

Somatostatin in the Periventricular Nucleus of the Female Rat: Age Specific Effects of Estrogen and Onset of Reproductive Aging

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1. Introduction

The functioning of the growth hormone (GH) and reproductive axis is known to be closely related: both GH overexpression and GH-deficiency are associated with dramatic decreases in fertility (Bartke, 1999; Bartke et al, 1999; 2002; Naar et al, 1991). Also, aging results in significant changes in functionality of both axes within a similar time frame.

In the rat, GH secretion patterns are clearly sexually dimorphic (Clark et al, 1987; Eden et al, 1979; Gattford et al, 1998). This has been suggested to result mainly from differences in somatostatin (SOM) release patterns from the median eminence (ME) (Gillies, 1997; Muller et al, 1999; Tannenbaum et al, 1990). SOM is synthesized in the periventricular nucleus of the hypothalamus (PeVN) and controls in concert with GH-releasing hormone (GHRH) the GH release from the pituitary (Gillies, 1987; Tannenbaum et al, 1990; Terry and Martin, 1981; Zeitler et al, 1991). An altered GH status is reflected in changes in the hypothalamic SOM system. For instance, the number of SOM cells (Sasaki et al, 1997) and pre-pro SOM mRNA levels (Hurley and Phelps, 1992) in the PeVN were elevated in animals overexpressing GH.

Several observations suggest that SOM may also affect reproductive function directly at the level of the hypothalamus. SOM synthesis in the hypothalamus and its release from the ME fluctuate over the estrous cycle. (Estupina et al, 1996, Zorrilla et al, , 1991). Central injections with SOM or a SOM analog (octreotide) decreased the number of gonadotropic cells in the pituitary (Lovren et al, 1998; Nestorovic et al 2002; 2003). Also, we previously showed that a single central injection with octreotide significantly attenuated the E₂-induced Luteinizing Hormone (LH) surge and significantly decreased the activation of Gonadotropin Releasing Hormone (GnRH) cells in the hypothalamus of female rats (van Vugt et al, 2004).

Age-related changes in fertility and fecundity are associated with selective changes at the level of the ovary and uterus (Meredith and Butcher, 1985; Nass et al, 1984; te Velde et al, 1998; Wise, 1982), pituitary gland (Brito et al, 1994; DePaolo et al, 1986; Krieg et al, 1995; Nass et al, 1984; Wise, 1982), and hypothalamus (Rubin et al, 1994; Wise, 1982; Wise et al, 2002). Reproductive aging is characterized by changes in the length of the reproductive

cycle. In female rats, for instance, the normal 4 or 5 day estrous cycle will lengthen with age and become irregular. This is followed by a period of repetitive pseudopregnancies and/or persistent estrus, while cyclicity ends with a state of persistent diestrus (Vom Saal, 1994). Although the general sequence of events during aging is predictable, the age at which the decline in fertility becomes evident varies considerably between individuals and rat strains (Vom Saal et al, 1994; Te Velde et al, 1998). Hence, especially during the initial stage when cyclicity is still regular, the relative contribution of the ovaries, pituitary and hypothalamus to reproductive aging is unclear.

One of the first, common changes appears to be an attenuation of the proestrous, ovulation-inducing luteinizing hormone (LH) surge (Wise et al, 2002), which can be demonstrated even before estrous cycles become irregular (DePaolo et al, 1986). The latter is strongly associated with the age at which rats become acyclic (Nass et al, 1984). Previous research suggested that changes in ovarian hormone release (DePaolo et al, 1986; Lu et al, 1985), pituitary hormone storage and/or responsiveness to ovarian or hypothalamic signaling (Brito et al, 1994; Keizer et al, 2001; Matt et al, 1998), or changes in hypothalamic signaling (Rubin et al, 2000; Downs and Wise, 2009; Wise et al, 2002) may underlie the age-related attenuation of the pituitary LH surge.

Evidence suggests that exposure to chronically elevated levels of circulating E_2 during life advances the decline in fertility with age (Lu et al, 1981; Rodrigues et al, 1993). 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 cell function (Baldino et al, 1988; Estupina et al, 1996; Knuth et al, 1983; Murray et al, 1999; Werner et al, 1988; Zorilla et al, 1991). Recent studies demonstrated a clear sex difference in the number and distribution of SOM peptide containing cells in the PEVN. In the female numbers were affected by ovariectomy and gonadal steroid treatment (Van Vugt et al, 2008).

During the early phase of reproductive aging, normal (or even elevated) levels of plasma estradiol (E_2), are correlated with a decline in somatotrophic axis activity (Chandrashekar and Bartke, 1993; Vom Saal et al, 1994; Wilshire et al, 1995). In 14 months old rats, hypothalamic SOM peptide content as well as basal and KCl-stimulated SOM release from the hypothalamus were increased compared to young animals (Ge et al, 1989). Compared to young female rats, SOM peptide levels in the ME are decreased at 25-29 months of age (Takahashi et al, 1987), suggesting increased SOM release from the ME with age. Altogether, these data point to the hypothalamic SOM system as a potential candidate to mediate some of the concurrent changes in the activity of the reproductive and GH axis with age.

In the light of the data described above, we set out to study the effects of E_2 exposure on hypothalamic SOM peptide levels at middle age when an attenuation of the LH surge can be found in regularly cycling females. To this end, we measured LH and P release in regularly 4-day cycling females at young (4 months) and middle-age (8.5 months) on proestrus as well as after a stimulus with a potent GnRH analog the following proestrus day. Subsequently, animals were ovariectomized to examine the effect of a physiological dose of E_2 on SOM-peptide containing cells in the PeVN at selected time points following estrogen exposure. Using this approach, we aimed to gain more insight in the mechanisms underlying the interaction between the somatotrophic and gonadotropic axis, i.e. a possible role for the hypothalamic SOM system. We hypothesize that SOM plays a role in the normal, physiological regulation of LH release in the female rat and suggest that changes in the response of PeVN SOM-ir with age may contribute to the hypothalamic changes that lead to an attenuated LH surge in middle-aged rats.

2. Material & methods

2.1 Animals

Virgin female (n=60) and male (n=8) Wistar rats (HsdCpb:WU, Wistar Unilever) were obtained from Harlan (Horst, NL) at 9 to 10 weeks of age. Rats were group housed (4/cage) under regular light-dark cycles (L/D 12:12, lights on at 3:00 h defined as 'zeitgeber time' 0, ZT0) with free access to standard food pellets (Hope Farms B.V., Woerden, NL) and water. Animals were housed individually from 1 week before cannulation onwards. Young and Middle-aged females were obtained from the same batch to reduce variation between animals. All experiments were approved by the animal experimental committee (DEC) of the Wageningen University.

2.2 Experimental design

To study changes in proestrous LH and P surge characteristics with age, 4-month-old ('young') and 8.5-month-old ('middle-aged') female rats with regular 4-day cycles were used for blood sampling and hormone analyses. Hourly blood samples were taken on proestrus to measure plasma LH and P profiles. To investigate pituitary LH and FSH responsiveness, a potent GnRH analog (Ovalyse[®]; des-Gly¹⁰-GnRH-ethylamide, Upjohn, Ede, The Netherlands) was used on the following proestrus. Ovalyse[®] (100 ng in 0.25 ml 0.9% NaCl (w/v) containing 1% BSA) was administered i.v. immediately after the first blood sample was drawn.

Subsequently, a group of cycling females was ovariectomized (OVX, Van der Beek et al, 1999) at 4.5 (young) or 9 mo (middle-aged) of age, and given a single s.c. injection with estrogen at ZT3 on day 13 following OVX. Animals were perfused 2, 8, 26 or 32 hrs later, i.e. at ZT5 and ZT 11. Brains sections were stained for SOM peptide as described previously (Van Vugt et al, 2008).

2.3 Estrous cycle length

Estrous cycles were monitored by daily vaginal lavage. Lavages were analyzed according to criteria described elsewhere (Freeman et al, 1994). In addition, receptive behavior (hopping and darting, ear wiggling and lordosis posture) was monitored daily. To this end, a naive male WU rat was introduced briefly in the female's home cage around ZT 11, 1 hr before dark onset to confirm a proestrous lavage typing.

Cycle length was defined by the last two monitored cycles before sampling. Most females displayed regular 4-day estrous cycles (70%). Cycle lengthening was observed in 8.5 mo old females: regular 4-day cycles decreased from 70% to 45%, while both 5-day cycles (from 10% to 24%) and acyclicity (from 0% to 10%) increased.

2.4 Cannulation and blood sampling

The right jugular vein of female rats was cannulated to obtain stress-free blood samples (Steffens, 1969, Van der Beek et al 1999, Van Vugt et al, 2004). After a recovery period of at least five days, ten hourly blood samples of 170 µl were taken on proestrus from ZT 5.5-14.5 for measurement of endogenous, preovulatory hormone profiles. To assess the amount of acutely releasable LH by the pituitary gland, ten hourly blood samples of 170 µl were drawn from ZT 5.5-14.5 on the following proestrus, following an i.v. injection of the GnRH analog Ovalyse[®], just after the first sample. Blood samples were collected in heparinized, air-dried

vials (25 IU heparin, Leo Pharma BV, Breda, NL) and centrifuged at 13,000 rpm for 5 minutes. Plasma was diluted 1:4 for LH and 1:20 for P analysis with PBS buffer (0.02M, pH 7.5) containing 0.1% BSA, and stored frozen at -20°C until RIA. LH and P plasma levels were determined by validated RIAs (Van der Beek et al, 1999, Van der Meulen et al, 1988). Only samples from animals that displayed regular 4-day estrous cycles were included in the analysis. The inter- and intra-assay coefficients of variation were determined using pooled rat serum, and amounted to respectively 12.1% and 10.8 % for the LH assay and 15.8% and 6.2% for P analysis.

2.5 Tissue processing, SOM immunocytochemistry & analysis

Forty-three regularly cycling female rats aged 4.5 (n=20) or 9 (n=23) months were ovariectomized and treated with estradiol benzoate before perfusion 2, 8, 26 or 32 hours later. The brains were processed for SOM immunocytochemistry as described in detail previously (Van der Beek et al, 1991; Van Vugt et al, 2008). Staining was performed in two separate runs (4.5 and 9 mo) and intra-assay variation was controlled for by including a group of young animals at 2 and 32 h after E_2 treatment in the second run. Every third brain section containing the PeVN was stained for SOM peptide by free-floating immunocytochemistry techniques. For staining, primary polyclonal rabbit antibody raised against SOM peptide (Somaar 080289, NIN, Amsterdam, NL) (Buijs et al, 1989) was used followed by detection with biotinylated goat anti-rabbit IgG and Avidin-Biotin Complex-elite (ABC; Vector Laboratories).

SOM-immunoreactive (-ir) neurons in the PeVN of the left side of the brain were counted using computer assisted analysis as described previously (Van Vugt et al, 2008). In addition to counting SOM-ir cells, also the amount/quantity of SOM-ir fibers (expressed in μm^2) was measured in these images in the young females only. To this end, both the fibers that were located closely to the SOM cells (the "PeVN region": measured in an area that had an absolute distance from the 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 that had an absolute distance from the ventricle of approximately 560 μm) were counted. The analysis threshold was determined in a representative selection of the images by measuring the mean gray level in an area devoid of SOM staining. Next, an upper and a lower threshold were determined (mean gray level + 3x S.D.; mean maximal gray level - 3x S.D. respectively) excluding SOM-ir cells and very light SOM-ir fibers.

2.6 Data processing

To determine the effects of age on the proestrus LH and P surge several profile characteristics were defined: i.e. basal levels, onset time, peak time, peak height and the total amount of LH and P released. Basal levels were defined as the average concentration of the first three blood samples (ZT 5.5, 6.5, and 7.5) per animal. In case of an early rise in LH levels, i.e. at ZT 7.5 (n=3), the first two blood samples were used to calculate basal levels. Onset time was defined as the sample hour (ZT; mean \pm SEM expressed as h:min \pm min) at which LH levels exceeded basal LH levels plus 3 \times the standard deviation, while LH levels continued to rise thereafter. Peak time of the LH surge was defined as the ZT hour at which the highest LH concentration was measured. The highest amount of LH measured at that time was defined as the peak height. The total amount of LH or P was defined by the cumulative value of hormone levels during the complete sampling period. LH levels

showed a clear distinction between 'early', Ovalyse® induced LH release and a second 'late' increase in LH levels resulting from endogenous proestrous GnRH release. Therefore, LH data after Ovalyse® administration were divided in ZT 5.5-8.5 ('early') and ZT 9.5-14.5 ('late'). For 'late' release, LH peak time and LH peak height were determined as described above. Finally, total LH levels during the complete sampling period were calculated. As proestrous P release continuously increased but did not peak in the time window evaluated, only basal level, preovulatory surge level, and total amount released were assessed.

2.7 Statistics

Hormone levels were expressed as mean \pm SEM and analyzed using SPSS (version 12.0). Differences were considered to be significant when $P < 0.05$. Basal LH levels, onset time and peak time of the LH surge, LH peak height, and basal P levels from proestrus measurements, as well as LH peak height and total LH levels following Ovalyse® were tested with the nonparametric Kruskal-Wallis test and were post-hoc tested using the Mann-Whitney test. Changes with age in the total amount of LH and P released during the proestrous surge, preovulatory P surge levels as well as LH peak height of the induced LH surge and total LH levels following Ovalyse®) were tested by one-way ANOVA. To compare the total number of SOM-ir cells between the different time points following E₂ treatment, one-way ANOVAs were used. A Bonferroni or Tukey HSD test was used as post hoc test.

3. Results

3.1 Proestrous LH profiles

The general profile of the LH surge was comparable between ages. Basal plasma LH levels averaged 0.3 to 0.4 ng/ml and onset of the surge occurred around ZT9.5, where after LH levels increased rapidly and reached peak levels around ZT12. Subsequently, LH levels gradually declined. We found a significant decrease in LH peak and total LH levels with age ($P = 0.041$ and $P = 0.035$, respectively) (Table 1). Pearson correlation tests showed that the magnitude of the LH surge (i.e. total LH levels) correlated with onset and height of the LH surge (LH onset time: $r = -0.527$ with $P < 0.001$; LH peak levels: $r = 0.924$ with $P < 0.001$).

age (mo)	n	LH surge					P surge		
		basal	onset time	peak time	peak height	total	basal	surge	total
4	12	0.3 \pm 0.1	9:43 \pm 0:30	12:06 \pm 0:20	12.8 \pm 1.4	40.2 \pm 4.5	24.9 \pm 2.6	339.4 \pm 28.8	414.2 \pm 35.9
8.5	9	0.4 \pm 0.1	9:37 \pm 0:23	11:37 \pm 0:19	8.3 \pm 1.4*	25.2 \pm 4.6*	23.2 \pm 5.0	329.0 \pm 64.6	398.6 \pm 78.7

Table 1. Proestrous LH and P surge characteristics of young and middle-aged 4-day cyclic WU rats. Basal levels are depicted as the average concentration ZT 5.5-7.5, onset time of the LH surge (ZT at which LH levels exceeded basal levels plus 3xSD), peak time of the LH surge (ZT at which the highest concentration was measured), peak height of the LH surge (highest concentration measured), preovulatory P surge levels (cumulative value from ZT 8.5-14.5), and the total amount of LH or P released during the surge (cumulative value during the complete sampling period). All data are expressed as group means \pm SEM in ng/ml (concentrations) or h:min (time). Significant differences ($P < 0.05$) between young and middle aged with ages are indicated with an asterisk.

3.2 Proestrous P levels

Plasma P levels increased gradually during proestrus, but did not show a distinct peak during the time window evaluated. Basal P levels defined by LH release characteristics showed no significant differences between groups and age did not affect P profiles (Table 1). Total P levels correlated significantly with basal P levels ($r=0.876$ with $P < 0.001$) and with P surge levels ($r=0.992$ with $P < 0.001$), in line with the observed elevation in P levels during the entire sampling period.

3.3 Pituitary responsiveness

Administration of Ovalyse® at ZT 5.5 resulted in a rapid and consistent increase in LH plasma levels irrespective of age (Table 2). Highest plasma LH levels were measured at 1 or 2 h after Ovalyse® administration and decreased thereafter (defined as the 'early', induced LH surge). After ZT 8.5, LH levels increased again (defined as the 'late', endogenous LH surge).

LH levels were of comparable magnitude at 1 and 2 h after Ovalyse® injection between 4 and 8.5 months old females. Also, the second, endogenous LH surge was comparable in magnitude (peak height and total LH levels) between groups and accompanied by a gradual increase in P levels comparable between ages.

age (mo)	n	LH following Ovalyse®					P following Ovalyse®		
		<ZT9		>ZT9		all samples	<ZT9	>ZT9	all samples
		peak height	total	peak height	total	total	total	total	total
4	12	30.0 ± 1.6	66.9 ± 2.5	14.8 ± 1.7	48.9 ± 5.4	115.9 ± 6.0	172.9 ± 12.2	365.5 ± 30.3	538.3 ± 42.0
8.5	10	31.0 ± 3.1	63.4 ± 7.2	16.3 ± 2.2	55.0 ± 7.7	118.4 ± 12.6	157.8 ± 17.9	338.2 ± 45.6	496.0 ± 61.9

Table 2. LH and P surge characteristics following Ovalyse® administration in young and middle-aged 4-day cyclic rats on proestrus. The surge was divided into a 'early' part (ZT<9; 'induced' surge) and a 'late' surge (ZT>9; 'endogenous' surge). Measured characteristics: peak height of the 'early' and 'late' LH surge (the highest concentration measured), and the total amount of LH or P released during the 'early' and 'late' and the entire sampling period (cumulative LH or P levels during the corresponding sampling periods). All data are expressed as group means ± SEM in ng/ml (concentrations).

3.4 SOM-ir cells and fibers in the PeVN

Total numbers of SOM-ir cells were roughly comparable between age groups (Figure 1). In the young animals, SOM-ir numbers were not significantly affected by time after E₂ treatment, although they appeared to be consistently lower at ZT5 compared to ZT11 (Figure 1A). In middle-aged rats, total numbers of SOM-ir cells were significantly lower at ZT 5 on day 1 compared to day 2 (Figure 1B).

SOM-ir cells within the PeVN showed a clear rostro-caudal distribution pattern, with maximal numbers of cells appearing in the more caudal part of the PeVN. The distribution in young females varied slightly over the different time points after E₂ treatment: maximal numbers of SOM-ir cells were found consistently in PeVN section 8 at ZT5, but in PeVN section 7 at ZT11 on both days (Figure 2A and B). In middle aged female, the rostro-caudal distribution pattern in the number of SOM-ir cells at ZT 5 on day 1 (Figure 2C) was absent, e.g. the number of SOM-ir cells was comparable between PeVN sections. Distribution

patterns at other time points were in general comparable with those found in the young rats, i.e. maximal numbers of SOM-ir cells in PeVN section 7 at ZT 11 and in PeVN section 8 at ZT 5 on day 2 (Figure 2D).

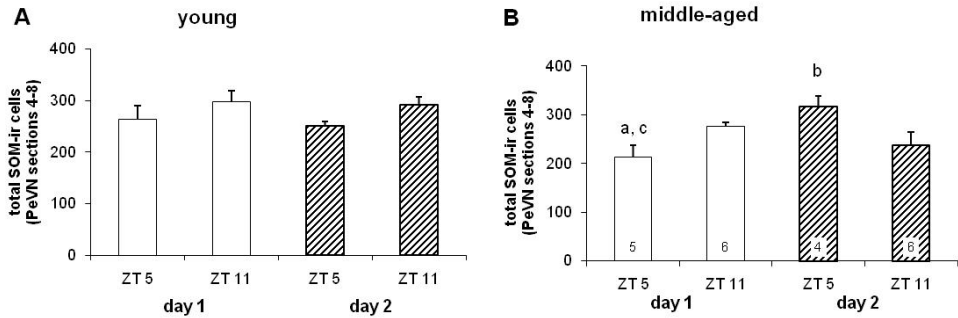


Fig. 1. Total number of SOM-ir cells (sum of PeVN sections 4-8) in young (4.5 months old) and middle aged (9 mo old) Wistar rats at different time points after E₂ treatment. ZT 5: 2 (day 1) or 26 (day 2) h after E₂ treatment, ZT 11: 8 (day 1) or 32 (day 2) h after E₂ treatment. n=5 for each young age group, numbers within base of bars indicate the number of animals.

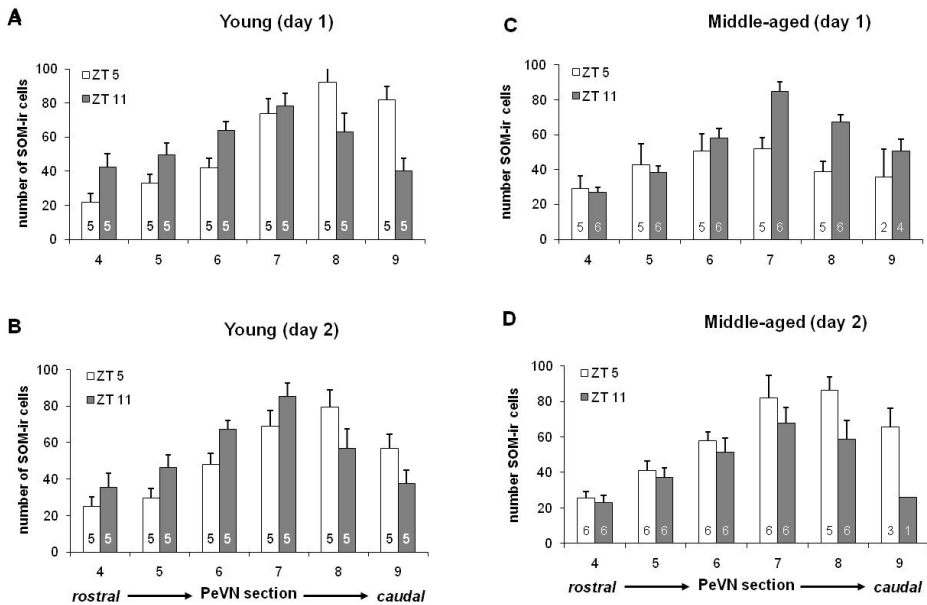


Fig. 2. Rostral to caudal distribution of SOM-ir cells in the PeVN of young (4.5 months old) (A and B) and middle aged (9 months old) OVX females at different time points after E₂ treatment. Numbers within base of bars indicate the number of animals.

In young animals, the area occupied by SOM-ir fibers in the PeVN region was significantly different at ZT5 on day 1 compared to ZT11 day 2 (Figure 3A). The total area of SOM-ir fibers, i.e. including the fibers projecting to the ME, was significantly different between ZT5 and ZT11 on both days (Figure 3B).

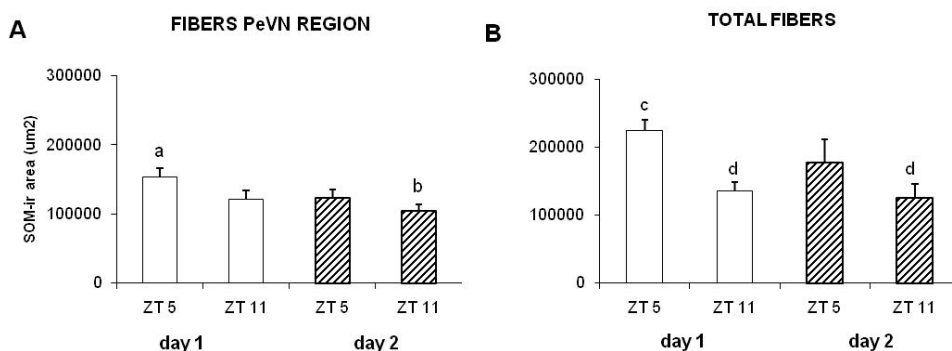


Fig. 3. Area of SOM-ir fibers in the PeVN region (A) or total SOM-ir area (B) in young (4.5 months old) OVX female Wistar rats at different time points after E₂ treatment. a significantly different from b ($p=0.047$; Bonferroni); c significantly different from d ($p \leq 0.05$; Tukey HSD). $n=5$ for each group.

4. Discussion

In the present study we showed that in adult female rats, the effects of E₂ on SOM-ir cell distribution and SOM-ir numbers in the PeVN were age dependent. Estrogen did not affect total numbers of SOM-ir cells in the PeVN of young female rats in line with our previous studies (Van Vugt et al, 2008) and those of others (Estupina et al, 1996). Other studies reported a decrease in SOM mRNA content in the PeVN following OVX which was reversed by E₂ treatment (Baldino et al, 1988; Zorilla et al, 1991). In these studies, however, animals were treated with E₂ for a prolonged period of time, whereas we studied the effect of a single physiological dose of E₂ on SOM peptide-containing cells on multiple time points following the estrogen exposure. Interestingly, the amount of SOM-ir fibers within the PeVN region of young females was decreased at 32 h compared to 2 h after E₂, which may suggest increased release of SOM peptide from the PeVN or decreased transport from cells to the fibers, apparently without affecting the amount of SOM peptide synthesized and/or stored in the PeVN cells. Prior to measuring SOM peptide responsiveness to an estrogen stimulus we showed that the attenuation of the LH surge at middle-age was not accompanied by a decrease in proestrus P levels or a decrease in pituitary LH responsiveness to a GnRH analog. These results clearly suggest that the attenuation of the LH surge is not initiated by alterations at the level of the ovary or pituitary gland, but rather the result of changes in response to ovarian feedback at the hypothalamic level as found for SOM peptide in this study. Subsequent experiments in the brain material obtained from these animals are now focusing on studying potential changes in hypothalamic estrogen and progesterone receptor immunoreactivity.

4.1 Reproductive aging and the pituitary gland

The attenuation of the natural LH surge at 8.5-months old is in accordance with previous reports concerning other rat strains (Brito et al, 1984; DePaolo et al, 1986; Krieg et al, 1995; Nass et al, 1984). Some studies suggested that the decrease in proestrus LH levels with age may follow changes at the level of the pituitary gland, such as changes in LH storage and/or release capacity (Matt et al, 1998; Wise et al, 1984). The results of the present study, however, suggest that this is not the case. Although the timing of GnRH analog administration was early (i.e. 3 to 4 hours before the natural LH surge occurred), no age-related differences in total and peak LH levels of the 'induced' LH surge (until ZT9) were observed. This implies that LH responsiveness to a bolus of GnRH is comparable between 4- and 8.5-month-old rats. Others did show that the acutely releasable pool of LH was reduced at the age of 9-12 months in cyclic Sprague-Dawley rats (Brann and Mahesh, 2005; Wise et al, 1984). In addition, pituitary responsiveness to GnRH *in vitro* is decreased in 10-12 month-old Long-Evans rats that show attenuated LH surges (Brito et al, 1994), and in pituitaries from 9- compared to 4-month-old Wistar rats that were tested in a superfusion system in our lab (Keizer et al, 2001). Since the age-related reduction in LH release after GnRH stimulation was more evident during the second and third stimulus in all studies, this suggests that the GnRH priming mechanism may be particularly affected.

Yet, we found no age-related differences in total LH levels of the 'late', 'endogenous' LH surge that results from endogenous GnRH release. Since the LH surge requires repeated pulses of GnRH to induce full pituitary priming, the absence of these age-related changes in this study suggest that GnRH priming is not significantly affected in our 8.5-month-old rats. The time between GnRH stimuli, however, differs between endogenous GnRH release (~1 hour between pulses) and our stimulus with the long-acting GnRH-analog Ovalyse® (~3 hours). Altogether, these results indicate that in our 8.5-month-old females, the attenuation of the LH surge is not caused by a diminished responsiveness of LH to initial GnRH signaling, although reproductive aging may eventually result in a decrease in the releasable pool of LH (Wise et al, 1984) and impaired GnRH priming (Brito et al, 1994; Keizer et al, 2001).

4.2 Reproductive aging and the ovary

In the present study we showed that proestrous P levels were comparable between 4- and 8.5-month-old rats, and thus do not underlie the observed attenuation of the LH surge. In contrast, another study (Miller and Riegler, 1980) showed that the attenuated preovulatory LH surge was accompanied by an attenuated P surge in 12-month-old cyclic Long-Evans rats. It has been suggested that attenuated P levels result from a decrease in proestrous LH levels, although increased responsiveness of the ovary to hCG stimulation in regular cyclic middle-aged compared has been reported for Long-Evans rats (Chern et al, 2000). Consequently, the lack of concurrent changes in P and LH release in our rats could be explained by an increased responsiveness to LH stimulation.

4.3 Reproductive aging and the hypothalamus

Based on these data, we hypothesize that the initial attenuation of the LH surge is indeed initiated by alterations at the hypothalamic level (i.e. GnRH release), and not at the pituitary gland (i.e. responsiveness to GnRH, GnRH priming) or the ovary (P levels).

A previous study by Rubin (Rubin, 1992) showed that the secretory capacity of the GnRH system is still intact in middle-aged rats, but that the LH secretion per GnRH burst during

the LH surge appears to decrease with age (Matt et al, 1998). This is thought to be the result of a decreased activity of the GnRH system, a reduced responsiveness to GnRH signaling, and/or a reduction in cellular LH with age (Matt et al, 1998; Rubin et al, 2000). Also, the GnRH neuroterminal-glial-capillary unit in the ME may be affected, influencing the regulation of GnRH release (Yin et al, 2009). There are no indications that the number of pituitary GnRH receptors is affected with age in female mice (Belisle et al, 1990) and our results after Ovalyse® administration suggest that the responsiveness to (robust) GnRH signaling is still intact. Indeed, several studies demonstrated that on proestrus the number of activated GnRH neurons (Wise et al, 2002; Rubin et al, 1994) and endogenous GnRH release (Rubin et al, 2000) are reduced in middle-aged female rats. The activity of GnRH neurons is regulated by many different neural signals (Smith and Jennes, 2001) and several of the systems involved in the regulation of the GnRH surge are also affected with age (Wise et al, 2002; Sahu et al, 1998; Gore et al, 2002; Mills et al, 2002). Taken together, this suggests that the input onto GnRH neurons may change with age, resulting in less activated GnRH neurons and reduced GnRH release that together with a reduction in endogenous GnRH priming could indeed lead to an attenuated LH surge. Previous studies have proposed a contribution of the suprachiasmatic nucleus (SCN) in the attenuation of the LH release surge (Wise et al, 2002; Downs and Wise 2009). Yet, we did not find any significant changes in timing of the LH surge (i.e. LH surge onset and/or peak levels) in middle-aged rats. Since a clear delay in timing of the LH surge at the age of 7-10 months has only been shown in Sprague-Dawley rats (Sahu et al, 1998; Wise, 1982), the age at which changes in SCN output influence the LH surge mechanism may be strain specific.

4.4 Effect of SOM on the reproductive axis

Previous studies provide evidence for a proposed central role of hypothalamic SOM neurons in the functional interaction between the somatotropic and gonadotropic axis. Octreotide, given during the "critical period" of the day (i.e. just prior to surge onset), completely abolished the E₂-induced LH surge and decreased GnRH cell activation (Van Vugt et al, 2004). Based on this, and the fact that SOM release may increase on proestrous afternoon (Estupina et al, 1983; Knuth et al, 1983; Zorilla et al, 1991), we hypothesize that in the cycling female rat, SOM release probably increases only after the "critical period", i.e. during the LH surge. Thus, we suggest that elevated levels of SOM on proestrous afternoon may be involved in the descending, rather than the ascending, phase of the preovulatory LH surge.

Our previous studies strongly suggest that SOM decreases LH release at least in part by decreasing hypothalamic GnRH neuron activation (Van Vugt et al 1994). 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 (Lovren et al. 1998). Here we propose three possible pathways via which SOM, originating from the PeVN, may affect GnRH neurons, resulting in a decreased LH release (see Figure 4).

The interactions between neurons in the hypothalamic areas involved in the regulation of LH (Preoptic Region, OVLT/POA) and GH (PeVN and Arcuate nucleus ARC) release are schematically depicted in Figure 4-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 E₂ on the LH surge (Miller et al, 2003; Zhen et al, 1997). These GABA-ergic cells originating from the OVLT/POA innervate SOM neurons in the PeVN and may

therefore also be involved in the regulation of GH release from the pituitary (Herbison et al, 1994; Murray et al, 1999; Rage et al, 1993; Willoughby et al, 1987). Also, within the PeVN, a small number of SOM neurons co-express GABA (Tanaka et al, 1997). GHRH neurons in the ARC are inhibited by SOM neurons originating from either the PeVN or the ARC (McCarty et al, 1992; Lanneau et al, 2000; Tannenbaum et al, 1990; Willoughby et al, 1989). Neuropeptide-Y (NPY) terminals originating from the ARC project to the preoptic region and ME, in which some of the axons make synaptic contacts with GnRH cell bodies and processes (Smith and Jennes, 2003). Also, NPY cells may project to SOM cells within the PeVN. NPY may hence be involved in the regulation of both LH and GH release from the pituitary.

4.4.1 Pathway 1: SOM projections to neurons in the OVL/POA

We showed that a centrally injected SOM analog decreased hypothalamic GnRH cell activation (Van Vugt et al, 2004), suggesting that SOM directly affects cells in the OVL/POA. The fact that SSTRs were demonstrated in the OVL/POA (Helboe et al, 1998; Schindler et al, 1996), and that lesions of the anterior hypothalamic area (including the PeVN) resulted in decreased SOM peptide levels in the POA (Epelbaum et al, 1977), suggests that SOM cells originating from the PeVN project to the OVL/POA. Possibly, GnRH neurons themselves express SSTRs, so 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 4-II). Alternatively, cells, other than GnRH-producing, in the OVL/POA may contain SSTRs. Neurons in the periventricular POA that project to GnRH neurons at the time of the preovulatory LH surge (Le et al, 1997; 1999; 2001) are a likely candidates. Although not identified yet, GABA-ergic cells may be (one of) these neurons containing SSTRs and projecting to the GnRH neurons (pathway B in Figure 4-II).

4.4.2 Pathway 2: SOM effects on LH release indirectly via NPY

NPY is very likely to influence the preovulatory LH surge: NPY synthesis and release are elevated just before the proestrous LH surge, and immunoneutralization of NPY prevents 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 (Smith and Jennes, 2003). As SSTRs were demonstrated on NPY cells in both the PeVN and ARC (Lanneau et al, 2000), SOM may inhibit NPY neurons 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 4-III).

4.4.3 Pathway 3: SOM effects pituitary LH release indirectly

Besides the decreased LH surge, we also found decreased plasma GH concentrations following the centrally injected SOM analog (Van Vugt et al, 2004). SOM was shown to directly decrease LH release (Yu et al, 1997) and affect gonadotroph cell number and morphology (Lovren et al, 1998). Moreover, 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 (Miller et al, 2003; Zhen et al, 1997) (pathway C in Figure 4-IV).

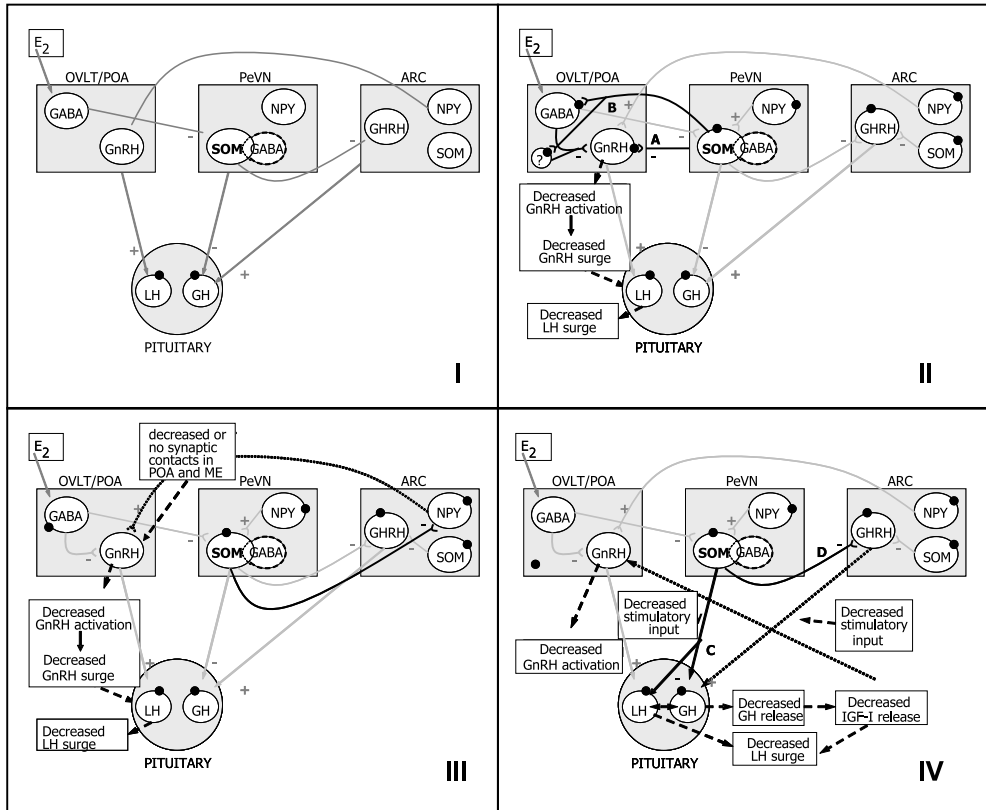


Fig. 4. Schematic drawings of the three proposed pathways via which SOM originating from the PeVN may decrease the LH surge. I: interactions between neurons in the OVLT/POA, PeVN and ARC as described in literature. II: direct effect of SOM on neurons in the OVLT/POA; A: directly on GnRH neurons; B: indirectly 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. Black circles represent SSTRs.

Alternatively, elevated SOM levels may inhibit GHRH neurons in the ARC (McCarty et al, 1992; Lanneau et al, 2000; Tannebaum et al, 1990; Willoughby et al, 1989), 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 (Childs, 2000; Childs et al, 2000; 1994), a decreased activation of GH cells may lead to decreased activity of LH cells, consequently resulting in a decreased LH release. In addition, decreased IGF-I levels, due to decreased plasma GH concentrations, may lead to both decreased LH release from the pituitary (Kanematsu et al, 1991) and decreased GnRH release from the ME (Miller et al 2003; Zhen et al, 1997) (pathway D in Figure 4-IV).

Although 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 direct effects of SOM at the level of the pituitary may be additional to the effects at the level of the hypothalamus concerning the proposed interaction with the reproductive axis. 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. In the light of our own data and data from literature, we propose that the role of SOM in the regulation of the descending phase of the LH surge, may involve, at least, a combination of pathways 1 and 2. In the cycling female rat, elevated plasma concentrations of E₂ and P on the day of proestrus may increase NPY levels in the ARC that, together with the removed inhibitory GABA-ergic tone (Smith and Jenness, 2003), stimulate GnRH cell activation, leading to the GnRH surge and, subsequently, the preovulatory LH surge. Secondly, the increased levels of gonadal steroids (Estupina et al, 1996, Van Vugt et al, 2008), and in addition, elevated levels of NPY (Rettori et al, 1990) 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).

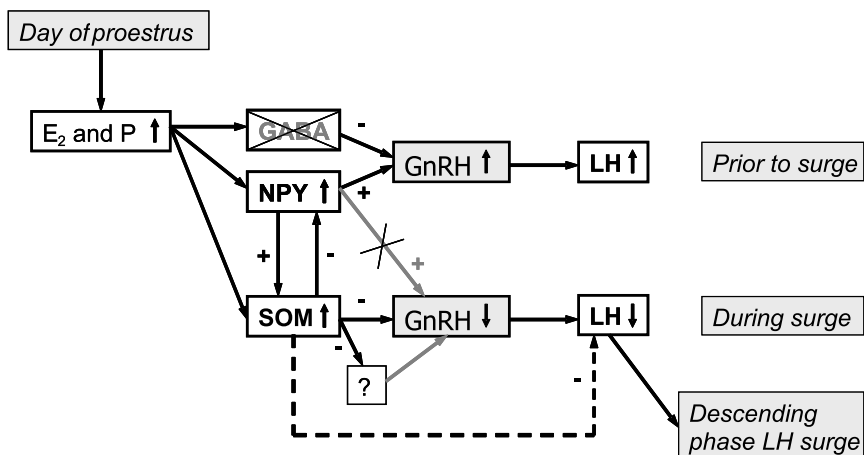


Fig. 5. Proposed mechanism via which hypothalamic SOM may be involved in the regulation of the descending phase of the preovulatory LH surge in the female rat

4.5 Age dependent effects of estrogen on PeVN SOM peptide

In young rats, the rostrocaudal distribution profiles of SOM-ir cells within the PeVN were comparable between ZT5 as well as ZT11 on the two subsequent days after E₂ treatment. In addition, the total SOM-ir fiber area, was consistently higher at ZT5 compared to ZT11 in these animals. These findings suggest that SOM peptide synthesized in the PeVN and released in the ME may be influenced by diurnal rhythms. Our data are supported by a study that reported a diurnal rhythm in SOM peptide content in the ME (Esquifino et al, 2004). Moreover, SOM peptide levels in the cortex, anterior hypothalamus and suprachiasmatic nucleus (SCN) (Fukuhara et al, 1993), and SOM release from the

hypothalamus (Berelowitz et al, 1982) were demonstrated to show circadian rhythmicity and suggest that the SCN may play a role in this diurnal change in SOM peptide transport from the PeVN to the ME. Thus, our data suggest that in the young female Wistar rat SOM cells in the PeVN are influenced by at least the SCN and E₂. E₂ may affect intrahypothalamic SOM projections within the PeVN or to other hypothalamic areas that contain SOM receptors (Beaudet et al, 1995; Hervieu et al, 1999), whereas SOM release from the ME may be influenced by the SCN. SOM content and release from hypothalamic explants is influenced by sex and age (Ge et al, 1989). The rostro-caudal distribution pattern of SOM-ir cells and the total number of SOM-ir cells in the PeVN was different in the middle-aged compared to young rats, but only 2 h after E₂ treatment. These findings suggest that with age, E₂ may become more crucial for the synthesis and/or storage of SOM peptide in the PeVN and affect the diurnal change in SOM levels within the PeVN.

The function of 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 (Clark et al, 1987; Pincus et al, 1996), suggesting that the shift in the rostro-caudal SOM cell distribution at ZT11, i.e. just before dark onset, may reflect this 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 compared to young females (Takahashi et al, 1987). Taking these findings into consideration, we suggest that the changes in SOM-ir levels within the PeVN may translate into changes in GH release patterns during aging in female rats.

5. Conclusion

In the present study we clearly demonstrate a significant attenuation of the LH surge at the age of 8.5 compared to 4 months old regular 4-day cycling females. This attenuation of the LH surge was not accompanied by changes in the releasable pool of LH, timing of the surge, GnRH priming or preovulatory P levels, supporting the notion that an attenuation of the LH surge may result from a change in the hypothalamic drive. Strikingly, we found clear changes in hypothalamic SOM peptide regulation following a physiological dose of estrogen in middle-aged animals. We hypothesize that the age dependent effects of an E₂ stimulus on SOM-ir cell distribution and SOM-ir numbers indicate alterations in the regulation of hypothalamic SOM peptide release in response to estrogen feedback could underlie an attenuation of the LH surge with age. These observations suggest that changes in the regulation of the GH axis with age indeed coincide with the process of reproductive aging in the female rat and suggest that the proposed interaction between these neuroendocrine axes may occur via alterations in hypothalamic somatostatin release.

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