 40 µm were sliced using a Vibratome (Leica) and were stored in 0.1 M Tris buffer containing 0.9% NaCl (TBS; pH 7.4) at 4ºC until further use.

Immunocytochemistry

One third of the coronal brain sections of each animal was double stained for GnRH and cFos peptide, using free-floating immunocytochemistry techniques (see also [97]). Sections were pretreated with 3% H_2O_2 (Merck) in TBS for 30 min, followed by extensive washing with TBS.

For the cFos staining, sections were incubated with the primary polyclonal rabbit antibody against cFos (SC-052, Santa Cruz Biotechnology, California, U.S.A.; #C076; final dilution 1:10,000), diluted in TBS containing 0.5% Triton-X-100 and 0.25% gelatin (Supermix) for 1 h at room temperature followed by 4 nights at 4ºC. This was followed by

incubation with biotinylated goat anti-rabbit IgG (GaR-bio; Vector Laboratories, Burlingame, U.S.A.; 1:400 in Supermix) for 1.5 h at room temperature, and Avidin-Biotin Complex-elite (ABC; Vector Laboratories; final dilution 1:1200 in Supermix) for 2 h at room temperature. Between incubation steps, sections were thoroughly washed with TBS. cFos immunoreactivity was visualized by incubation with 0.05% 3,3'-Diaminobenzidine (DAB; Sigma Chemical Company, St. Louis MO, U.S.A.) in TBS containing 0.2% nickelammoniumsulphate and 0.03% H_2O_2 for 20 min.

For the double staining, cFos-stained sections were washed in graded series of methanol, including 100% methanol with 0.3% H_2O_2 (short wash steps, each lasting 5 min; 45 min in total). After extensive washing with TBS, the sections were incubated with the primary polyclonal rabbit antibody against GnRH (#PLR 005, Eurodiagnostics, Apeldoorn, The Netherlands; final dilution 1:10,000 in Supermix) for 1 night at room temperature. This was followed by incubation with GaR-bio (1:400 in Supermix) for 1.5 h at room temperature, and ABC (final dilution 1:1200 in Supermix) for 2 h at room temperature. Between incubation steps, sections were thoroughly washed with TBS. GnRH immunoreactivity was visualized by incubation with 0.05% DAB in TBS containing 0.1% imidazole and 0.03% H_2O_2 for 8 min.

Stained sections were mounted on albumin coated slides, dried, dehydrated and coverslipped in DEPEX (BDH Laboratory Supplies, Poole, England). Activated (cFos-immunoreactivity-containing) and non-activated GnRH-immunoreactive (GnRH-ir) neurons were counted in different areas of the hypothalamus using a light microscope.

Radioimmunoassay

LH and GH were determined in a double-antibody radioimmunoassay for rat-LH (see also [150]) or rat-GH using materials supplied by NIDDK (for LH: rLH-I-9 as label and anti-rLH-s-11 as antiserum; for GH: rGH-I-6 as label and anti-rGH-S-5 as antiserum), and using Sac-cel® (donkey anti-rabbit, Welcome Reagents, Beckenham, U.K.) as a second antibody. The levels of LH and GH are expressed in terms of NIDDK-rLH-RP-2 and NIDDK-rGH-RP-2, respectively. The assay sensitivity was 0.2 ng/tube for LH and 0.9 ng/tube for GH at the 90% B/B_0 level. The intra-assay variation was determined using pooled rat serum and was 8.5% for both LH and GH.

Data analysis

To determine the effects of the somatostatin analog octreotide on LH release, surge characteristics of each saline- or octreotide-treated rat were compared. These included: (1) baseline levels as determined in blood samples collected at 08:30 h (ZT 6.5); (2) the time of peak onset defined as the time at which plasma LH levels had increased to twice the baseline level and remained at this level or higher; (3) the time of peak LH levels, i.e. the time at which the highest hormone levels were determined; (4) the magnitude of the peak LH release (ng/ml plasma at peak time); (5) the number of animals that showed an LH peak (incidence of peak), and (6) the total amount of LH released, determined as the cumulative value of hormone levels during the entire sampling period (area under the curve; AUC). To include data from animals that did not show samples with values higher than twice the baseline level (i.e. n=1 in the saline group and n=7 in the octreotide group), for statistical analysis the parameters peak onset, peak time and peak height were chosen to be the value of the last sample. Three animals (n=1 in the saline and n=2 in the octreotide group) showed several samples, however not successive, in which LH was increased to higher than twice the baseline level. From these animals, the sample with the highest value was decided to be the peak onset, as well as peak time and peak height.

To determine the effect of the somatostatin analog on GH release, the total amount of GH released, i.e. the cumulative value of hormone levels during the complete sampling period (AUC), was compared between saline- and octreotide-treated animals.

Brain sections were divided into three separate areas, based on the distribution of GnRH cells [97, 151], i.e. the diagonal band of broca (DBB) (area B, 2-6 sections), the organum vasculosum of the lamina terminalis (OVLT) (area C, 3-6 sections), and the medial preoptic area (MPO) (area D, 3-5 sections). These are the areas containing the GnRH neurons that are predominantly responsible for the generation of the GnRH surge, inducing the preovulatory LH surge [152]. In these areas, the total number of GnRH-ir cells as well as the number of activated GnRH-ir cells (i.e. GnRH cells containing a cFos-positive nucleus) was determined.

Data were analyzed using the SPSS statistical analysis system. To compare basal LH levels, peak LH value, both LH and GH AUC, and perfusion LH values between saline- and octreotide-treated rats, separate Oneway ANOVA's were used. To compare LH surge onset time and LH peak time between treatments, Mann-Whitney U tests were used, and for the incidence of an LH peak, a Fisher Exact test was used. Furthermore, to compare

both the total number of GnRH-ir cells and the number of activated GnRH-ir cells between the two treatments, Oneway ANOVA's were used. Differences were considered to be significant when $p<0.05$.

RESULTS

Effect of octreotide treatment on LH and GH release

Repeated estradiol benzoate (EB) treatment induced small but significant LH surges in 6 out of 7 OVX animals that were i.c.v. injected with saline. LH levels started to rise at ZT 11.5 ± 0.8 and peaked at ZT 12.3 ± 0.7 with an average LH peak height of 3.24 ± 0.66 ng/ml (Table 1). Variations in peak onset as well as peak time and height were comparable to previous studies using this animal model [150, 153].

A single i.c.v. injection with the somatostatin analog octreotide significantly suppressed the EB-induced LH surge (Figure 1). Octreotide had no effect on basal plasma LH levels, but significantly decreased LH peak height, AUC and the incidence of an LH peak. Also, the onset time of the LH peak and the LH peak time were significantly delayed after treatment with the SOM analog (Table 1). Moreover, plasma LH values at the time of perfusion (ZT 11.5-12.0) were significantly lower in octreotide-treated rats than in saline-treated rats (data not shown; values were 2.7 ± 0.6 and 5.5 ± 0.9 respectively; p=0.02), demonstrating that the effect of the central octreotide treatment on LH release was similar in both experimental parts.

Table 1. LH surge characteristics in E_2-treated OVX female rats

	Basal levels*	Peak onset+	Peak time+	Peak height*	Incidence of Peak#	AUC*
saline	0.82 ± 0.06	11.5 ± 0.8[a]	12.3 ± 0.7[b]	3.24 ± 0.66[c]	6/7[d]	17.11 ± 2.56[c]
octreotide	1.00 ± 0.09	14.5 ± 0.5	14.7 ± 0.3	1.24 ± 0.28	3/10	9.14 ± 0.97

* Means ± SEM expressed as plasma LH levels in ng/ml.
+ Means ± SEM expressed as the ZT.
# Number of animals out of the total in that group showing an LH surge.
[a] p=0.003 (Mann-Whitney U-test).
[b] p=0.002 (Mann-Whitney U-test).
[c] p=0.001 (one-way ANOVA).
[d] p=0.02 (Fischer Exact test).

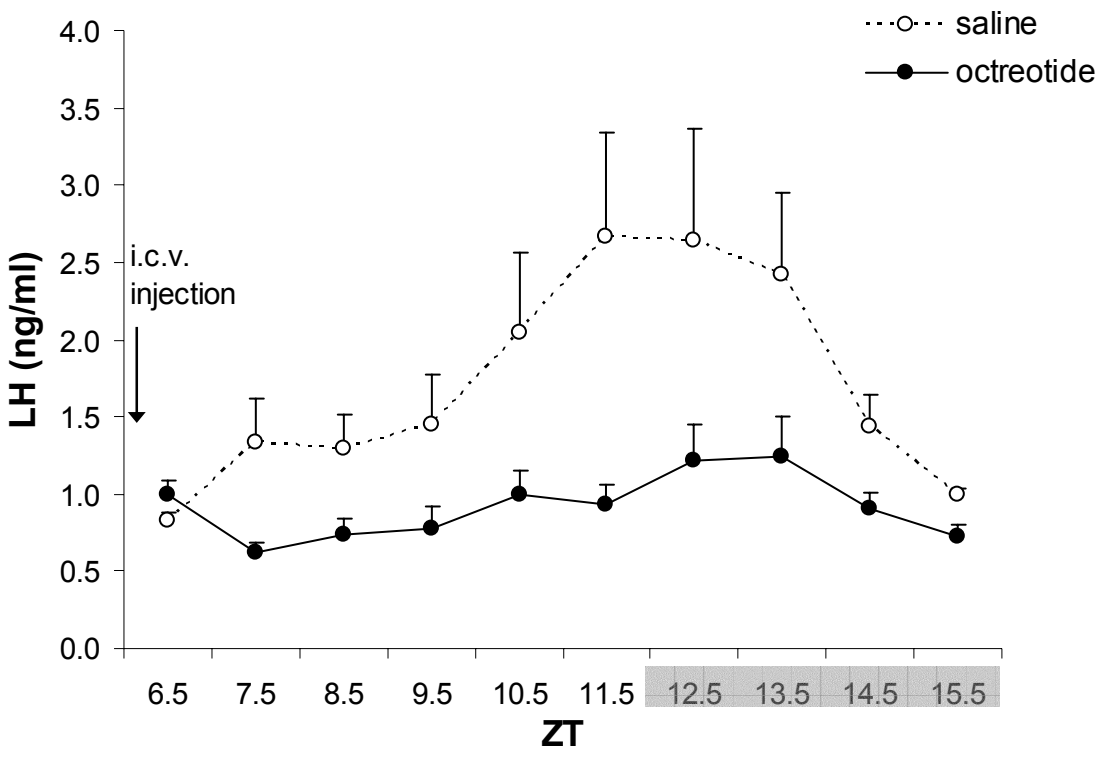


Figure 1. Mean ± SEM plasma LH concentrations in OVX+EB rats that received a single i.c.v. injection with saline (open circles; n=7) or the SOM analog octreotide (closed circles; n=10).

To test the physiological significance of octreotide, we also measured plasma GH levels. GH secretion patterns showed high individual variations in the saline-treated rats. Not all control rats showed a clear GH peak in this short period of sampling. However, we found an overall lowering of plasma GH levels after octreotide treatment, i.e. the GH AUC was significantly lower in octreotide-treated animals compared to saline-treated animals (Figure 2).

Effect of octreotide treatment on hypothalamic GnRH cells

The total number of GnRH-ir cells in the brain areas DBB (area B), OVLT (area C) and MPO (area D) of OVX+EB rats was slightly increased in octreotide-treated rats compared to saline-treated rats (data not shown; values were 168 ± 12 and 146 ± 12 respectively), albeit not significant.

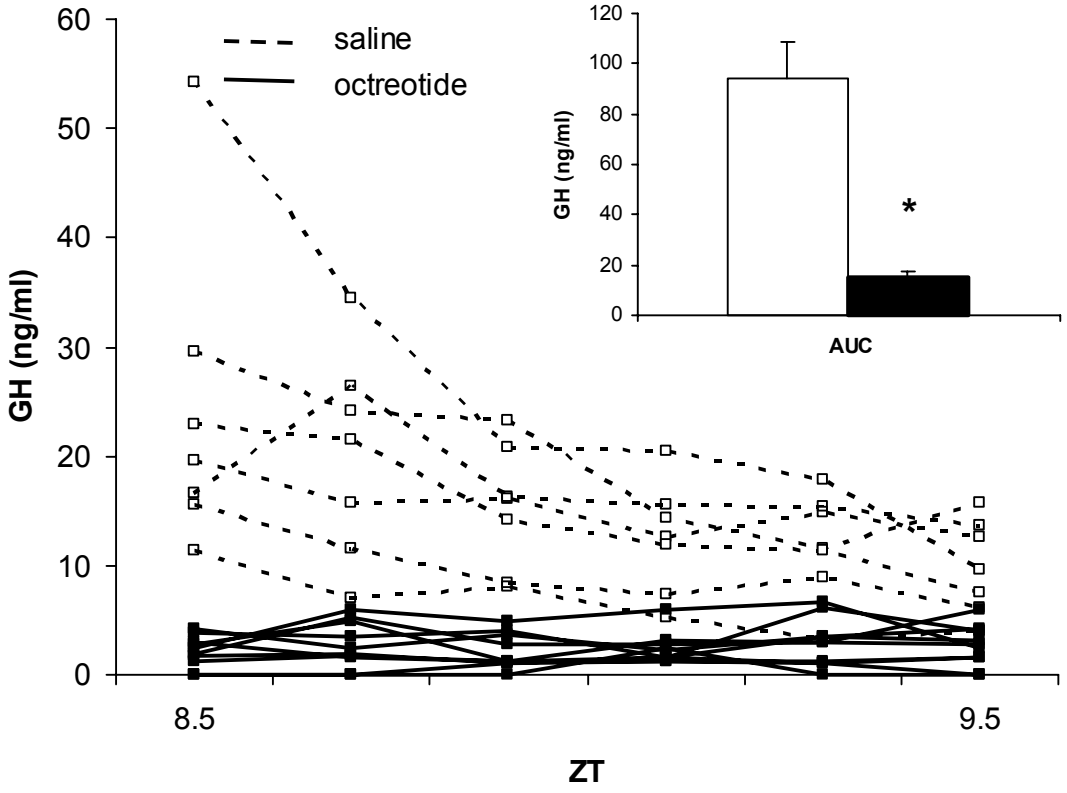


Figure 2. Plasma GH concentrations of all individual OVX+EB rats that received either a single i.c.v. injection with saline (open squares; n=7) or the SOM analog octreotide (closed squares; n=10) and the total amount of GH released during the complete sampling period (AUC; insert; white bar: saline-treated, black bar: octreotide-treated). AUC (mean ± SEM) was significantly decreased in octreotide-treated rats compared to saline-treated rats (p=0.000).

The total percentage of activated (cFos-ir containing) GnRH-ir neurons was significantly lower in octreotide-treated OVX+EB rats than in saline-treated OVX+EB rats (Figure 3). Although the percentage of activated GnRH-ir neurons after octreotide treatment was reduced in all areas (B-D), when evaluated per area, the difference compared to saline-treated rats was only statistically significant in area C. Percentages in saline- and octreotide-treated rats were respectively 2.7 ± 1.6 and 0.56 ± 0.37 for area B (p=0.25), 40.6 ± 8.0 and 15.8 ± 4.3 for area C (p=0.02), and 41.3 ± 9.3 and 22.9 ± 5.6 for area D (p=0.12).

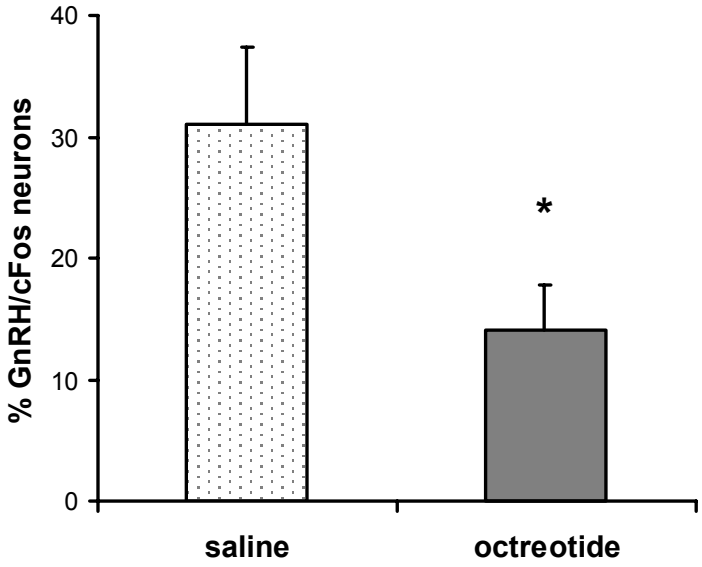


Figure 3. Mean ± SEM percentage of activated (cFos-ir containing) GnRH-ir neurons in brain areas B-D of OVX+EB rats. The percentage of activated GnRH-ir neurons was significantly decreased in octreotide-treated (n=8) rats compared to saline-treated rats (n=9) (p=0.042).

DISCUSSION

The present study clearly demonstrates that a single central injection with a somatostatin (SOM) analog (octreotide) just prior to surge onset significantly decreased the estradiol (E_2)-induced LH surge in ovariectomized female rats. Moreover, 7 out of the 10 animals that were treated with octreotide showed no apparent LH surge at all. These findings are in agreement with both *in vitro* and *in vivo* studies showing that SOM is able to decrease LH secretion in male rats [70, 146]. However, in these studies prolonged treatment, inducing high levels of SOM were used, whereas we applied the SOM analog only once within the so-called “critical period” [153, 154]. Moreover, in previous studies male rats, which do normally not express an LH surge were used. The present results suggest that SOM, apart from possible effects on basal LH release, may also affect episodic preovulatory LH release during the reproductive cycle in the female.

In female rats, both hypothalamic SOM peptide and preproSOM mRNA levels fluctuate during the estrous cycle [33, 89, 155]. Hypothalamic preproSOM mRNA levels [155] as well as SOM release from the median eminence (ME) [33] are maximal at

proestrous afternoon, whereas SOM peptide levels in the pre-optic area (POA), posterior hypothalamus and ME are decreased on the day of proestrus compared to all other days of the estrous cycle [89]. These data may suggest that in cycling female rats SOM release from the hypothalamus peaks on proestrous afternoon. In addition, our present data clearly show that centrally applied SOM decreases LH release, suggesting that SOM may be involved in the regulation of the preovulatory LH surge in female rats.

We injected the SOM analog in the lateral ventricle of the brain, assuming that the inhibiting effect on LH release is caused by direct effects of SOM at the hypothalamic level leading to changes in the activity of hypothalamic GnRH cells and/or GnRH release from the ME. Indeed, we found a significant decrease in the percentage of activated GnRH neurons (i.e. the neurons containing a cFos-positive nucleus) after octreotide treatment. We studied the activity of GnRH neurons during the ascending phase of the LH surge, as it is well known that at that phase a positive correlation exists between the percentage of activated GnRH cells and plasma LH concentrations [97, 156-158]. We particularly evaluated the GnRH cells in the areas that are known to become activated during the preovulatory LH surge, i.e. the DBB, OVLT and MPO [51, 97, 152, 158]. Interestingly, the decrease in activated GnRH neurons following octreotide treatment was most prominent at the level of the OVLT, the region in which the first activation of GnRH neurons during LH surge onset occurs [51, 97, 152]. Our data strongly suggest that the decreased LH release we found after a single central injection with the SOM analog octreotide is, at least partially, the result of a direct effect on hypothalamic GnRH neuron activation, most likely resulting in a reduction in GnRH release, leading to the observed decrease in LH release from the pituitary.

On the day of proestrus, rising levels of E_2 together with the feedback of progesterone (P) lead to an increase in GnRH release, evoking the LH surge. In the rat, the descending phase of the LH surge is most likely the direct result of a decrease in GnRH release from the hypothalamus [159, 160]. We hypothesize that the increasing levels of hypothalamic SOM on proestrous afternoon may be involved in this process. In view of the fact that in the present study the LH surge was completely abolished in 7 out of 10 animals after octreotide administration in the “critical period”, we suggest that somatostatin may be involved in the descending rather than the ascending phase of the LH surge. However, the exact physiological role and mechanism of action of SOM in the regulation of the preovulatory LH surge remains to be determined.

Apart from a decrease in LH release, we also found a decrease in GH release from the pituitary within 2.5 hours after the central administration of octreotide. Physiological regulation of GH release involves, besides direct projections of periventricular SOM cells to the ME [90, 93, 94, 101, 161], projections of hypothalamic SOM cells to the GHRH cells in the arcuate nucleus of the hypothalamus (ARC). Via these projections, SOM can decrease GHRH release from the ME leading to a decrease in GH release from the pituitary [100, 114, 130, 131, 162, 163]. Possibly, also the octreotide we injected into the lateral ventricle may have affected the GHRH cells located very close to the third ventricle in the ARC [164], resulting in a decreased GH release. However, we can not exclude the possibility that the centrally injected SOM analog also reached the pituitary by passing the blood-brain barrier (BBB), and directly affected both pituitary gonadotrophs and somatotrophs via the portal vessel system. It has been shown that several SOM analogs can pass the BBB by a saturable transport mechanism [165]. Moreover, several *in vivo* studies have shown that SOM may directly affect the gonadotropic cells in the pituitary [71, 147, 148]. Anterior pituitary cells express all five SOM receptors ($SSTR_{1-5}$), but LH cells predominantly express $SSTR_2$, whereas GH cells mainly express $SSTR_4$ and $SSTR_5$ [82]. E_2 is able to upregulate $SSTR_2$ and $SSTR_3$ [78, 166, 167], suggesting that these SSTRs on the pituitary LH cells may be upregulated by the increasing E_2 levels on the day of proestrus. Octreotide has a high binding affinity for both $SSTR_2$ and $SSTR_5$ and moderate for $SSTR_3$, but has only a low binding affinity for $SSTR_1$ and $SSTR_4$ [78, 80]. Consequently, a direct effect of octreotide on LH and GH release from the pituitary can not be excluded in the present experimental setup. In addition, several studies showed that a subset of somatotrophs is multipotential and does not only produce GH, but also LH and FSH mRNA. Moreover, these cells express both GHRH and GnRH receptors [58, 168-170], indicating that also effects at the level of the pituitary may have played an additional role. However, the fact that we found an effect of centrally injected octreotide at the level of the hypothalamus, i.e. a significant decrease in activated GnRH cells, supports the idea that octreotide, at least partially, may have also affected the control of LH and GH release at the level of the hypothalamus.

In the hypothalamus, $SSTR_1$ and $SSTR_2$ are present in high numbers, expression of $SSTR_3$ is moderate, and $SSTR_4$ and $SSTR_5$ expression is very low [171]. SOM cells are widely distributed in the rat brain [80], but only SOM cells in the rostral part of the periventricular nucleus of the hypothalamus (PeVN) are responsible for SOM release from

the ME that is involved in the regulation of GH release from the pituitary [90, 93, 94, 161]. In the PeVN $SSTR_1$ and $SSTR_2$ mRNA, colocalized with SOM cells, has been demonstrated [79, 80, 172], whereas in the ARC $SSTR_{1-4}$ have been found [79, 80, 173-175]. GHRH neurons were found to be predominantly co-expressed with $SSTR_1$ and $SSTR_2$, hardly with $SSTR_3$ and $SSTR_4$ and not with $SSTR_5$ [172, 176]. Also in the hypothalamic areas that contain GnRH neurons SSTR expression has been demonstrated: $SSTR_{1-3}$ expression has been found in the anterior hypothalamus [81, 173], $SSTR_{1+2}$ in the MPO [80, 81] and $SSTR_1$ in the OVLT [79]. As octreotide shows highest affinity for $SSTR_2$, $SSTR_3$ and $SSTR_5$, which are found in or near areas that contain GnRH and GHRH neurons, the changes in LH and GH release as found in the present study could very well be the result of a direct action of this SOM analog at the level of the hypothalamus leading to changes in the activity of GnRH and GHRH neurons. The hypothalamic circuitry involved in these effects, however, remains to be established.

To our knowledge, no published data exist on possible direct innervation of GnRH by SOM cells, or on the presence of specific SSTRs on GnRH cells in the OVLT and/or MPO. Although studies in the ewe proved evidence for a direct input from the ventromedial nucleus (VMN) to GnRH neurons [177], in the rat, fibers that arose from the VMN were found to project to the periventricular POA (pePOA) [178]. The pePOA itself does not contain GnRH neurons [151, 157], but was found to contain neurons that become activated concurrent with the activation of GnRH neurons surrounding the OVLT at the time of the preovulatory LH surge, and project to these GnRH neurons at this time [152, 157, 178-180]. Possibly, the neurons projecting from the pePOA to GnRH cells at the time of the preovulatory LH surge are gamma-aminobutyric acid (GABA)-, neurotensin- and/or glutamate-containing neurons, as these neurons are located in the POA and were found to innervate GnRH neurons in the OVLT and POA [181]. Thus, if SOM is involved in the regulation of the LH surge by affecting the activity of GnRH cells, its effect in the female rat is more likely to be indirect. As $SSTR_1$ is located in both the POA [80] and in the OVLT [79], SOM may very well directly affect the activity of neurons in these areas. Indeed, lesions of the anterior hypothalamic area (AHA) resulted in reduced SOM levels in the POA [93], suggesting that SOM cells from the AHA, i.e. the hypothalamic region that contains SOM cells projecting to the ME to regulate GH release, project also to the POA to send signals to GnRH cells in this region.

In conclusion, a single i.c.v. injection of a somatostatin analog during the so-called "critical period" resulted in a significant attenuation of the E_2-induced LH surge in OVX rats. Our results strongly suggest that this was the direct result of, at least partially, a significant inhibition of the activation of GnRH cells in the hypothalamus. These results, together with the observed changes in hypothalamic SOM activity and release over the estrous cycle, as previously described in the literature, led us to propose the hypothesis that SOM may be involved in the normal physiological regulation of the descending phase of the proestrous LH surge.

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CHAPTER 6

Somatostatin peptide distribution in the periventricular nucleus of reproductively aging female rats: effects of estrogen and strain

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ABSTRACT

The growth hormone (GH) and reproductive axis are known to be functionally related. For instance, the decline in GH axis activity with age is correlated with changes in the reproductive axis with age. It is hypothesized that somatostatin (SOM), the neurohormone originating from the periventricular nucleus (PeVN) that inhibits GH release, may play a central role in this interaction. We studied the effect of a single physiological dose of estradiol benzoate (EB) on hypothalamic SOM peptide content in young (4.5 months old) and middle-aged (9 months old) female rats, already showing early signs of reproductive aging.

Female rats of the Wistar and (UxRP)F1 strain were ovariectomized (OVX) at the age of 4.5 or 9 months and were treated with EB 13 days later. Brains were collected 2 and 8 h (respectively at ZT 5 and ZT 11 on day 1), and 26 and 32 h (respectively at ZT 5 and ZT 11 on day 2) after EB treatment. Brains were sliced and sections containing the PeVN were stained for SOM peptide. Subsequently, the number of SOM cells and SOM fiber immunoreactivity (-ir) were counted automatically using a computer assisted analysis system.

EB treatment neither affected total SOM cell numbers nor the characteristic distribution of SOM cells within the PeVN in young animals of both sub-strains. In young Wistar, but not in (UxRP)F1 females, SOM cell distribution within the PeVN was different between ZT 5 and 11 on both days. SOM fiber-ir within the PeVN itself was significantly decreased at 32 h compared to 2 h after EB treatment in young Wistars. Interestingly, total hypothalamic SOM fiber-ir, including the fibers outside the PeVN that project to the median eminence, was consistently higher at ZT 5 compared to ZT 11 on both days in these females. In middle-aged Wistar rats, the number of SOM cells was significantly increased at 26 h compared to 2 h after EB treatment. Also, the number of SOM cells at 2 h after EB treatment was significantly higher in middle-aged (UxRP)F1 compared to Wistar rats. SOM cell distribution within the PeVN was completely different in the middle-aged Wistar compared to the young females only at 2 h after EB treatment. In contrast, SOM cell distribution in the PeVN in middle-aged (UxRP)F1 rats was different from the young animals at all time points following EB treatment.

The present study shows that in adult female rats, the effects of estrogen on SOM cell distribution, SOM cell numbers and SOM fiber-ir in the PeVN are both age and strain dependent. Moreover, our data demonstrate that already during the early stage of

reproductive aging, significant changes occur in SOM neurons in the PeVN of these female rats.

INTRODUCTION

The growth hormone (GH) and reproductive axis are known to be closely related: Both GH overexpression and GH-deficiency cause dramatic changes in reproductive function, i.e. reduced fertility in animals that overexpress GH [54, 55, 61] and complete infertility in animals that lack GH [53, 54]. Also, changes with age occur concurrently in both axes, indicating that the functionality of these two axes may be closely related.

In the rat, GH secretion patterns are clearly sexually dimorphic [4, 6, 7]. This has been suggested to result mainly from differences in somatostatin (SOM) release patterns from the median eminence (ME) [19, 23, 25]. SOM is synthesized in the periventricular nucleus of the hypothalamus (PeVN) and acts together with GH-releasing hormone (GHRH), synthesized in the arcuate nucleus of the hypothalamus (ARC), to regulate GH release from the pituitary: SOM inhibits and GHRH stimulates GH secretion [19, 83-85].

In addition to well described effects of altered GH status on reproduction at the level of the ovaries [53, 54, 56, 61, 65] and the pituitary [38, 53, 54, 60, 61], there are several indications that changes also occur at the level of the hypothalamus. In transgenic mice overexpressing GH, decreased gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus *in vitro* in males [53], and an altered distribution of GnRH-containing neurons in the hypothalamus of females were reported [65]. Also, in GH-deficient animals preproSOM mRNA levels in the PeVN were decreased [66], whereas preproSOM mRNA levels [66] and the number of SOM cells [65] in the PeVN were elevated in animals that overexpress GH. An altered endogenous GH status is thus correlated with changes at the hypothalamic level.

Interestingly, SOM may affect reproductive function at the level of the pituitary: in male rats, SOM was found to inhibit the GnRH-induced release of LH *in vitro* [70] and to decrease plasma LH *in vivo* [146]. In addition, multiple central injections with SOM or a SOM analog resulted in smaller and pycnotic gonadotropic cells and in decreased gonadotropic cell numbers in the pituitary of both male and female rats [71, 146-148]. Moreover, we recently reported that a single central injection with a SOM analog significantly attenuated the estrogen-induced LH surge and significantly decreased the activation of GnRH cells in the hypothalamus of female rats [182]. Altogether, these data

suggest that hypothalamic SOM neurons may play a role in the changes that occur in the reproductive axis following changes in the GH axis.

During adulthood the decline in reproductive function in the female, despite normal or even elevated levels of plasma estradiol (E_2), is correlated with a decline in somatotropic axis activity [48, 77, 183]. In 14 months old rats, hypothalamic SOM peptide content and both basal and KCl-stimulated SOM release from the hypothalamus were higher in both sexes compared to younger animals [44]. Moreover, the SOM concentration in the ME was lower in 25-29 months old than in young female rats [40], suggesting increased SOM release from the ME with age.

Evidence exists that exposure to chronically elevated levels of circulating E_2 during life advances the decline in fertility with age [184, 185]. Moreover, it is known that E_2 affects hypothalamic SOM content and release, although the literature is somewhat controversial on the precise role of E_2 on SOM [28, 29, 31-33] (*chapter 3 and 4*). Recently, it was found in our laboratory that the LH surge was significantly decreased in cycling female rats already at 9 months of age [50]. In the light of the data described above, we studied the effects of acute E_2 feedback on hypothalamic SOM peptide levels in the very same animals during early reproductive aging. Therefore, we compared the effect of a physiological dose of E_2 on SOM peptide-containing cells in the PeVN of young (4.5 months) and middle-aged (9 months) short-term ovariectomized female rats. We used two different rat sub-strains: Wistar rats, predominantly displaying 4-day estrous cycles, and (UxRP)F1 rats that predominantly display 5-day estrous cycles by lengthening of diestrous. The (UxRP)F1 rats are hence exposed to elevated plasma E_2 levels approximately one extra day each estrous cycle, and, in addition, were shown to exhibit impaired fertility and fecundity at an earlier age than the Wistar strain [48, 186]. Using this approach, we aimed to gain more insight in the mechanism underlying the interaction between the somatotropic and gonadotropic axis, i.e. a possible role for the hypothalamic SOM system.

MATERIALS AND METHODS

Experimental design

Forty-three regularly (4-day) cycling female Wistar rats (Harlan, Horst, The Netherlands) aged 4.5 (n=20) or 9 (n=23) months and 42 regularly (5-day) cycling female

(UxRP)F1 rats (a locally bred hybrid of two Wistar sub-strains) of 4.5 (n=20) or 9 (n=22) months old were individually housed in a room with controlled lighting (LD 12:12; lights on at 5:00 h (zeitgeber time (ZT) 0)), temperature (21°C) and humidity (70%). Standard pelleted food and water were available *ad libitum*. All experiments were approved by the Wageningen University Animal Ethics Committee.

All females were bilaterally ovariectomized (OVX) under Ketamine/Rompun® (60 mg/kg and 3.3 mg/kg body weight, respectively) anesthesia that was given intraperitoneally (i.p.). On day 13 after OVX, each rat was given a single subcutaneous (s.c.) injection with estradiol benzoate (EB; 6.25 µg in 0.2 ml cottonseed oil) at ZT 3, and was perfused 2, 8, 26 or 32 hours later.

Prior to perfusion animals were given an overdose of Nembutal® (CEVA sante animale B.V., Maassluis, The Netherlands) anesthesia (i.p.; 1.5 ml/kg body weight). The rats were perfused transcardially with 250 ml saline followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate buffer (4% PFA; pH 7.4). Brains were removed from the skull immediately after perfusion and postfixed in the same fixative for 25 h at 4ºC. Prior to sectioning, tissue blocks containing the hypothalamus were embedded in 20% gelatin and fixed in 4% PFA for 4 h at 4ºC [122]. Coronal sections of 40 µm were sliced using a Vibratome (series 1000 clasic; Vibratome Company, St. Louis, MO, USA) and were stored in 0.1 M Tris buffer (TBS; pH 7.4) containing 1% polyvinylpyrrolidone-40 (PVP-40; Sigma), 30% ethylene glycol (Sigma) and 30% sucrose (Sigma) (“cryprotectant” [123]) at 4ºC until immunocytochemistry.

Young (4.5 months old) and middle-aged (9 months old) females were obtained from the same batch to reduce variation between animals. As a consequence, the experiments with young and middle-aged animals were performed in two separate runs. Therefore, two extra groups of young (4.5 months old; perfused at 2 (n=6) and 32 (n=6) h after EB treatment) female Wistar rats were added to the second experiment (i.e. middle-aged females), to be able to compare stainings.

Immunocytochemistry

One third of the brain sections containing the PeVN was stained for SOM peptide using free-floating immunocytochemistry techniques [97]. Sections were pretreated with TBS containing 0.1% sodiumborohydride for 20 min, followed by extensive washing with TBS. Thereafter sections were incubated in TBS containing 3% H_2O_2 for 30 min, followed

by extensive washing with TBS. For the staining, sections were incubated with the primary polyclonal rabbit antibody raised against SOM peptide (Somaar 080289, NIH, Amsterdam, The Netherlands; final dilution 1:30,000) [98] diluted in TBS containing 0.5% Triton-X-100 and 0.25% gelatin ("supermix") for 1 night at 4ºC. This was followed by incubation with biotinylated goat anti-rabbit IgG (GaR-bio; Vector Laboratories; 1:400 in supermix) for 1.5 h at room temperature, and Avidin-Biotin Complex-elite (ABC; Vector Laboratories; final dilution 1:1500 in supermix) for 1.5 h at room temperature. Between incubation steps, sections were thoroughly washed with TBS. SOM immunoreactivity was visualized by incubation with 0.05% DAB in TBS with 0.1% imidazole and 0.03% H_2O_2 for 10 min.

Stained sections were mounted on albumin coated slides, dried, dehydrated and coverslipped in DEPEX (BDH Laboratory Supplies, Poole, England).

Image analysis

Images of the left side of the brain only containing the PeVN were captured using a microscope (5x10 magnification) with a digital black-and-white CCD camera (Sony, XC-77CE) connected to a computer. No differences in SOM peptide content were found between the left and right side of the hypothalamus (personal observations). The PeVN as we selected it consisted of 6 consecutive sections at 80 µm intervals. The first section of the PeVN was decided to be the second-last section in which the suprachiasmatic nucleus (SCN) was still visible (plate 24 according to Paxinos and Watson [99]). The last section of the PeVN was the last section before the ventral medial nucleus (VMN) first appeared (plate 25 according to [99]) (these selected sections correspond with PeVN sections 4-9 as described in *chapter 2* of this thesis).

A background correction was made by capturing each image at the same gray level (i.e. the average gray level of the entire image), after which images were analyzed using an image analysis program (Scion Image Beta, version 3B; Scion Corporation, Maryland, USA) using gray level threshold discrimination as previously described [50]. The threshold for the analysis of the number of SOM cells was determined in a representative selection of the images by measuring the mean gray level in an area that contained SOM fibers, but no SOM cell bodies. Using these background measurements, the threshold was determined to be the mean maximal gray level. SOM-immunoreactive (-ir) neurons, i.e. the

cell bodies without fibers that consisted of a minimal size of 5 pixels, could subsequently be counted automatically.

In addition to counting SOM-ir cells, we also measured SOM fiber-immunoreactivity (-ir) in these images. To this end, both the fibers that were located closely to the SOM cells (the "PeVN region": measured in an area with absolute distance from the third ventricle of approximately 200 µm) and all fibers that originated from SOM cells in the PeVN, including those projecting to the ME ("total fibers": measured in an area with absolute distance from the third ventricle of approximately 560 µm) were counted. The threshold for the analysis of SOM fibers was determined in a representative selection of the images by measuring the mean gray level in an area that contained neither SOM cell bodies nor SOM fibers. For the SOM fiber-ir measurements, an upper and a lower threshold were determined, as calculated by the mean gray level + 3x S.D. and the mean maximal gray level – 3x S.D., respectively. Using these upper and lower thresholds, both SOM-ir cells (from very light to very dark stained) and very light SOM fibers were excluded, hence only true SOM fibers (expressed in μm^2) were taken into account.

Statistical analysis

Data were analyzed using the SPSS statistical analysis software program. To compare the total number of SOM-ir cells between the different time points after EB treatment within one group (same strain and age), or between different groups (different strain or age) at the same time point, Oneway ANOVAs were used. For posthoc tests, a Bonferroni or Tukey HSD test was used. Differences were considered to be significant when $p<0.05$.

PeVN section 9 was not present in several animals. Therefore we decided to use only sections 4-8 for statistical analysis. As described above, brains of young and middle-aged animals were stained in two separate runs. After testing, it appeared that brain sections of the first run (young females) were more intensely stained compared to the second run (middle-aged and young control females). Although this difference in staining intensity that was detected had no consequences for the number of SOM-ir cells (the sum of SOM-ir cells in PeVN sections 4-8 in young Wistar rats was not significantly different between the first and second run), SOM fiber-ir could not be identified in the sections that were stained in the second run, i.e. the middle-aged females. Therefore, only in the young females, SOM fiber-ir was analyzed as described above.

RESULTS

SOM-ir cells in the PeVN

Total numbers (i.e. the sum of PeVN sections 4-8) of SOM-ir cells did not change in time upon EB treatment in the young animals of either sub-strain, although total numbers of SOM-ir cells were consistently lower at ZT 5 compared to ZT 11 in the young Wistars (n.s.; Figure 1).

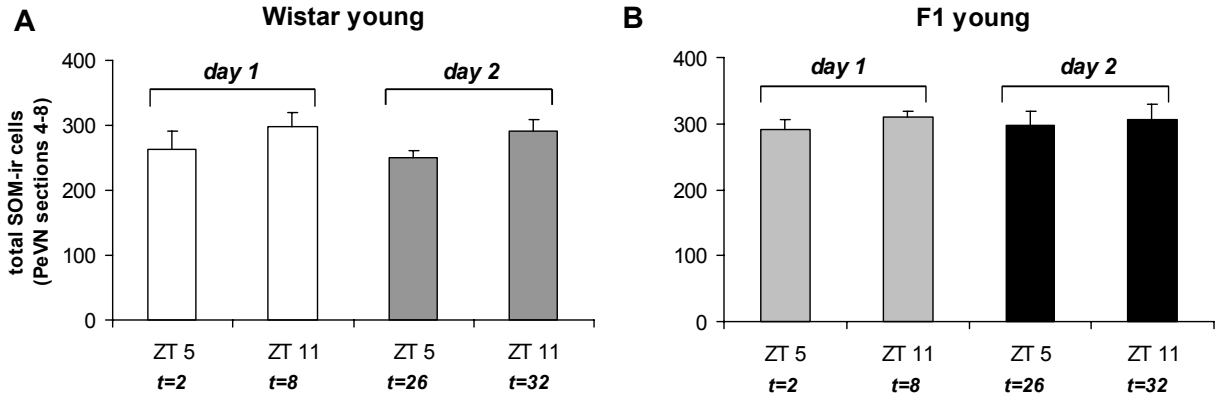

Figure 1. Total number of SOM-ir cells (sum of PeVN sections 4-8) in young (4.5 months old) Wistar (A) and (UxRP)F1 (B) OVX females at 2, 8, 26, or 32 h after EB treatment. Bars represent mean ± SEM. n=5 for each group.

SOM-ir cells within the selected PeVN area (PeVN sections 4-9) showed a clear rostral to caudal distribution, with maximal numbers of cells appearing in the more caudal part of the PeVN (Figure 2A-D). In the young (UxRP)F1 rats, the numbers of SOM-ir cells were more comparable between sections compared to the young Wistar rats, showing a clear “peak” in SOM cell localization. Distribution patterns of SOM-ir cells within the PeVN in young Wistar rats varied slightly between the different time points after EB treatment: maximal numbers of SOM-ir cells were found consistently in PeVN section 8 at ZT 5 (i.e. at t=2 and t=26 after EB), but in PeVN section 7 at ZT 11 (i.e. at t=8 and t=32 after EB) (Figure 2A and B). Maximal numbers of SOM-ir cells in young (UxRP)F1 rats were found in PeVN section 7 at all time points after EB treatment (Figure 2C and D).

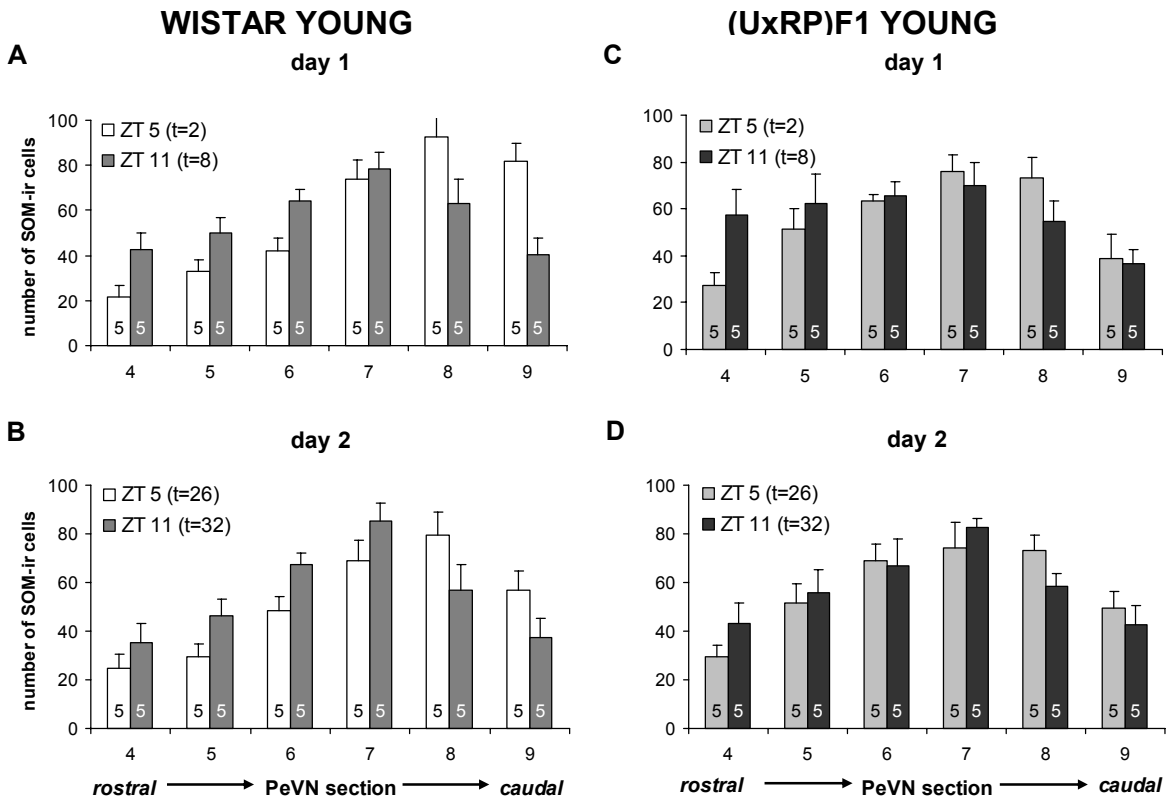

Figure 2. Rostral to caudal distribution of SOM-ir cells in the PeVN of young (4.5 months old) Wistar (A and B) and (UxRP)F1 (C and D) OVX females at different time points after EB treatment. Bars represent mean ± SEM. Numbers within bars indicate the number of animals.

In the middle-aged Wistar rats, the total number of SOM-ir cells was significantly lower at ZT 5 on day 1 (i.e. at t=2) compared to day 2 (i.e. at t=26) (Figure 3A). The distinct rostro-caudal distribution of SOM-ir cells in the PeVN was not present at ZT 5 on day 1 (t=2) in the middle-aged females: the number of SOM-ir cells was comparable between all PeVN sections. This in contrast to the peak in SOM-ir cell number in section 8 found in young Wistar rats at the same time point after EB treatment. Distribution patterns at the other time points after EB treatment were largely comparable with those found in young Wistar rats, i.e. maximal numbers of SOM-ir cells in PeVN section 7 at ZT 11 (t=8 and t=32 after EB) and in PeVN section 8 at ZT 5 on day 2 (t=26 after EB) (Figure 4A and B).

In middle-aged (UxRP)F1, the total number of SOM-ir cells at ZT 5 on day 1 (t=2) was significantly higher than in middle-aged Wistar rats (Figure 3). In the middle-aged (UxRP)F1 rats the total number of SOM-ir cells did not change significantly in time upon EB treatment (Figure 3B), as found in young (UxRP)F1 females. The rostro-caudal SOM-ir

cell distribution in the PeVN of the middle-aged (UxRP)F1 rats, however, was completely different from young F1 rats at ZT 5 on day 1 (t=2) and at ZT 11 on day 1(t=8) and day 2 (t=32): maximal numbers of SOM-ir cells were found in PeVN section 6 at t=2, and in section 5 at t=32 compared to section 7 found in the young animals (Figure 4 C and D).

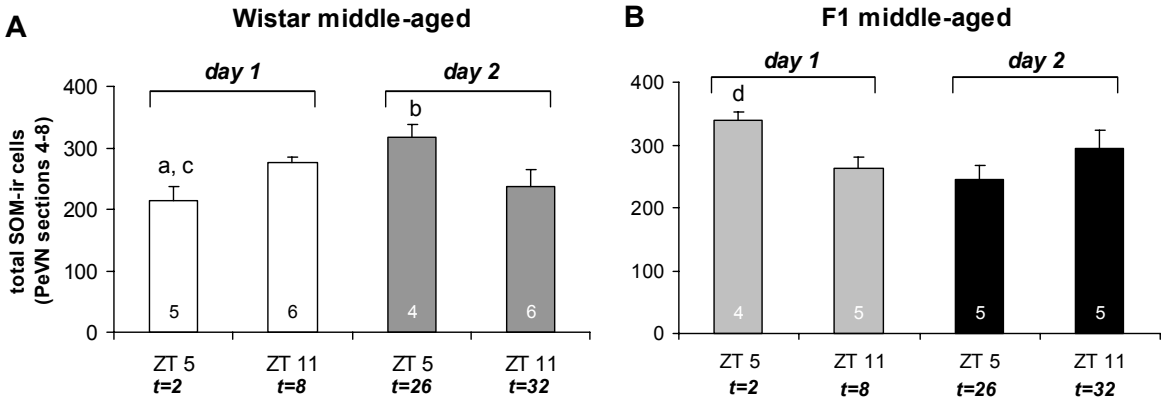

Figure 3. Total number of SOM-ir cells (sum of PeVN sections 4-8) in middle-aged (9 months old) Wistar (A) and (UxRP)F1 (B) OVX females at different time points after EB treatment. a significantly different from b (p=0.026; ANOVA); c significantly different from d (p=0.01; Bonferroni). Bars represent mean ± SEM. Numbers within bars indicate the number of animals.

SOM fiber-ir in the PeVN

SOM fiber-ir in the PeVN region itself was significantly lower at t=32 than at t=2 after EB treatment in young Wistar rats (Figure 5A). Also, total SOM fiber-ir (i.e. SOM fibers within the PeVN as well as fiber bundles projecting to the ME) was significantly higher at t=2 than at t=8 and t=32 after EB treatment in the young Wistar females (Figure 5B).

In young (UxRP)F1 female rats, neither SOM fiber-ir in the PeVN nor total SOM fiber-ir was changed significantly following EB treatment (data not shown). Total SOM fiber-ir at t=2 after EB treatment was significantly higher in young Wistar than in young (UxRP)F1 female rats (data not shown; values were 224886 ± 16027 μm^2 for Wistar and 162455 ± 7921 μm^2 for (UxRP)F1; p=0.008; ANOVA).

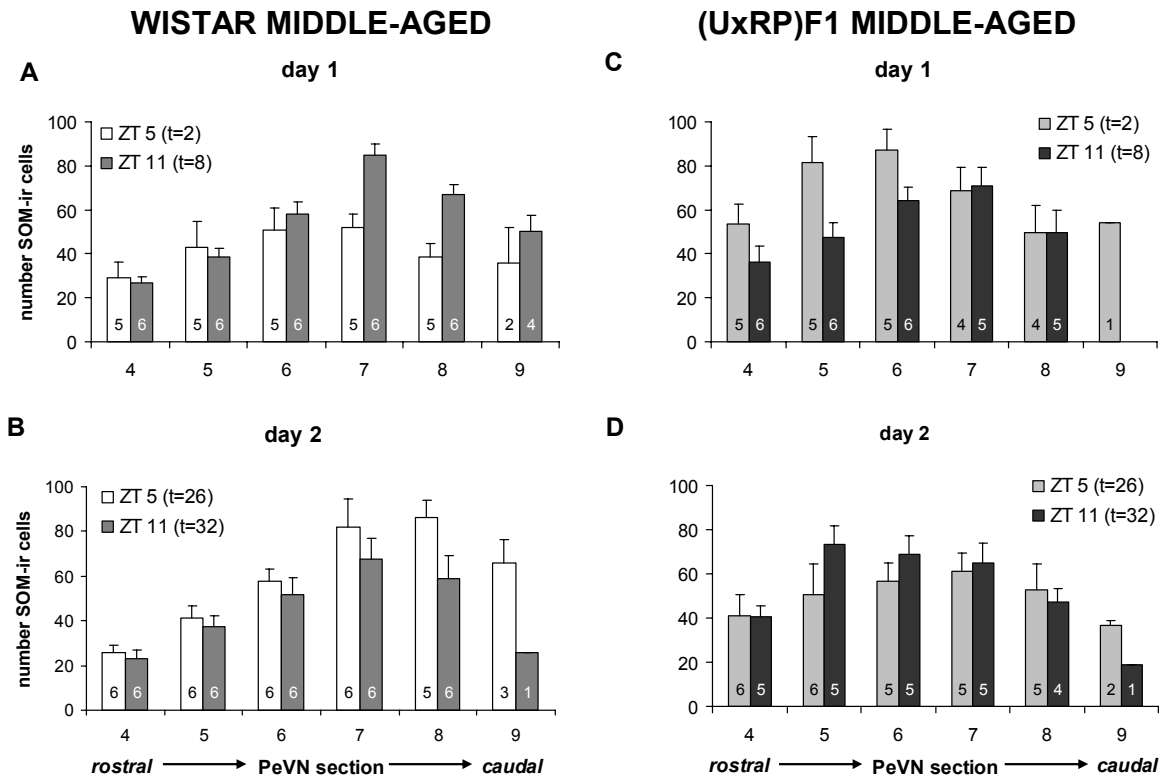

Figure 4. Rostral to caudal distribution of SOM-ir cells in the PeVN of middle-aged (9 months old) Wistar (A and B) and (UxRP)F1 (C and D) OVX females at different time point after EB treatment. Bars represent mean ± SEM. Numbers within bars indicate the number of animals.

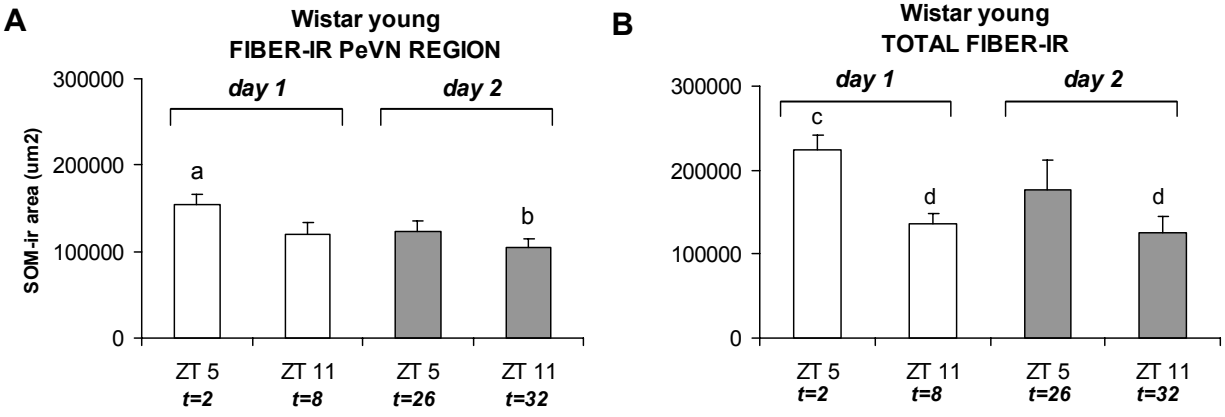

Figure 5. SOM fiber-ir in the PeVN region (A) or total SOM fiber-ir (B) in young (4.5 months old) OVX female Wistar rats at different time points after EB treatment. a significantly different from b (p=0.047; Bonferroni); c significantly different from d ($p \leq 0.05$; Tukey HSD). Bars represent mean ± SEM. n=5 for each group.

DISCUSSION

In the present study we show that in adult short-term OVX female rats, the effects of estrogen on SOM-ir cell distribution, SOM-ir cell numbers and SOM fiber-ir in the PeVN are both age and strain dependent.

EB did not affect the total number of SOM-ir cells in the PeVN of young female rats, which is in line with our previous studies (*chapter 3 and 4* of this thesis) and a paper that also reported no effects of E_2 on both hypothalamic SOM content and release [33]. Other reports showed that a decrease in SOM mRNA content in the PeVN after OVX can be reversed by E_2 [28, 29]. The discrepancy with our data may be due to the fact that in the latter studies animals were treated with E_2 for a prolonged period of time, whereas we studied the effect of a single physiological dose of EB on SOM-ir. Interestingly, SOM fiber-ir within the PeVN region itself in young female Wistars was gradually, but significantly decreased 32 h compared to 2 h after a single physiological dose of EB, suggesting changes in release or transport of SOM peptide from the PeVN following EB treatment, however, without affecting the amount of SOM peptide synthesized and/or stored in the PeVN cells themselves.

In young Wistar rats, the rostro-caudal distribution of SOM-ir cells within the PeVN appeared to be similar at 2 and 26 h, and at 8 and 32 h after EB treatment. Both t=2 and t=26 correspond to ZT 5, whereas t=8 and t=32 correspond to ZT 11, on two consecutive days. Also, total SOM fiber-ir, i.e. all fibers originating from SOM cells in the PeVN including those projecting to the ME, was consistently higher at ZT 5 compared to ZT 11 in these animals. These findings suggest that SOM peptide synthesized in the PeVN and released in the ME may show a diurnal rhythm, rather than be directly affected by circulating E_2 concentrations. These observations are supported by the reported diurnal rhythm in SOM peptide content in the ME [46].

The rostro-caudal distribution of SOM-ir cells in the PeVN was different in middle-aged, compared to young Wistar rats, at 2 h but not at 8, 26 and 32 h after EB treatment. These findings suggest that during (early) aging, estrogen may become more crucial for the synthesis and/or storage of SOM peptide in the PeVN of the Wistar female. Interestingly, the diurnal change in SOM cell distribution, as found in young Wistars, was only evident on day 2 after EB treatment in the middle-aged Wistar females. Estrogen may hence become important for the maintenance of the diurnal change in SOM levels within the PeVN during (early) aging.

The function of such a diurnal change in SOM levels in the PeVN remains speculative. A few studies reported more pronounced GH secretory bursts in cycling female rats after the onset of darkness [5, 6], suggesting that the shift in the rostro-caudal SOM cell distribution at ZT 11, i.e. just before dark onset, may reflect the reported shift in GH secretion pattern. Although to our knowledge no data exist on light/dark-related GH secretory patterns during aging, mean plasma GH levels and mean peak GH levels were found to be decreased already in 11 month old female rats compared to the young females [40]. Taking these findings into consideration, we suggest that the changes in SOM-ir levels within the PeVN may affect GH release patterns during aging in female rats.

Interestingly, SOM cells in the PeVN of young (UxRP)F1 rats did not show this diurnal change in immunoreactivity. Moreover, the SOM cells in the (UxRP)F1 were more evenly distributed from rostral to caudal within the PeVN compared to the young Wistar rats. Furthermore, in the middle-aged (UxRP)F1 rats the rostro-caudal SOM-ir cell distribution within the PeVN was completely different from that found in the young rats and EB treatment appeared to have no effect on this, or on the total number of SOM-ir cells in the PeVN. These observations suggest that even between rat sub-strains the hypothalamic SOM system, regulating GH release, may be very differently organized.

Characteristics of hypothalamic regulation of reproduction and changes with age in female Wistar and (UxRP)F1 rats were studied extensively in our laboratory. Studies in the very same animals that were used in the present study showed that in 9 months old females of both sub-strains the preovulatory LH surge was attenuated, which is illustrative of reproductive aging. Interestingly, the results indicated that hypothalamic regulation of reproduction differs between the sub-strains. Analysis of plasma E_2 and progesterone (P) levels, and hypothalamic P receptor concentrations in these animals supported the notion that the (UxRP)F1 females may become reproductively aged at an earlier age than the Wistar rats [50]. Although we did not study plasma GH levels in these 4.5 and 9 months old females, our present data suggest that changes in hypothalamic regulation of GH release occur already at a relative early age. Moreover, our data indicate that changes in hypothalamic regulation of both GH and LH release occur simultaneously in the female rat.

We found an increased number of SOM-ir cells in middle-aged (UxRP)F1 females compared to female Wistars of the same age. Elevated SOM peptide concentrations and release are associated with aging [17, 46], suggesting that the (UxRP)F1 female rats may indeed show signs of aging earlier in life than Wistar rats, as previously suggested [48, 50,

186]. Our results may imply that the absence of any rostro-caudal change in SOM-ir levels in the PeVN, which are not affected by ZT and/or a physiological EB stimulus, may be one of the characteristics of aging in the (UxRP)F1 sub-strain. In view of the fact that we previously demonstrated that SOM decreases the EB-induced LH surge and hypothalamic GnRH cell activation in young Wistar females [182], our present data suggest that age-related changes in the hypothalamic SOM system may directly affect reproductive function in the female rat.

ACKNOWLEDGEMENTS

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CHAPTER 7

Summary and General Discussion

The somatotropic and gonadotropic axis are clearly closely related, as described in detail in *chapter 1*. Interestingly, both elevated and decreased concentrations of plasma GH lead to a dramatic decline in reproductive function [53, 55, 56], suggesting that GH release, and consequently its hypothalamic regulatory mechanisms, need to be in balance for optimal reproductive function. The aim of this thesis was to test the hypothesis that somatostatin plays a central role in the interaction between the somatotropic and gonadotropic axis in the female rat. In this chapter we will summarize and discuss the sex differences in the hypothalamic SOM system and the actions of SOM on the reproductive axis. Based on our findings as described in this thesis, and on data from the literature, we will propose a possible mechanism via which SOM may act on both the somatotropic and gonadotropic axis. Finally, we will discuss the consequences of an altered GH status on reproductive function in general, and during reproductive aging in particular.

7.1. SUMMARY OF FINDINGS

To discuss the implications of the possible effects of hypothalamic SOM on reproductive axis activity, detailed information on the functioning of the hypothalamic SOM system itself is required. As most studies to date were performed in male rats, we first aimed to study the hypothalamic SOM system in the female rat in more detail. To this end, we described the highly characteristic location and distribution of SOM cells within a strictly defined PeVN area (*chapter 2*). We concluded that in the female rat SOM cells are affected by the gonadal steroids E_2 and P in specific subregions of the PeVN, and the SOM cells may hence have specific functions concerning the sexual dimorphic regulation of GH release.

Next, we studied the acute effect of a single GH injection on SOM cells in the PeVN of female rats. Our findings suggest that SOM cells in the female PeVN may contain GH-receptors (GH-Rs), like in male rats, as we demonstrated that GH acutely induces SOM cell activation in the female rat. Gonadal steroids clearly play a role in the hypothalamic feedback actions of GH in the female: E_2 may modulate the sensitivity of SOM cells to GH. However, our data also indicate that other ovarian factors may be involved; we suggested an additional role for progesterone (P) (*chapter 3*).

Based on these findings, we focused on the effect of the ovarian steroids E_2 and P on SOM neurons in the PeVN of female rats. We found that E_2 and P act synergistically,

rather than alone, to affect SOM peptide synthesis and/or storage in cells in the female PeVN (*chapter 4*).

We tested the hypothesis that hypothalamic SOM neurons may play a central role in the somatotropic and gonadotropic interaction by examining the effect of a central injection with a SOM analog on hypothalamic GnRH neurons and LH release. We demonstrated that SOM inhibits the E_2-induced LH surge by, at least in part, decreasing GnRH cell activation, suggesting that indeed also at the level of the hypothalamus the two axes interact (*chapter 5*).

Given the fact that the decrease in GH axis activity is correlated with the decline in reproductive function with age, despite similar or elevated plasma E_2 concentrations, we studied the acute effects of a single physiological dose of E_2 on hypothalamic SOM cells in young and reproductively aging ("middle-aged") female rats. Middle-aged female rats in which the preovulatory LH surge was already attenuated [50], also showed changes in hypothalamic SOM levels. Moreover, our data suggest that during the early stages of aging the somatotropic axis may be characterized by an altered distribution of SOM cells and elevated SOM peptide levels within the PeVN, despite the presence of physiological concentrations of E_2 (*chapter 6*).

7.2. SEXUAL DIFFERENCES IN THE HYPOTHALAMIC SOM SYSTEM

Synthesis, storage and release

Based on our findings described in *chapter 3 and 4* in this thesis and on data from the literature, we can conclude that adult female rats possess more SOM-producing neurons (*chapter 3*), but that the synthesis, and possibly also the storage, of SOM peptide is higher in adult male rats (*chapter 3 and 4*)[23, 30, 31]. Interestingly, both basal and K^+-stimulated SOM release from the ME was demonstrated to be higher in female compared to male rats [31, 45]. These data support the proposed differences between the sexes in SOM release with respect to the regulation of GH release [25, 35]: In the female rat, many SOM neurons in the PeVN may constantly synthesize and secrete small amounts of SOM to maintain the suggested relatively low and tonic release of SOM into the portal blood vessel system. In male rats, high levels of SOM peptide are secreted from the ME in a burst approximately every 3 h. This requires a hypothalamic SOM system that is able to release a very high amount of SOM peptide at once. Therefore, synthesis and storage of

SOM peptide may be constantly high in a specific group of SOM neurons in the male PeVN. The synthesis, storage and release of SOM peptide hence differs between adult male and female rats. This supports the previously proposed central role of the hypothalamic SOM system in the establishment of the sexual dimorphism of GH release from the pituitary [19, 23, 25].

Subregions of the PeVN

We found that the acute effects of GH on SOM cells in the PeVN were distinctly different between the sexes: In male rats the number of SOM cells was increased specifically in the medial part of the PeVN, while in female rats, the number of SOM cells was decreased specifically in the posterior part of the PeVN. Interestingly, increased SOM cell activation following a single GH injection was most abundant in the anterior PeVN in male rats, whereas in females, increased SOM cell activation was comparable between all PeVN subregions (*chapter 3*). Thus, not only the acute feedback effects of GH on the number of periventricular SOM cells, but also the site of impingement differs between adult male and female rats. Moreover, these observations may imply that the negative "short-loop" feedback of SOM on its own release [20] may occur in different PeVN subregions in male and female rats. The organization of SOM neurons within the PeVN may therefore differ between the sexes, which may result in the different SOM release patterns from the ME, and consequently, in the sexually dimorphic GH release patterns.

Effects of gonadal steroids

A role for gonadal steroids in the regulation of SOM synthesis and/or release seems reasonable, as concentrations of SOM mRNA, peptide and release fluctuate during the estrous cycle [33, 88, 89], concurrent with plasma E_2 and P levels [103]. Although many studies have focused on the possible effects of E_2 on hypothalamic SOM mRNA, SOM peptide and SOM release levels, the precise role of E_2 on the hypothalamic SOM system with respect to the regulation of GH release in the female rat remains unclear. Our data suggest that in the female rat E_2 may decrease the sensitivity of SOM neurons in the PeVN to acute GH feedback (*chapter 3*). Absence of E_2 may hence result in a more "male-like" hypothalamic SOM system, and consequently a "male-like" GH release pattern (*chapter 1*). In the female rat, estrogen may thus dictate, at least in part, the regulation of GH release at the level of the hypothalamic SOM system.

Yet, the conflicting data from the literature as well as our own data (*chapter 3*) provide evidence for the fact that E_2 may not be the only gonadal factor that influences the hypothalamic SOM system in the female. Indeed, our data show that E_2 and P act synergistically, rather than alone, to alter the activity of SOM cells leading to changes in SOM peptide concentrations in the female PeVN (*chapter 4*). Interestingly, the collaborating effects of E_2 and P on SOM cell number and SOM peptide concentrations were most abundant in the medial and posterior PeVN, suggesting that the gonadal steroids together dictate SOM synthesis, storage and release in specific PeVN subregions in the female rat. The effects of E_2 and P on SOM synthesis and release as reported in literature and found in our own studies are summarized in Table 1.

Surprisingly, the decreased SOM release following OVX could not be restored by E_2 treatment [31, 33], which is remarkable as SOM release from the ME was found to be maximal on the day of proestrus [33]. Thus, the elevated plasma E_2 levels on this day of the estrous cycle are not likely to be responsible for the increased SOM release. Hence, given the facts that E_2 and P act synergistically to affect SOM cells in the PeVN (*chapter 4*), and that peak levels of P occur on proestrous afternoon, we suggest that SOM release from the ME may be directed by the cooperative effects of E_2 and P, rather than the rising levels of plasma E_2 alone.

Effect of:	**OVX**		**E_2**		**P**		**E_2 and P**	
SOM mRNA in PeVN	**# cells**	**content**	**# cells**	**content**	**# cells**	**content**	**# cells**	**content**
	≡ *(ch. 3)*	≡ *[31]* ↓ *[28, 29+, 32+]*	≡ *(ch. 3)*	↑ *[28*, 29*, 32]*	n.d.	n.d.	n.d.	n.d.
SOM peptide in PeVN	↓ *(ch. 3)*	≡ *[31]*	≡ *(ch. 3+4)*	≡ *(ch. 4)*	≡ *(ch. 4)*	≡ *(ch. 4)*	↑ (n.s.) *(ch. 4)*	↑ *(ch. 4)*
SOM release	↓ *[31, 33]*		≡ *[33]*		n.d.		n.d.	

Table 1. Overview of the effects on the hypothalamic SOM system in the female rat following OVX, or gonadal steroid treatment following OVX. +: OVX period of 3 weeks or longer; *: long-term E_2 treatment; n.d.: no data; n.s.: not significant.

7.3. EFFECTS OF SOM ON THE REPRODUCTIVE AXIS

This thesis provides further evidence for the proposed central role of hypothalamic SOM neurons in the functional interaction between the somatotropic and gonadotropic axes. A SOM analog, given during the “critical period” (i.e. just prior to surge onset), completely abolished the E_2-induced LH surge and decreased GnRH cell activation (*chapter 5*). Based on this, and the fact that SOM release may increase on proestrous afternoon [33, 88, 89], we hypothesize that in the cycling female rat, SOM release during a normal cycle increases only after the “critical period”, i.e. during the LH surge. Thus, we suggest that the elevated levels of SOM on proestrous afternoon may be involved in the descending, rather than the ascending phase of the preovulatory LH surge.

Our data described in *chapter 5*, strongly suggest that SOM decreases LH release, at least in part, by decreasing hypothalamic GnRH neuron activation. However, the mechanism behind this action remains speculative. Moreover, indirect effects of SOM cannot be excluded, as SOM was demonstrated to directly affect gonadotropic cell number and morphology [71]. We propose three possible pathways via which SOM, originating from the PeVN, may affect hypothalamic GnRH neurons, resulting in a decreased LH release (see Figure 1*II*-*IV*).

The interactions between neurons in the hypothalamic areas involved in the regulation of LH (OVLT/POA) and GH (PeVN and ARC) release are schematically depicted in figure 1-*I*. ***GnRH*** neurons in the OVLT/POA are innervated by gamma-aminobutyric acid (***GABA***)-ergic cells, which are thought to be involved in the negative feedback of E_2 on the LH surge [181, 187]. The GABA-ergic cells, originating from the OVLT/POA, also innervate ***SOM*** neurons in the PeVN and may therefore also be involved in the regulation of GH release from the pituitary [141, 142, 188, 189]. Also, within the PeVN, a small number of SOM neurons co-express GABA [190]. ***GHRH*** neurons in the ARC are inhibited by SOM neurons originating from either the PeVN or the ARC [114, 162, 163, 191]. Neuropeptide-Y (***NPY***) terminals originating in the ARC project to the preoptic region and ME, where some of the axons make synaptic contacts with GnRH cell bodies or processes [181]. NPY cells located within the PeVN may project to SOM cells in this hypothalamic nucleus. NPY may hence also be involved in the regulation of both LH and GH release from the pituitary.

Pathway 1: directly on neurons in the OVLT/POA

We showed that a centrally injected SOM analog decreased hypothalamic GnRH cell activation (*chapter 5*), suggesting that SOM directly affects cells in the OVLT/POA. The fact that SSTRs were demonstrated in the OVLT/POA [79-81], and that lesions of the anterior hypothalamic area (including the PeVN) resulted in decreased SOM peptide levels in the POA [93], suggests that SOM cells originating from the PeVN project to the OVLT/POA. Possibly, GnRH neurons themselves express SSTRs: $SSTR_2$ gene expression was found in GnRH neurons in adult male and female mice (Prof. A. Herbison, personal communication). Hence, SOM may directly inhibit GnRH cell activation, leading to the supposed decrease in GnRH release, and hence to decreased LH release from the pituitary (pathway A in Figure 1-*II*). Indeed, SOM-immunoreactive fibers were found in close apposition to GnRH cells in adult female rats (Van der Beek *et al*., unpublished data). Moreover, SOM was found to acutely inhibit GnRH neuron electrical excitability (Prof. A. Herbison, personal communication). These data suggest that SOM cells may indeed directly project to GnRH neurons and inhibit GnRH cell activity.

Alternatively, cells in the OVLT/POA, other than GnRH neurons, may contain SSTRs. Neurons in the periventricular POA that project to GnRH neurons at the time of the preovulatory LH surge [157, 179, 180] are likely candidates. Although not proven yet, GABA-ergic cells may be among those neurons containing SSTRs and projecting to the GnRH neurons (pathway B in Figure 1-*II*).

Pathway 2: indirectly via NPY

NPY is very likely to positively affect the preovulatory LH surge: NPY synthesis and release are elevated just before the proestrous LH surge, and immunoneutralization of NPY severely attenuates the steroid-induced LH surge. The effects of NPY on LH release may, at least in part, take place at the hypothalamic level, as NPY terminals synapse on GnRH cell bodies and processes [181, 192]. As SSTRs were demonstrated on NPY cells in both the PeVN and ARC [191], SOM may inhibit NPY neuronal activity, resulting in a decreased stimulating signal to GnRH cells, which in turn decreases GnRH cell activation and release, leading to the observed decreased LH surge (Figure 1-*III*).

Pathway 3: indirectly via the pituitary

Besides the decreased LH surge, we also found decreased plasma GH concentrations following the central injection of the SOM analog (*chapter 5*). SOM was shown to directly decrease LH release [70] and to affect gonadotroph cell number and morphology [71]. In addition, both gonadotrophs and somatotrophs express SSTRs. Hence, SOM may directly decrease both LH and GH release from the pituitary. The decrease in GH release leads to decreased IGF-I release, which may subsequently result in a decreased GnRH release from the ME [67, 68] (pathway C in Figure 1-*IV*).

Alternatively, elevated SOM levels may inhibit GHRH neurons in the ARC [114, 162, 163, 191], resulting in decreased GH release from the pituitary. As somatotroph and gonadotroph cell co-expression in the pituitary is maximal on the day of proestrus [58, 168, 169], a decreased activation of GH cells may lead to decreased activity of LH cells, resulting in a decreased LH release. In addition, decreased IGF-I levels, due to decreased plasma GH concentrations, may lead to both decreased GnRH release from the ME [67, 68] and decreased LH release from the pituitary [63] (pathway D in Figure 1-*IV*).

Physiological role of SOM in the regulation of reproduction

Although (in)direct effects of SOM at the pituitary level in the regulation of the LH surge (pathway 3) cannot be excluded, in the cycling female rat this pathway seems very unlikely to be the primary one with respect to hypothalamic regulation of the preovulatory LH surge. We suggest that the possible effects of SOM at the level of the pituitary may be additional to the direct effects at the level of the hypothalamus with respect to the interaction with the reproductive axis. Altered hypothalamic SOM concentrations causing changes at the pituitary level that in turn affect hypothalamic GnRH neurons, probably take place at a different time scale compared to the intrahypothalamic interactions and may therefore be involved in long-term, rather than short-term effects (i.e. in the regulation of the preovulatory LH surge) on reproductive function.

Also, the suggested role for NPY in the hypothalamic regulation of the LH surge in the female rat (pathway 2) is probably one of the factors in a complex regulatory mechanism. NPY may play a facilitating, rather than a crucial role in the normal regulation of the preovulatory LH surge. In view of our own data and data from the literature, we propose that the role of SOM in the regulation of the descending phase of the LH surge,

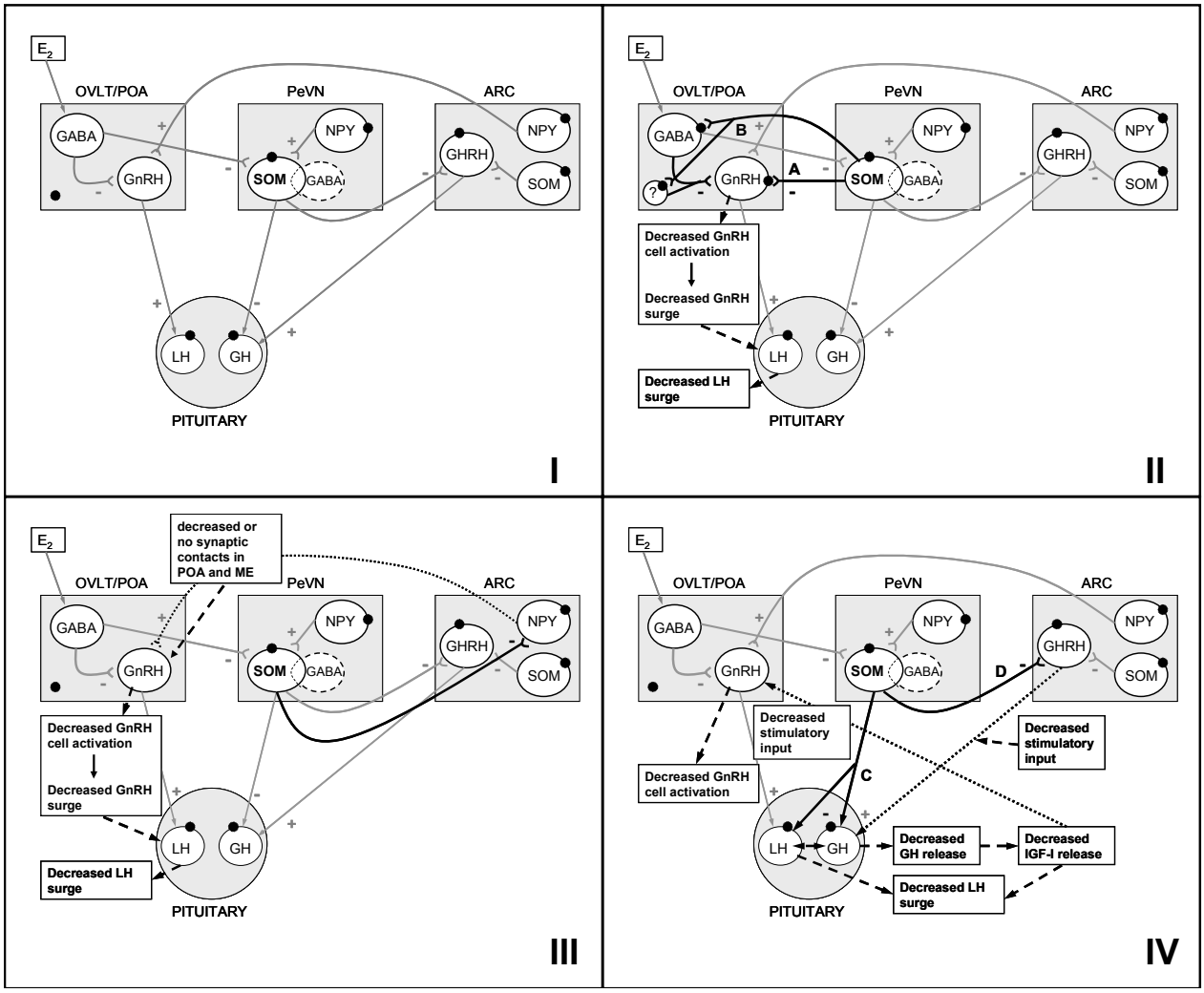

Figure 1. Schematic drawings of possible pathways via which SOM, originating from the PeVN, may decrease GnRH cell activation and the LH surge. **I**: Interactions between neurons in the OVLT/POA, PeVN and ARC as described in the literature. **II**: Direct effect of SOM on neurons in the OVLT/POA; **A**: directly on GnRH neurons; **B**: indirectly via cells projecting to GnRH neurons. **III**: Indirect effect of SOM on GnRH neurons via NPY cells in the ARC. **IV**: Indirect effect of SOM on GnRH neurons via the pituitary; **C**: direct effect of SOM on LH and GH cells; **D**: indirect effect of SOM on pituitary cells via GHRH neurons in the ARC. For more details: see text. ●: presence of SSTRs.

may involve, at least, a combination of pathways 1 and 2. In the cycling female rat, elevated plasma concentrations of E_2 and P on the day of proestrus may increase NPY levels in the ARC that, together with the removed inhibitory GABA-ergic tone [50, 181], stimulate GnRH cell firing, leading to the GnRH surge and, subsequently, the preovulatory LH surge. Secondly, the increased levels of gonadal steroids [33](*chapter 2, 3, and 4*), and in addition, elevated levels of NPY [117] may increase SOM release from the ME. Elevated concentrations of SOM, in turn, inhibit either neuron activity in the OVLT/POA, or

NPY and its stimulating effects on GnRH neurons, or both, leading to decreased GnRH cell activation and subsequently release, finally resulting in a decrease in plasma LH levels (see Figure 2).

In this model, however, the stimulating effects of elevated plasma E_2 and P levels on SOM release are assumed to be delayed compared to the effects of these gonadal steroids on GABA and NPY neurons. We found that E_2 reduces the sensitivity of SOM cells to GH feedback (*chapter 3*). The rising levels of E_2 on the day of proestrus may thus decrease SOM cell activity, leading to a decreased SOM release but, increased storage of SOM peptide. On proestrous afternoon, plasma E_2 concentrations already start to decrease, which may permit an increase in the release of the stored SOM peptide, resulting in the increased SOM release on proestrous afternoon.

Apart from steroid feedback, SOM synthesis and release show a diurnal rhythm, which is likely directed by the suprachiasmatic nucleus (SCN, the so called "biological clock"): Interestingly, the distribution of SOM cells within the PeVN as well as SOM fiber-immunoreactivity (including SOM fibers projecting to the ME) differs between morning (ZT 5) and afternoon (ZT 11) in young female Wistar rats (*chapter 6*). Also, SOM peptide concentrations in the anterior hypothalamus, SCN [193], in the ME [46], and SOM release from the hypothalamus [194] show circadian rhythmicity. Altogether, these data support the suggested increased SOM release during the afternoon.

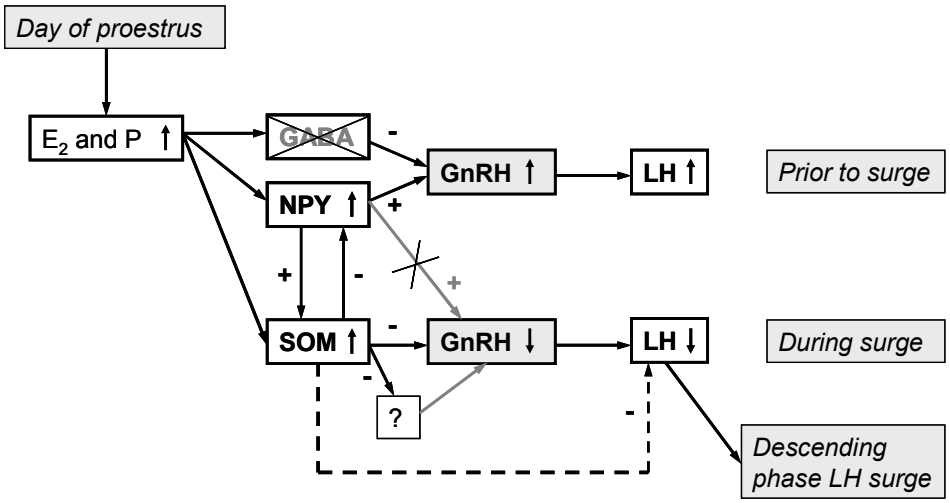

Figure 2. Proposed mechanism via which hypothalamic SOM may be involved in the regulation of the descending phase of the preovulatory LH surge in the adult female rat. See text for details.

7.4. CONSEQUENCES OF ALTERED GH STATUS ON FEMALE REPRODUCTION

Both GH-deficiency and excessive GH release are correlated with changes in hypothalamic SOM concentrations in rodents [65, 66]. Based on our proposed role of SOM in the regulation of LH release, the decreased proestrous and GnRH-induced LH surge in animals with an altered GH status [53, 54, 65] could very well be caused by changes at the level of the hypothalamic SOM system.

The gradual decline in plasma GH levels with age may be due to increased SOM release [17, 44]. Moreover, the decline in somatotropic activity is concurrent with (early) reproductive aging. Female rats that show an attenuated preovulatory LH surge already at the age of 9 months [50], also show changes in the specific SOM cell distribution within the PeVN (*chapter 6*). The above suggests that also during aging SOM may impinge on both axes and affect both GH release and reproductive function. During early reproductive aging, changes at the level of the hypothalamic SOM level may hence, at least in part, contribute to the decreased GnRH cell activation and LH surge [51, 52].

During aging, changes may also occur in the diurnal rhythm of SOM synthesis and release. Already at the age of 9 months, the observed diurnal change in SOM cell distribution in the PeVN of Wistar females disappears (*chapter 6*). Also, peak levels of SOM peptide content in the ME are shifted in time in 18-20 months old male rats [46], indicating that age-related changes in hypothalamic SOM content and release may be related to changes in the SCN. Age-related changes in the SCN may thus contribute to changes in SOM synthesis and release, resulting in the decline in GH release with aging.

Fertility problems are common in women with altered plasma GH levels, caused by e.g. acromegaly (characterized by excessive GH release, in most cases caused by GH-secreting adenomas) or Polycystic Ovarian Syndrome (PCOS, also associated with a disturbed GH status). To our knowledge, no data exist on possible changes in hypothalamic SOM concentrations in these women. However, our data presented in this thesis and data from the experimental literature indicate that in these women an altered hypothalamic SOM neuron activity may underlie, at least in part, the fertility problems that are correlated with an altered GH status.

The age at which fertility ends is characterized by large individual differences in both humans and rats. Our laboratory previously reported significant differences in both

the regulatory aspects of the reproductive axis as well as in the changes with age in reproductive function between two rat sub-strains. The fact that we found differences in SOM cell activity in these very same animals may further stress the importance of the SOM system to these individual and age-related changes in reproductive function. Consequently, also in women, individual variation in the activity of the GH axis, as a result of differences in the hypothalamic SOM system activity, may underlie individual characteristics of reproductive function in general.

In summary, structural changes in GH release, either caused by genetic changes, tumors, or age, result in, or are likely caused by, changes in the hypothalamic SOM system. We showed that SOM is able to decrease the LH surge in female rats and demonstrated that age-related changes in reproductive function occur concurrently with age-related changes in the hypothalamic SOM system, suggesting that decreased reproductive function in females with an altered GH status may be due to the direct inhibiting effects of a highly activated hypothalamic SOM system. **Hence, we conclude that hypothalamic SOM neurons may indeed be the "cross-link" between the somatotropic and gonadotropic axis.**

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Nederlandse samenvatting

***Groeihormoon** is, zoals de naam al zegt, nauw betrokken bij allerlei processen in het lichaam die de groei van een dier regelen. Opmerkelijk is echter dat dieren die extreem veel, of juist heel weinig tot geen groeihormoon produceren, kampen met ernstige voortplantingsproblemen. Dit wijst erop dat groeihormoon, naast het sturen van groei, ook bij de regulatie van andere systemen betrokken is, zoals de **voortplanting**. In dit proefschrift wordt beschreven hoe groeihormoon de voortplanting mogelijk kan beïnvloeden door interactie tussen de regulatoire circuits in de **hersenen** van de vrouwelijke rat.*

De hersenen

De hersenen bestaan uit een groot aantal gebieden waarin zenuwcellen, *neuronen,* liggen. Deze neuronen bestaan uit een *cellichaam*, waarin de celkern zich bevindt, en meerdere *uitlopers*. Via deze uitlopers kan een neuron in contact staan met bijvoorbeeld andere neuronen. Elk neuron is gespecialiseerd in het maken van één of meerdere zogenaamde transmitters of *neuro-hormonen*, chemische stoffen die een reactie teweeg kunnen brengen bij bijvoorbeeld andere neuronen. Via de uitlopers worden deze neuro-hormonen naar hun 'doel' getransporteerd.

De *hypothalamus*, een gebied aan de onderkant van de hersenen, is belangrijk voor de regulatie van veel lichaamsfuncties. Het gebied, dat zelf weer uit meerdere deelgebieden bestaat, staat in nauw contact met de *hypofyse*, een klein kliertje dat onderaan de hersenen 'hangt' waarin een aantal hormonen wordt gemaakt en/of opgeslagen. Deze hormonen kunnen in de bloedbaan worden afgegeven en zo de werking van andere organen en weefsels in het lichaam beïnvloeden. Neuro-hormonen uit de hypothalamus bereiken, via de uitlopers van de neuronen waarin ze gemaakt zijn, de hypofyse via speciale bloedvaten (de *portale vaten*) en kunnen de afgifte van hormonen uit de hypofyse aan het bloed remmen of stimuleren.

Groeihormoon

Groeihormoon (GH) is één van de hormonen die in de hypofyse gemaakt wordt. De afgifte van GH wordt voornamelijk gereguleerd door twee neuro-hormonen uit de hypothalamus: *somatostatine (SOM)* en *groeihormoon-releasing hormoon (GHRH)*. SOM, dat de afgifte van GH remt, wordt gemaakt door neuronen die in de *periventriculaire nucleus (PeVN)* liggen, één van de deelgebieden in de hypothalamus. GHRH stimuleert

de afgifte van GH en wordt gemaakt door neuronen in de *nucleus arcuatus (ARC)*, eveneens gelegen in de hypothalamus.

De afgifte van veel hormonen, zo ook GH, aan het bloed door de hypofyse is niet constant, maar *pulsgewijs*. Deze pulsgewijze afgifte van GH is het gevolg van de pulsgewijze afgifte van SOM en GHRH in de portale vaten. Deze regelmatige pulsen van SOM en GHRH zijn compleet uit fase met elkaar, dus tijdens een piekafgifte van SOM, is de afgifte van GHRH zeer laag, en andersom. Dit resulteert in een hoge afgifte van GH tijdens een GHRH puls terwijl de afgifte van GH vrijwel geheel onderdrukt wordt tijdens een SOM puls. Echter, het hier geschetste afgiftepatroon wordt zo gereguleerd in de mannelijke rat. In de vrouwelijke rat is veel minder onderzoek gedaan naar de hypothalame regulatie van GH, maar aangenomen wordt dat in de vrouwelijke rat de afgifte van SOM meer constant is, terwijl GHRH in onregelmatige pulsen afgegeven wordt. Hierdoor zou het typische 'vrouwelijke' GH afgifte patroon ontstaan, namelijk met GH pulsen die lager en onregelmatiger zijn dan in de mannelijke rat. Het feit dat het verschil in SOM afgifte het meest opvallend is tussen beide seksen, suggereert dat SOM zeer bepalend is voor de regulatie van het specifieke afgifte patroon van GH uit de hypofyse.

Het vrouwelijke voortplantingssysteem

Het voortplantingssysteem is een samenspel tussen de hypothalamus, de hypofyse en de geslachtsorganen (*gonaden*), in de vrouwelijke rat de *ovaria*. Bepaalde neuronen in de hypothalamus maken het *gonadotropin-releasing hormoon (GnRH)*, dat de hypofyse aanzet tot het maken en afgeven van het *luteïnizerend hormoon (LH)* en het *follikel-stimulerend hormoon (FSH)*. Deze 'gonadotropine' hormonen stimuleren de ovaria. In de ovaria beginnen er dagelijks één of meerdere *eicellen* met hun omringende cellen (de *follikel*) te groeien onder invloed van onder andere LH en FSH. Deze groeiende follikels gaan zelf ook hormonen produceren: *oestradiol (E_2)* en *progesteron (P)* (de *gonadale hormonen*), welke vervolgens zowel de hypothalamus als de hypofyse kunnen beïnvloeden via een *terugkoppelings ('feedback') mechanisme*. Op deze manier zijn E_2 en P in staat de afgifte van hormonen door de hypothalamus en hypofyse te reguleren, en indirect dus ook hun eigen afgifte. Lage concentraties van E_2 remmen op deze manier de afgifte van GnRH vanuit de hypothalamus en dus de afgifte van LH en FSH vanuit de hypofyse. Echter, op het moment dat de follikel volgroeid is, is door toename in het aantal hormoon producerende cellen de hoeveelheid E_2 en P in het bloed inmiddels zeer hoog,

waardoor de afgifte van GnRH, en als gevolg daarvan de afgifte van LH en FSH, juist wordt gestimuleerd. De grote hoeveelheid LH die nu in één keer wordt afgescheiden, wordt de 'LH piek' genoemd. Follikels die volgroeid zijn exact op het moment dat deze LH piek plaatsvindt, *ovuleren* (ook wel *'eisprong'* of *'ovulatie'* genoemd) vervolgens, waarna de vrijgekomen eicel bevrucht kan worden.

Groeihormoon en voortplanting

Het feit dat uit eerdere wetenschappelijke studies is gebleken dat zowel een overmaat als een tekort aan GH afgifte nadelige gevolgen heeft voor de voortplanting, geeft aan dat het groeihormoonsysteem en het voortplantingssysteem nauw verbonden zijn. Bekend is dat GH invloed heeft op bepaalde processen in de ovaria, zoals het stimuleren van de follikelgroei, en op de hypofyse cellen die LH en FSH maken. Er zijn echter aanwijzingen dat ook binnen de hypothalamus de twee systemen op elkaar inspelen. Dit wil zeggen dat het groeihormoonsysteem het voortplantingssysteem zou kunnen beïnvloeden op het niveau van de hypothalamus, dus via SOM en/of GHRH. Gezien de bepalende rol van SOM in de hypothalame regulatie van GH afgifte, zou het goed mogelijk kunnen zijn dat SOM ook de factor is die, vanuit de hypothalamus, zowel de GH afgifte als het voortplantingssysteem reguleert. Op deze manier zouden veranderingen in het GH systeem, via veranderingen in SOM afgifte, kunnen leiden tot veranderingen in het functioneren van het voortplantingssysteem. Het onderzoek zoals beschreven in dit proefschrift, richt zich op de vraag *of* en *hoe* SOM betrokken is in de regulatie van de voortplanting in de volwassen vrouwelijke rat.

Hypothalame regulatie van GH afgifte in de vrouwelijke rat

Om de mogelijke rol van SOM in de vrouwelijke voortplanting te kunnen onderzoeken, is het noodzakelijk om voldoende kennis te hebben van het hypothalame SOM systeem zelf. Aangezien dit tot nog toe voornamelijk in de mannelijke rat is onderzocht, is het eerste deel van dit proefschrift gewijd aan het 'in kaart brengen' van het hypothalame SOM systeem in de volwassen vrouwelijke rat.

In ***hoofdstuk 2*** wordt het PeVN gebied, met daarin de SOM-producerende neuronen, voor zowel de mannelijke als vrouwelijke rat gedetailleerd beschreven. De SOM neuronen blijken zeer specifiek gelokaliseerd te zijn binnen de PeVN.

SOM remt en GHRH stimuleert de afgifte van GH vanuit de hypofyse, maar ook GH kan via een feedback mechanisme zijn eigen afgifte regelen, door in de hypothalamus de betreffende neuronen te stimuleren dan wel af te remmen. In de mannelijke rat heeft GH een direct feedback effect op SOM neuronen, dit wil zeggen dat SOM neuronen gevoelig zijn voor GH zelf. Of en hoe SOM neuronen 'reageren' op GH zegt dus iets over de hypothalame regulatie van GH afgifte. Daarom hebben we in vrouwelijke ratten gekeken naar het effect van één enkele GH injectie op SOM neuronen in de PeVN (***hoofdstuk 3***). Een deel van de SOM neuronen bleek al zeer snel na de injectie 'geactiveerd' te zijn, wat wil zeggen dat het GH een reactie teweeg heeft gebracht in deze neuronen. Deze resultaten suggereren dat ook in de vrouwelijke rat GH een direct terugkoppelingseffect heeft op SOM neuronen in de PeVN. Opvallend was dat E_2 dit terugkoppelingseffect enigzins lijkt te remmen, wat aangeeft dat de gonadale hormonen mogelijk een modulerende rol hebben in de specifieke hypothalame regulatie van GH afgifte in de vrouwelijke rat.

Uit eerdere wetenschappelijke studies en uit onze resultaten is gebleken dat de gonadale hormonen een rol spelen in het hypothalame SOM systeem in de vrouwelijke rat. Echter, welke rol E_2 en P precies hebben, is tot op heden niet geheel duidelijk aangezien de resultaten elkaar in enkele gevallen tegenspreken. In ***hoofdstuk 4*** hebben we daarom de effecten van E_2, P, of beide op de SOM neuronen in de PeVN beschreven. Onze resultaten laten zien dat de gonadale hormonen E_2 en P alleen samen een effect hebben op de hoeveelheid SOM die aangemaakt en/of opgeslagen wordt door de neuronen.

Het effect van SOM op het voortplantingssysteem

Nu we meer inzicht hadden in het hypothalame SOM systeem in de vrouwelijke rat, konden we ons richten op de vraag of SOM uit de PeVN inderdaad direct effect kan hebben op het voortplantingssysteem in de vrouwelijke rat. In ***hoofdstuk 5*** beschrijven we het effect van SOM, dat in de hypothalamus geïnjecteerd wordt, op de GnRH neuronen in de hypothalamus en op de hoeveelheid LH die afgegeven wordt door de hypofyse. SOM blijkt zowel de activatie van GnRH neuronen als de afgifte van LH af te remmen. Deze resultaten wijzen er sterk op dat het groeihormoonsysteem inderdaad het voortplantingssysteem kan beïnvloeden vanuit de hypothalamus, oftewel via SOM afgifte.

Naast de rol die het groeihormoonsysteem speelt in het vrouwelijke voortplantingssysteem, is bekend dat ook tijdens *veroudering* deze twee systemen nauw

bij elkaar betrokken zijn. In ***hoofdstuk 6*** hebben we het hypothalame SOM systeem bestudeerd in vrouwelijke ratten van 9 maanden oud. Al op deze leeftijd vinden er namelijk veranderingen plaats in het voortplantingssysteem, zoals een verlaging van de LH piek. Wij vonden in deze dieren ook veranderingen in het hypothalame SOM systeem, wat suggereert dat ook tijdens veroudering de hypothalame regulatie van GH afgifte en van de voortplanting in de vrouwelijke rat nauw met elkaar verbonden zijn.

Conclusies

Het onderzoek dat beschreven is in dit proefschrift ondersteunt de veronderstelling dat het hypothalame SOM systeem verschilt tussen de mannelijke en vrouwelijke rat. Kort samengevat suggereren onze resultaten dat in het PeVN gebied van vrouwelijke ratten meer SOM-producerende neuronen liggen dan in mannelijke ratten. Opvallend is echter dat in mannelijke ratten juist meer SOM wordt aangemaakt en afgegeven; door minder neuronen dus. Deze resultaten ondersteunen de hypothese dat de verschillen in GH afgifte tussen mannelijke en vrouwelijke ratten veroorzaakt worden door verschillen in het hypothalame SOM systeem.

De in dit proefschrift beschreven resultaten leveren verder bewijs voor het feit dat het hypothalame SOM systeem het vrouwelijke voortplantingssysteem direct kan beïnvloeden. Veranderingen in het voortplantingssysteem die geassocieerd worden met veranderingen in GH afgifte patronen, zoals dwerggroei en gigantisme, zouden dus het directe gevolg kunnen zijn van veranderingen in SOM afgifte. Ook subtielere veranderingen in het GH afgifte patroon, en dus in het hypothalame SOM systeem, zouden zo het voortplantingssysteem kunnen beïnvloeden.

SOM neuronen in de PeVN kunnen dus inderdaad een centrale rol spelen in de wisselwerking tussen het groeihormoon- en voortplantingssysteem.

Ook vrouwen met (onverklaarde) vruchtbaarheidsproblemen hebben vaak een verstoord GH afgifte patroon. Terwijl de oplossing nu in eerste instantie vooral op ovarium niveau gezocht wordt, wijzen onze resultaten erop dat de oorzaak van deze vruchtbaarheidsproblemen mogelijk op hypothalamus niveau ligt. Een verschuiving van de aandacht naar de hersenen zou wellicht kunnen leiden tot een effectievere behandeling van vrouwen met dit soort vruchtbaarheidsproblemen.

Dankwoord

DANKWOORD

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Harmke

Wageningen, september 2004

Curriculum Vitae

CURRICULUM VITAE

Harmke Hulot van Vugt werd geboren op 6 maart 1976 te Utrecht en groeide op in Arnhem. Na het behalen van het VWO diploma in juni 1995 aan het Katholiek Gelders Lyceum te Arnhem, begon zij aan de studie Biologie aan de toenmalige Landbouwuniversiteit Wageningen in september van datzelfde jaar. Tijdens deze studie deed zij een afstudeervak neuro-endocrinologie bij de leerstoelgroep Fysiologie van Mens en Dier van de Landbouwuniversiteit en verrichtte zij een stage in de richting van de immunologie bij het departement Pediatrische Immunologie en Neonatologie in het Wilhelmina Kinderziekenhuis (Universitair Medisch Centrum) te Utrecht. In januari 2000 behaalde zij haar doctoraal examen.
Van februari 2000 tot juni 2004 was Harmke werkzaam als Assistent in Opleiding (AIO) aan de leerstoelgroep Fysiologie van Mens en Dier van de Wageningen Universiteit, waarvan de resultaten in dit proefschrift beschreven zijn.

PUBLICATIONS

Full papers

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TRAINING AND SUPERVISION PLAN, Graduate School WIAS

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WIAS Introduction Course, Wageningen (2003)

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International conferences, seminars, workshops, and presentations

WIAS seminar 'The Placenta', Wageningen (2000)

WIAS seminar 'Stress and Adaptation', Wageningen (2000)

WIAS Science day, Wageningen (2000, 2001, 2002, 2003)–poster presentation

Endo-Neuro Meeting (ENM), Doorwerth (2000, 2001)

Society For Neuroscience (SFN), San Diego, USA (2001)–poster presentation

Platform Voortplantingsonderzoek Landbouwhuisdieren (PLV), Utrecht (2001, 2003)–oral presentation

Seminar 'Add Life to Your Years', Utrecht (2002)

European Neuroendocrine Association (ENEA), Munich, Germany (2002)–poster presentation

Endo-Neuro-Psycho Meeting (ENP), Doorwerth (2002)

SFN, New Orleans, USA (2003)–oral presentation

Workshop on Steroid Hormones and Brain Function, Breckenridge, USA (2004)–oral presentation

In-Depth Studies (4.6 cp)

Disciplinary and interdisciplinary cources, and advanced statistic courses

Advanced Course in Neuroscience, Utrecht/Amsterdam (2000)

In Situ Hybridization, Breda (2001)

Design of Animal Experiments, Wageningen (2002)

Professional Skills Support Courses (3.5 cp)

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Course Supervising MSc thesis work, Wageningen (2000)

Laboratory Use of Isotopes, Velp (2001)

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Supervising practicals and excursions, supervising MSc theses

Practical Course Endocrinology (2000, 2001, 2002, 2003, 2004)

Supervision of 5 MSc students (2000-2003)

This PhD project was part of the research program of Graduate School WIAS (Wageningen Institute of Animal Sciences) of Wageningen University

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Back: photo of a female Wistar rat by E. Rijntjes

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