

**SEXUALLY DIMORPHIC DISTRIBUTION OF SST2A RECEPTORS ON GHRH NEURONES IN  
MICE: MODULATION BY GONADAL STEROIDS**

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## ABSTRACT

The ultradian pulsatile pattern of growth hormone (GH) secretion is markedly sexually dimorphic in rodents as in primates but the neuroanatomical mechanisms of this phenomenon is not clear. In the arcuate, nucleus of the hypothalamus GH-releasing hormone (GHRH) neurones receive somatostatinergic inputs through sst2A receptor (sst2A-R) and the percentage of GHRH neurones bearing sst2A-R is higher in female than in male GHRH-eGFP mice. In the present study we hypothesised that sst2A-R expression on GHRH neurones is modulated by gonadal steroids and constitute a mechanism for the sexually differentiated GH secretion.

The distribution of sst2A-R on GHRH neurones was evaluated by immunohistochemistry, in adult GHRH-eGFP mice gonadectomised and treated for 3 weeks with oestradiol or testosterone implants.

In gonadectomised females supplemented with testosterone, sst2A-R distribution on GHRH neurones was reduced to the level seen in intact males whereas oestradiol implants were ineffective. Conversely, orchidectomy induced a female “sst2A phenotype” which was reversed by testosterone supplementation. Changes in the hepatic expression of GH-dependent genes MUP-3 and Prl-R reflected the altered steroid influence on GH pulsatile secretion.

In the ventromedial-arcuate region, GHRH and sst2-R, as well as GHRH and somatostatin expression as measured by real time PCR, were positively correlated in both sexes. In contrast, the positive correlation between ventromedial-arcuate GHRH and periventricular somatostatin expression in males was reversed to a negative one in females. Moreover, the positive correlation between periventricular somatostatin and ventromedial-arcuate sst2-R expressions in males was lost in females.

These results suggest that, in the adult mouse, testosterone is a major modulator of sst2A distribution on GHRH neurones. This marked sex difference in sst2A-R distribution may constitute a key element in the genesis of the sexually differentiated pattern of GH secretion possibly through testosterone-modulated changes in somatostatin inputs from hypophysiotropic periventricular neurones.

## INTRODUCTION

Many neuroendocrine systems communicate via a pulsatile rather than a continuous signal exchange allowing an optimised response in target tissues. The secretion of GH from pituitary somatotrophs occurs with an ultradian pulsatile pattern, which is sexually dimorphic and dictates sex differences in adult body size, as well as a dimorphic expression of inducible enzymes of the liver, a major target organ of GH. Besides interacting at the pituitary level to drive the release of GH from somatotroph cells, the antagonistic neurohormones growth hormone releasing hormone (GHRH) and somatostatin, also exert functional interactions at the level of the hypothalamus. Indeed, somatostatin immunoneutralisation increases GHRH levels in portal blood vessels (1) as well as GH nadir values (2) supporting the notion that somatostatin neurones may directly influence GHRH cell population. In particular, in the arcuate nucleus of the mouse hypothalamus GHRH neurones receive somatostatinergic inputs through the sst2A receptor subtype (sst2A-R) (3).

The sexually dimorphic patterns of GH secretion are well assessed in Rat where male individuals exhibit narrow GH pulses with a frequency of about one pulse every 3 to 4 hours and prolonged nadir values below 1-2 ng/ml whereas females exhibit lower-amplitude pulses with an irregular frequency and nadir values of 5-20ng/ml (4, 5). Such patterns of release are established with the onset of the puberty where major hormonal modulations occur. The underlying mechanisms for such a dimorphism in the ultradian secretory pattern of GH remain unclear. Experimental evidences indicate that a neonatal exposure or deprivation of specific sex steroid hormones induce permanent alterations of the adult GH secretory profiles. During development, testosterone appears to play an important organizational role in generating the high-amplitude GH pulses whereas oestradiol is responsible for the elevated basal GH levels observed in the female. Among these organizational effects, periventricular hypothalamic somatostatin peptide and mRNA levels are higher in male than in female rats and mice (6, 7). In addition, a sexual dimorphism has been described for expression of sst1 but not sst2 somatostatin receptor subtype in the hypothalamus (8-10). Although it is well documented that the neonatal gonadal steroid milieu is an important determinant of the adult pattern of GH secretion and body growth, the effect of gonadal steroid is not limited to the perinatal critical period of imprinting. In adults, acute treatments with either oestradiol or testosterone feminise or masculinise the GH secretory profiles of male and female rats respectively (11, 12).

Recent observations also stress the importance of somatostatin in maintaining sexually dimorphic pattern of GH secretion in the mouse species. Baseline GH values are increased in male somatostatin knockout mice and these changes are associated with demasculinisation of GH dependent genes (13). Moreover, hypothalamic somatostatin is also necessary to maintain GHRH mRNA levels

in a gender-dependent fashion in mice (14). Using GHRH-GFP transgenic mice to identify GHRH neurones, we recently observed that sst2A receptor distribution on GHRH neurones differs markedly between the sexes, the percentage of GHRH neurones decorated with sst2A receptors being three fold higher in females (78%) than in males (26%) (15). In the present study, we sought to determine whether this sexually differentiated pattern of sst2A receptor immunostaining on GHRH-eGFP neurones could be altered by manipulation of the gonadal steroid environment, directly or through neurohormonal modulation. For that purpose, gonadectomies were performed in young adult male and female mice, with or without oestradiol or testosterone substitution, respectively, in order to demasculinise or defeminise (16) GH pulsatility. Sst2A receptor immunolabelling distribution on GHRH-eGFP neurones, as well as sst2-R and GHRH and somatostatin expressions were then determined in the hypothalamus by confocal immunohistochemistry and real-time PCR, respectively.

## **MATERIALS AND METHODS**

### ***Animals***

Hemizygous male and female GHRH-eGFP transgenic mice (17) were obtained from breeding pairs of GHRH-eGFP transgenic and C57Bl/6J mice. The offspring were genotyped by PCR amplification of tail DNA. Animals were housed on a 12h/12h light/dark cycle at  $22 \pm 2^\circ\text{C}$  and were given ad libitum access to regular chow and water. Female oestrus cycles were followed by histological examination of vaginal smears. Only female mice that exhibited normal oestrus cycles were used and sham female mice were killed on dioestrus.

After gonadectomy and until the day of killing animals were monitored every three days for body weight increase.

All animal procedures complied with French laws regarding animal experimentation (Decree N° 87-848, October 19, 1987 and the Ministerial Decree of April 19, 1988) and recommendations of a local ethical committee.

### ***Surgery and hormone treatments***

The influence of steroid hormones on the expression of sst2A receptor on GHRH neurones in adult mice was assessed by comparing six groups of animals for each sex: adult males treated with either vehicle, testosterone or oestradiol, and adult gonadectomised males treated with either vehicle, testosterone or oestradiol. The mirror experiment was conducted with female mice.

Castrations (and sham castrations on control animals) were performed at 5 to 7 weeks of age under forene gas anaesthesia (Abbot Laboratory, Centravet, Plancoët, France) delivered by a pump (200 ml/min, 2% forene) (Univentor 400 anaesthesia unit, Phymep, Paris, France). Two weeks after surgery, animals were randomly assigned to a treatment group and received either a  $17\beta$ -oestradiol filled, a testosterone filled (Sigma, St Quentin Fallavier, France) or an empty silastic implant. Oestradiol or testosterone implants were made by packing 4mm of each crystalline steroid into silastic tubing (i.d. 1.57 x o.d. 3.18mm) from Corning laboratories. Both ends of the silastic tubing were glued with a piece of silicone sheeting (Perouse Plastic, Bornel, France). Silastic implants were placed under gas anaesthesia in the midscapula region. Three weeks after implantation, animals were divided in two groups to be killed, one for immunohistochemistry, the other for tissue collection.

### ***Immunohistochemistry, image acquisition and processing***

On the day of killing, animals were anaesthetised with a mix of ketamine and xylazine (0.5 mg and 0.1 mg respectively /15g BW, i.p.; Sanofi, Libourne, France) and perfused transcardially with 4% paraformaldehyde in 0.1 M Na/K phosphate buffer (PB), pH 7.4. Brains were cryoprotected by overnight immersion in a 30% sucrose solution at 4 °C, frozen in liquid isopentane (-45 °C) and kept at -80° C until use. Using a cryostat, sectioning was performed at the level of the hypothalamus, and thirty-micrometre thick coronal sections encompassing the entire extent of the arcuate nucleus were collected one by one in wells containing Tris buffered saline pH 7.4 (TBS).

To assess the distribution of sst2A receptor on GHRH positive neurones, one out of three sections for one hypothalamus representing 10 to 12 sections per animal, was then processed for immunocytochemistry performed on free floating sections in TBS at room temperature. Sst2A receptors were immunolocalised using a fully characterised antiserum raised in rabbit against the C-terminal segment 330-369 of the human protein (18, 19). During the immunohistochemical procedure control and experimental groups were processed simultaneously in the same reagents at room temperature. Free floating sections were rinsed in 0.1M TBS and preincubated for 30 min in 5% normal donkey serum (NDS) in TBS containing 0.3% Triton X-100. Sections were incubated overnight in 1:2000 rabbit anti-sst2A receptor antibody diluted in TBS containing 0.5% NDS and 0.3% Triton X-100. Sections were then rinsed in TBS and incubated for 45 min in 1:300 biotinylated donkey anti-rabbit IgG (Vector laboratory, Burlingame, CA, USA) diluted in TBS containing 0.5% NDS and 0.3% Triton X-100. Finally, sections were incubated for 30 min in 1:4000 Streptavidin Cy3 (Jackson Laboratories, West Grove, PA, USA) diluted in TBS. Sections were then rinsed in TBS, mounted on glass slides and coverslipped with a vectashield solution (Vector Laboratories).

Blind-coded immunolabelled sections were analysed by confocal microscopy using a Leica DMRA microscope equipped with a TCS SP2 Leica confocal imaging system with Ar 488-nm and HeNe 543-nm lasers (Leica, Heidelberg, Germany). For each animal, all sections encompassing the entire extent of the arcuate nucleus were analysed. For each section, a zone of 10µm depth was determined at the same z-axis distance from the top and bottom of the section. Within this 10µm zone, single optical sections using a 40x Plan-Apochromat oil-immersion lens objective NA: 1.4 (Leica, Heidelberg, Germany) were scanned every 2µm to locate each GHRH-eGFP positive cell. For each individual GHRH-eGFP neurone the presence at the membrane of the sst2A was determined using numerical zoom. The number of GHRH-eGFP cells and of GHRH-eGFP/sst2A receptor double-labelled cells are recorded for each section and summed for one animal. Data are presented as mean±sem per animal.

### *Tissue collection*

On the day of killing, blood was collected from the jugular vein on EDTA after having anaesthetised the animals with a mix of ketamine:xylazine (0.5 mg and 0.1 mg respectively /15g BW, i.p.; Sanofi, Libourne, France). Plasma was obtained by centrifugation and kept frozen at  $-80^{\circ}\text{C}$  until steroids concentrations were assayed. Animals were then killed by decapitation and brains were quickly removed. Two hypothalamic fragments containing arcuate and periventricular areas were excised: a mediobasal one containing the ventromedial-arcuate region (VMH-Arcuate), and a more dorsal and anterior segment adjacent to the third ventricle comprising the periventricular nucleus (PeN) (20). Hypothalamic fragments were immediately placed and disrupted in lysis buffer and stored at  $-80^{\circ}\text{C}$  until total RNA extraction and real-time PCR for assaying sst2-R, GHRH and somatostatin expression. A sample of liver was also quickly removed, placed in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  to quantify the expression of two GH regulated genes, a member of the major urinary proteins (MUP-3) and the prolactin receptor (PrL-R) by real time PCR.

### *Hormone assays*

Completeness of male gonadectomy and female ovariectomy as well as the efficacy of steroids filled silastic implants were verified by measuring circulating plasma steroid hormone levels from blood samples withdrawn at the time of killing. Testosterone and oestradiol levels were measured in plasma using a mouse testosterone RIA (Diagnostic Systems Laboratories Inc., Webster, Texas, USA) and a mouse oestradiol RIA (DiaSorin, S.p.A., Saluggia, Italy).

### *Tissue processing and quantification by real time PCR*

Hypothalamic and liver RNAs were extracted with a silicium membrane method using an RNeasy Mini extraction Kit (Quiagen, Courtaboeuf, France) and quantified by spectrophotometry (Spectronics, Milton Roy, New York, USA). First-strand cDNA was prepared by reverse transcription (RT) using 1 $\mu\text{g}$  of total mRNA (liver) or 0.5  $\mu\text{g}$  hypothalamic samples in a 20 $\mu\text{l}$  final reaction mixture containing 200U of MMLV reverse transcriptase (Invitrogen, Carlsbad, CA), 0.167  $\mu\text{g}$  of random primer (dN6, Promega, Charbonnière, France), 12.5 nmoles dNTP (Promega) and 20U of RNAsin (Promega).

Real-time PCR was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France). Amplification reactions were performed in a

20µl final volume using a Taqman Universal Master Mix reagent kit for somatostatin, GHRH, sst2-R and Prl-R primers or a SYBR Green PCR Master Mix reagent kit for MUP-3 (Applied Biosystems, Courtaboeuf, France). MUP-3 primer sequences for gene amplification were designed using Primer Express (Applied Biosystems) and 18S rRNA was used for standardisation.

PCR was initiated after activation of the Amplitaq Gold enzyme (Applied Biosystems) in the reaction mixture by heating for 10 min at 95°C. All genes were amplified by a first denaturation step of 15 sec at 95°C, followed by an annealing and amplification step of 1 min at 60°C for 40 cycles. Relative gene expression levels were calculated according to the comparative CT method which normalises the copy number of target genes to that of an endogenous reference gene (21). Based on exponential amplification of the target and reference genes, the amount of amplified molecules at the threshold cycles (CT values) was given. Normalised target gene expression relative to 18S rRNA is obtained by calculating the difference in CT values, the relative change in target transcripts being computed as  $2^{-\Delta CT}$ . In order to validate the comparative CT method of relative quantitation, the efficiencies of each target gene amplification and the efficiency of the active reference amplification (endogenous control 18S) were measured and shown to be approximately equal.

### *Statistical analysis*

Results are expressed as mean ± S.E.M. The data were analysed by ANOVA followed by Student's least significant difference test. For correlation studies, multivariate tests were performed and analysed for pairwise correlations, using Stat View 4.0 (Abacus concept Inc., Berkeley, CA, USA).



## RESULTS

### *Hormone Assays*

Plasma oestradiol and testosterone concentrations in the different groups of mice are given on Table 1. As expected, circulating gonadal steroids were undetectable in the gonadectomised groups. In the steroid supplemented groups, oestradiol concentrations were in the range for oestrus values, and testosterone were in the high physiological range (22). Oestradiol was more effective than testosterone on body weight, independently of sex and castration (Table 2).

### *Modulation of liver GH regulated genes by steroids*

The steroid status of adult mice was manipulated by performing gonadectomy and testosterone or oestradiol treatments, in female and male mice. To assess the efficacy of these treatments on ultradian GH secretory rhythms, we took advantage of the established role of GH secretory dynamics in regulating the sexually differentiated expression of particular hepatic genes (23). As shown in Fig. 1, MUP-3, a male predominant gene transcript, was 2.9-fold more expressed in male than in female mice (ratio MUP-3/18S= 14.1 in male versus 4.9 in female,  $p < 0.001$ ), as expected. This ratio was demasculinised by orchidectomy, without any further modulation by oestradiol treatment and the effects of castration reversed by testosterone treatment only. Reciprocally, MUP-3 expression pattern was defeminised by testosterone treatment in both intact and ovariectomised female mice. Surprisingly, in intact male (sham) mice, testosterone and oestradiol treatments exerted a slight but significant demasculinising effect on MUP3 ratio.

Conversely, a female predominant transcript, Prl-R, was 3.5 fold more expressed in females than in males (ratio Prl-R/18S= 2.74 in females versus 0.79 in males,  $p < 0.001$ ), as originally published in mice (24). In males, Prl-R expression was totally demasculinised by castration (without further effects by oestradiol) and this effect was completely antagonised by testosterone treatment. In both intact and ovariectomised females, Prl-R expression was defeminised by testosterone treatment but not by oestradiol.

### *Modulation of sst2A receptor distribution by steroids*

In the arcuate nucleus, the number of GHRH-eGFP neurones was 35 % higher in male than in female animals ( $1323 \pm 63$  in male versus  $981 \pm 78$  in female,  $n=19$ ,  $p < 0.005$  by three way ANOVA,

sex\*castration\*hormone treatment) whereas the proportion of GHRH neurones bearing sst2A receptors were three fold more numerous in females than in males (Figs 2, 3). The number of GHRH neurones was unaffected by gonadal steroid manipulation but the proportion expressing sst2A-R was highly sensitive to gonadal steroids.

In males, sst2A decoration of GHRH-eGFP neurones rose from 21% in sham-operated mice to 71% in orchidectomised animals ( $p<0.001$ ), while testosterone treatment reversed this phenomenon in orchidectomised animals (23%) and had no effect on the proportion of GHRH-eGFP neurones expressing sst2A-R in sham operated males (24%). Oestradiol supplementation did not affect sst2A receptor distribution in sham male mice but fully reversed the effect of the orchidectomy.

For female animals, ovariectomy did not significantly affect sst2A receptor distribution on GHRH-eGFP neurones, while testosterone supplementation defeminised sst2A receptor distribution in both intact and gonadectomised female mice (81% in sham female versus 26% in sham + testosterone,  $p<0.01$  or 20% in Ovx + testosterone,  $p<0.01$ ). Oestradiol treatment did not significantly affect the proportion of GHRH-eGFP neurones expressing sst2A-R in sham-operated or ovariectomised females.

#### *Modulation of ss2 receptor, GHRH and somatostatin expression by steroids*

As shown in Table 3, neither sex nor castration affected sst2-R and somatostatin gene expression in VMH-Arcuate or PeN regions, or GHRH gene expression in the former region as measured by real time PCR (Table 3). By one way ANOVA, the only intra group difference was observed for oestradiol supplementation which increased GHRH expression in the VMH-Arcuate region in orchidectomised animals when compared to the testosterone-supplemented orchidectomised group.

Three-way-ANOVA analysis (sex\*castration\*hormone treatment) revealed an interaction between sex and hormone treatments for somatostatin gene expression in the VMH-Arcuate region and sst2-R expression in PeN. In both cases, testosterone was inhibitory while oestradiol increased mRNA expressions.

In both males and females, there was a positive correlation between GHRH and sst2-R expression (Fig. 4, upper panels) and GHRH and somatostatin expression in the VMH-Arcuate region (Fig. 4, middle panels). In contrast, the correlations between VMH-Arcuate GHRH and PeN somatostatin relative gene expressions differed significantly with sex, being positive in males and negative in females (Fig. 4, lower panels). Similarly, the correlation between somatostatin gene expression in VMH-Arcuate and PeN regions was positive in males ( $r=0.448$ ,  $p<0.001$ ) but negative ( $r=-0.338$ ,  $p<0.05$ ) in females (not shown).

## DISCUSSION

The present results indicate that testosterone modulates sst2A receptor decoration of GHRH neurones. In parallel, steroid environment changes the activity of two populations of intrahypothalamic somatostatin neurones: those from the PeN and the Arcuate region. Previous experimental evidences already suggested the involvement of somatostatin in mediating the sexual dimorphism of GH secretion. For example, intermittent administration of somatostatin can masculinise female GH secretory patterns (25), whilst deletion of somatostatin feminises GH secretion in mice (13). In addition to its effects on pituitary GH secretion, there is good evidence for an intra-hypothalamic impact of somatostatin on GHRH neurones, not least the observations that the GH rebound seen on withdrawal of somatostatin is dependent on active GHRH secretion (26), and that somatostatin receptors are expressed on GHRH neurones in the arcuate nucleus. (27).

Gonadal steroids exert a complex role in programming the sex-dimorphic patterns of the GH axis, with both neonatal and pubertal imprinting effects that permanently affect GH secretion in adults (28). In the mouse, there is some evidence for a dimorphic pattern of ultradian GH secretion, though technical difficulties in chronic blood sampling in non restrained animals have limited the reports of direct evidence for this (13, 14, 29). Studies rely either on differences in distributions of large numbers of single samples (13, 14), or on indirect measures, such as the expression of GH-dependent hepatic genes (23, 24).

Whilst much of the mechanistic information outlined above has been gained from studies in rats, transgenic GHRH-eGFP mice offer an opportunity to study identified GHRH neurones (17). Combining this model with the immunodetection of sst2A receptors, we recently reported a marked sexual dimorphism in the distribution of sst2A receptors on GHRH neurones (15). In this previous study performed on a limited number of animals we did not observe a significant sex difference in the number of GHRH-eGFP cells. However, herein, by comparing a greater number of mice of each sex, a 35% greater number was observed in male mice, in accordance with two previous reports (30) (31).

The steroid regulation of hypothalamic somatostatin receptors is complex. For instance, arcuate nucleus sst receptor levels are increased upon oestradiol treatment in female rats (32) and sst1- but not sst2-R gene expression exert a sexual dimorphism in this species (8). In mice, however, sst2 receptor is necessary for GH negative feed-back on arcuate GHRH neurones (3). In rodents, sst2-R mRNA can be spliced in two isoforms sst2A and sst2B, both expressed in brain and pituitary (33).

However, no sst2B expression is detected in the arcuate nucleus/median eminence complex, indicating that sst2A is the isoform associated with GHRH neurones (34).

In adult rat, short term exposure to oestradiol demasculinises the male pattern of spontaneous and GHRH-induced GH secretion, as well as rate of somatic growth (11). However, testosterone or dihydrotestosterone administration to adult females leads to a male-like secretory pattern (12, 35). In order to determine whether the sexually differentiated pattern of sst2A receptor on GHRH-eGFP neurones was determined by gonadal steroid environment, we carried out gonadectomy/steroid supplementation experiments in normal or gonadectomised GHRH-eGFP mice of both sexes and examined the effects on sst2A receptor distribution on their GHRH neurones.

Our results point to a specific regulatory role for testosterone on sst2A receptor expression in GHRH neurones. Testosterone supplementation defeminised the pattern of sst2A receptor distribution on GHRH neurones both in sham and ovariectomised female mice, while castration in females, did not affect it. Furthermore, removal of endogenous testosterone by gonadectomy in males induced a female-like sst2A receptor distribution on GHRH neurones. These results do not rule out a potential effect of oestradiol when given to males, since this steroid was able to counteract the effects of gonadectomy on sst2A receptor distribution. This is in accordance with reports in the rat in which dihydrotestosterone administration fully remasculinised GH secretory pattern of ovariectomised females, but failed to do so in intact ones (35).

Our results could be explained by a direct effect of testosterone on sst2A receptor expression. However, such steroid effects could also be exerted in other parts of the hypothalamus or at the pituitary level. For example, oestrogens could counteract the defeminising effects of androgens by acting on somatostatin neurones reducing the inhibitory tone mediated by somatostatin. We do not favour this explanation since, at least in the rat, somatostatin periventricular neurones do not express oestrogen receptors (36). Alternatively, and by analogy with the rat, excessive levels of circulating oestradiol could directly activate oestrogen alpha receptors subtypes, since 70 % of GHRH neurones do bear this receptors in that species (37, 38). It is also possible that sex differences in hypothalamic connections, established neonatally, contribute to sexual dimorphic GH responses, since sex differences in GH pattern do remain detectable after gonadectomy (39).

Steroid implants induced their expected effects on growth rate and plasma steroid levels. Since direct measurement of GH secretory pattern is not easily accessible in mice, we took advantage of the firmly established role of pulsatile GH secretory rate in regulating sexual differentiation of the liver

(13, 24, 40). Because GH secretory pattern exhibits high narrow GH pulses in male rodents, liver MUP-3 mRNA levels are greater in males than in females (24). In our experiments, Pr1-R expression was defeminised in females treated with testosterone, and demasculinised in males, whether orchidectomised or treated with oestradiol. MUP-3 expression followed a similar pattern. These findings are consistent with published observations that acute exposure to testosterone or dihydrotestosterone are sufficient to defeminise GH ultradian pulsatile pattern measured in adult ovariectomised rats (12, 35).

Despite the evident sexual dimorphisms in the functional GH axis, significant difference between males and females in the expression of sst2-R, GHRH and somatostatin transcripts in VMH-Arcuate and PeN extracts were not observed. This may be due to variation within individual mice in each groups, or to variability within GH pulse cycles, since variations in hypothalamic somatostatin and GHRH mRNA levels are related to the regular ultradian oscillations in GH secretory episodes (41). It could also be due to the tissue dissection which “lump” together different hypothalamic nuclei and neuronal populations which might be differently affected in both sexes. Finally, it could be due to strain differences. Low et al (13) recently reported that male somatostatin mRNA levels, in the whole hypothalamus of a 5 generation 129/Sv x C57Bl backcross, were twofold those of female mice. However, the absence of dimorphism reported in somatostatin hypothalamic expression in the GHRH-eGFP transgenic strain, herein, is also found by Luque and Kineman for wild type (C57Bl/6J) male and female mice (14). In addition, Kuwahara et al (31) reported no significant sex differences in the immunoreactivity of somatostatin PeN neurones in C57Bl/6J mice, up to one year of age after which a major increase is observed.

Oestradiol treatment positively affected GHRH mRNA expression in orchidectomised males as compared to testosterone-treated ones. In mice and rats, contradictory data are available for GHRH regulation by steroids. In the adult rat, gonadectomy as well as either testosterone or oestradiol supplementation induce little or no change in GHRH mRNA expression (42, 43). Other studies pointed to stimulated GHRH expression by testosterone (28) without any change in the number of GHRH neurones (28, 30). A common problem to all studies, including our own, is that both GHRH and somatostatin are subject to acute and multiple feedback regulation by GH and steroids. Thus, it is difficult to distinguish direct effects of oestradiol on GHRH synthesis, from indirect effects via altered GH signalling. Concerning somatostatin regulation, it seems clearly established that testosterone is the main stimulator of somatostatin in the PeN nucleus of rodents (7, 28, 30).

Correlations between the expression of GHRH and somatostatin and sst2-R genes suggested a positive link between GHRH and somatostatin, and between GHRH and sst2-R in male and in female VMH-Arcuate region. Interestingly, VMH-Arcuate GHRH and periventricular somatostatin gene expressions were negatively correlated in females, but positively in males. This suggests that dimorphic sst2A receptor distribution on GHRH neurones reflects modifications in periventricular hypophysiotropic somatostatinergic neurones in male and female, probably regulated by testosterone, while for arcuate somatostatinergic neurones, regulation remains the same in both sexes. Other observations support this hypothesis. In the rat arcuate nucleus double immunohistochemistry failed to find evidence for androgen receptors in GHRH neurones (44), in contrast to periventricular area where the majority of somatostatinergic neurones express androgen receptors both at the mRNA and protein levels (45, 46). This further supports our contention that, in mouse, the most likely candidates for mediating the effects of testosterone on GHRH cells are the hypophysiotropic periventricular somatostatinergic neurones, even if the origin of somatostatin fibres innervating GHRH neurones in the mouse is unclear. In mice, deafferentation of mediobasal hypothalamus prevents the defeminising effect of dihydrotestosterone on GH secretory pattern (35) and GH-mediated negative autofeedback involves signalling between PeN and Arcuate neurones through sst2 receptors (3). Very recently, Luque and Kineman pointed out the gender dependent role of endogenous somatostatin in regulating GH axis in mice especially at the level of the hypothalamus. In particular, they showed that GHRH mRNA levels are lower in female somatostatin knockout mice compared with their male counterparts promoting the speculation that hypothalamic somatostatin may in fact be required to maintain optimal GHRH neuronal activity in a gender dependent fashion (14).

In summary, our results demonstrate that, in mice, sst2A receptor distribution on GHRH neurones and the sexually dimorphic pulsatile pattern of GH secretion are both regulated by testosterone, probably through modulation of hypophysiotropic somatostatinergic neurones. This may be achieved by androgen-induced increase of periventricular somatostatin release. It has been shown that somatostatin is able to induce sst2A receptor internalisation (47) in the arcuate nucleus *in vivo*. Such a mechanism may be involved in the steroid modulation of the sexually dimorphic sst2A receptor distribution on GHRH neurones.

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## FIGURE LEGENDS

### Figure 1. Hepatic gene expression of MUP-3 and Prl-R in male and female GHRH-eGFP mice according to steroid environment.

GH-dependent hepatic gene expression of MUP-3 and Prl-R were quantified by real-time PCR on 7 to 8 animals per experimental group. Gene expression levels are expressed in reference to 18S mRNA in the same sample. Sham operated (Sham), orchidectomised (Orch), ovariectomised (Ovx) or supplemented or not with oestradiol (E) or testosterone (T).

\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ : versus sham animals of the same sex; °:  $p < 0.05$ , °°  $p < 0.001$ : versus castrated animal of the same sex.

### Figure 2. Representative micrographs illustrating the effects of orchidectomy in males and testosterone treatment in females on sst2A receptor decoration of GHRH-eGFP neurones

Confocal micrographs of GHRH-eGFP positive (a, d, g, j) and sst2A receptor immunoreactive (sst2A-IR) (b, e, h, k) and overlay (c, f, i, l) cells in intact male (a-c), orchidectomised male (d-f), intact female (g-l) and testosterone-treated female (j-l). Asterisks indicate sst2A receptor labelling surrounding green GHRH cell bodies. Scale is indicated in the lower right panel (scale bar, 10  $\mu\text{m}$ ).

### Figure 3. Effect of steroid environment on sst2A receptor distribution on GHRH-eGFP neurones.

Sst2A receptor distribution on GHRH-eGFP was quantified on 3 males and 3 females in each experimental group. Data are means  $\pm$  SEM of the number (nb) of GHRH-eGFP neurones decorated by sst2A receptor antibody in sham operated (Sham), orchidectomised (Orch), ovariectomised (Ovx) or supplemented or not with oestradiol (E) or testosterone (T).

\*\*\*:  $p < 0.001$  versus sham animals of the same sex; °:  $p < 0.05$ , °°:  $p < 0.01$ , °°°  $p < 0.001$ : versus gonadectomised animals of the same sex.

### Figure 4. Correlations between expression of GHRH and sst2 genes in VMH-arcuate (Arc) region, and GHRH and somatostatin (SRIH) genes in VMH-arcuate and periventricular regions.

Relative gene expression are expressed as number of copies target gene/ number of copies 18S  $\times 10^5$ .

Pairwise correlations were calculated from data originating from 44 males and 50 females. Regression coefficients are given on each plot.

\*:  $p < 0.05$ , \*\*:  $p < 0.01$  and \*\*\*\*:  $p < 0.0001$ .

Male: black squares Sham, open squares Orch, black triangles Sham + T, open triangles Orch T, black lozenges Sham + E, open lozenges Orch + E.

Female: black circles Sham, open circles Ovx, black triangles Sham + T, open triangles Ovx + T, black lozenges Sham + E, open lozenges Ovx + E.