The Influence of Gonadal Steroid Hormones on Immunoreactive Kisspeptin in the Preoptic Area and Arcuate Nucleus of Developing Agonadal Mice with a Genetic Disruption of Steroidogenic Factor 1

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Key Words

Kisspeptin · Sex difference · Brain · Estradiol · Genetic factor · Steroidogenic factor 1 · SF-1 knockout mice

Abstract

Kisspeptin, a regulator of reproductive function and puberty in mammals, is expressed in the rostral (anteroventral) periventricular nucleus (AVPV) and arcuate nucleus (Arc), and its expression is at least partially regulated by estradiol in rodents. The aim of the present study was to determine contributions of genetic factors and gonadal steroid hormones to the sexual differentiation of kisspeptin-immunoreactive (kisspeptin-ir) cell populations in the AVPV and Arc during postnatal development using agonadal steroidogenic factor 1 (SF-1) knockout (KO) mice. To examine the effects of gonadal hormones on pubertal development of kisspeptin neurons, SF-1 KO mice were treated with estradiol benzoate (EB) from postnatal day (P)25 to P36, and their brains were examined at P36. No sex differences were observed in the SF-1 KO mice during postnatal development and after treatment with EB – which failed to increase the number of kisspeptin-ir cells at P36 to the levels found in wild-type (WT) control females. This suggests that specific time periods of estradiol actions or other factors are needed for sexual dif-

ferentiation of the pattern of immunoreactive kisspeptin in the AVPV. Kisspeptin immunoreactivity in the Arc was significantly higher in gonadally intact WT and SF-1 KO females than in male mice at P36 during puberty. Further, in WT and SF-1 KO females, but not in males, adult levels were reached at P36. This suggests that maturation of the kisspeptin system in the Arc differs between sexes and is regulated by gonad-independent mechanisms.

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Introduction

Kisspeptin (Kiss1) and its receptor, G protein-coupled receptor 54 (GPR54, Kiss1r), have been implicated in reproduction and sexual maturation as key regulators of gonadotropin-releasing hormone (GnRH) secretion in mammals including humans [1–10]. The neuroanatomy of *Kiss1*-expressing neurons differs between mammalian species [11]. In the rodent brain, two populations of *Kiss1*-expressing neurons reside in the rostral (anteroventral) periventricular nucleus (AVPV) and in the arcuate nucleus (Arc) of the hypothalamus [12–15]. Regulation of *Kiss1* expression differs between these neural populations. In the AVPV, neurons expressing kisspeptin can be detected by

immunohistochemistry (IHC) on postnatal day (P)10 in male and female mice [16]. Afterwards, the number of kisspeptin-immunoreactive (kisspeptin-ir) neurons progressively increases in a sex-specific manner until the onset of puberty, so that mature female mice have approximately 10 times more kisspeptin-ir neurons than males [16]. Several lines of evidence suggest the involvement of steroid hormones in the sexual differentiation of kisspeptin neurons. Colocalization studies showed that *Kiss1*-expressing neurons in the AVPV coexpress all major receptors for steroid hormones [estrogen receptor- α (ER α) and - β (ER β), androgen and progesterone receptors] [12, 17, 18]. Studies in rodents have shown that the sex-specific development of *Kiss1* neurons in the AVPV depends on both organizational and activational effects of gonadal steroid hormones. Treatment with androgens during the first postnatal week masculinized a number of *Kiss1* mRNA-expressing neurons in adult female rats [15, 19], and neonatal castration of male rats blocked masculinization of a number of kisspeptin-ir neurons [19], suggesting that a male phenotype is a consequence of permanent organizing actions of gonadal hormones on developing *Kiss1* neurons in the AVPV. The development of the full female complement of kisspeptin-ir neurons in the AVPV in gonadectomized wild-type (WT) mice depends on the exposure to estrogens during puberty from P22 to P30 [20], although a study by Kim et al. [21] suggested that at the level of mRNA (but not peptide) expression, feminization of *Kiss1*-expressing neurons might start earlier. In adulthood, *Kiss1* mRNA and kisspeptin expression in the AVPV depends on activation effects of gonadal steroids, as mRNA (in situ hybridization) and peptide (IHC) are decreased after gonadectomy and restored by estradiol replacement [12, 13, 18, 20]. The effects of gonadal steroid hormones have been reinforced by studies with ER α knockout (KO) and aromatase KO (ARKO) mice [12, 13, 22, 23].

Kiss1 mRNA and kisspeptin immunoreactivity in the Arc can be detected during early fetal development in mice and rats [24, 25], and it persists throughout prenatal and postnatal development [25–27]. Several sex differences in *Kiss1* mRNA and kisspeptin expression in the Arc in the developing brain have been reported in gonadally intact rodents. During embryonic development and in adulthood, sex differences in the mRNA content from dissected hypothalami and in the number of *Kiss1* mRNA-containing cells have been reported in mice [27]. *Kiss1* mRNA levels during neonatal, prepubertal and pubertal development [28] and in adulthood [14] have been reported to be higher in female than in male rats. Similarly, sex differences have been reported also at the peptide lev-

el from the neonatal period to adulthood in rats [26] and during early postnatal development from P10 to P25 in mice [23]. Previous studies have shown that gonadal steroid hormones regulate *Kiss1* mRNA levels and kisspeptin immunoreactivity in the Arc. *Kiss1* mRNA levels increased after gonadectomy and decreased by estradiol, testosterone and dihydrotestosterone replacement in mice and rats [12–15, 19]. However, at the peptide level, decreased levels of immunoreactive kisspeptin following gonadectomy were restored with estradiol or dihydrotestosterone treatment in adult mice [23]. The requirement for estradiol to induce kisspeptin immunoreactivity in the Arc has also been suggested by studies in mice with ER α -ablated *Kiss1* neurons [22] and in ARKO mice [23], both of which had diminished kisspeptin immunoreactivity during postnatal development. Similarly to kisspeptin neurons in the AVPV, neonatal exposure to estradiol benzoate (EB) significantly decreased kisspeptin immunoreactivity in the Arc in female rats [29].

Early studies demonstrated that central mechanisms regulating puberty onset are not gonad dependent [30, 31]. These observations have been confirmed by recent studies showing profound changes in *Kiss1* mRNA levels and kisspeptin immunoreactivity in constantly low peripheral estrogenic environments [26] or low levels of gonadal steroid hormones [32] as well as by RT-PCR, in situ hybridization and IHC studies showing that *Kiss1* expression in the Arc might be regulated by other factors such as members of the polycomb group protein family [33], neuropeptides [34–41], trophic factors [42] and neurotransmitters [43, 44]. Despite the progress toward an identification of the gonad-independent factors involved in the regulation of *Kiss1* expression in the Arc during development in recent years, the role of these factors in the sexual differentiation of *Kiss1* expression in the Arc requires further clarification.

Gonadal steroid hormones and genes on sex chromosomes are two major factors influencing brain sexual differentiation in mammals [45–47]. Two genetic mouse models have provided a better understanding of contributions of sex chromosomes to brain sexual differentiation and function, namely the ‘four core genotype’ model [48–51] and mice with disruption of the steroidogenic factor 1 (*Nr5a1*) gene (SF-1 KO) [52, 53]. In SF-1 KO mice, genital ridges disintegrate early during embryonic development [54], before the initiation of steroidogenesis in fetal testes [55]. Therefore, these mice are not exposed to endogenous gonadal steroid hormones and, when compared to hormonally manipulated WT control mice, they represent a unique model for studying genetic and

hormonal influences on brain sexual development separately [47].

The aim of the present study was to determine whether there are any sex differences in the production of immunoreactive kisspeptin in the AVPV and Arc after birth in agonadal SF-1 KO mice either without hormones or after treatment with estradiol. In addition, it was our goal to determine if a peripubertal window of estradiol exposure from P25 to P36 for the development of kisspeptin immunoreactivity is sufficient to induce normal levels of immunoreactive kisspeptin in agonadal SF-1 KO mice in the AVPV area.

Materials and Methods

Animals and Brain Recovery

Heterozygous mice with a disrupted *sf-1* (*Nr5a1*) allele (SF-1^{+/-}) were backcrossed for more than 10 generations to C57BL/6J mice to produce a congenic line. All mice were housed under standard laboratory conditions at the University of Ljubljana Veterinary Faculty in a 12:12-hour light/dark cycle (lights off at 18:00 h) with a phytoestrogen-free diet (No. 2916; Harlan Teklad, Milan, Italy) and water ad libitum. All animal experiments were conducted according to ethics principles and in accordance with the EU directive (2010/63/EU). The experiments were approved by the Veterinary Commission of Slovenia and the Animal Care and Use Committee at Colorado State University.

SF-1^{+/-} mice were mated to produce homozygous SF-1 KO and control WT offspring. Due to a lack of gonads during fetal development, SF-1 KO mice develop female reproductive organs and external genitalia regardless of genetic sex. In the present study, SF-1 KO mice were sexed according to their chromosomal sex, i.e. XY = SF-1 KO males and XX = SF-1 KO females. To ensure survival of SF-1 KO mice, all newborn pups were injected subcutaneously daily for 6–7 days with 50 µl of a corticosteroid cocktail in corn oil (400 µg/ml hydrocortisone, 400 ng/ml dexamethasone and 500 ng/ml fludrocortisone acetate; all from Sigma, Steinheim, Germany). The mice were genotyped by PCR assay of tail DNA on day 6 or 7 after birth as previously described [56]. Female WT littermates or female pups from other C57BL/6J litters born within 3 days were used as a source of adrenal transplants; these techniques have been reported previously [57]. After adrenal transplantation, SF-1 KO mice received 3 more corticosteroid injections on days 9, 12 and 16 until weaning on P21. WT mice used for controls were subjected to the same corticosteroid treatment protocol as were SF-1 KO mice. After weaning, mice of the same chromosomal sex were group housed (2–3 per cage) until the time of sacrifice (gonadectomized WT and SF-1 KO mice were never housed with the gonadally intact WT mice, due to the possible aggressive behavior of WT males).

Some WT mice were gonadectomized before puberty (P21) to prepare controls with a gonadal status comparable to that of SF-1 KO mice. For gonadectomies, WT mice were anesthetized with a mixture of ketamine [Vetoquinol Biowet, Gorzów Wielkopolski, Poland; 100 µg/g body weight (BW)], xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 µg/g BW) and acepromazine

(Fort Dodge Animal Health, Fort Dodge, Iowa, USA; 2 µg/g BW), and ovaries and testes were removed through a single or bilateral incision, respectively. After gonadectomy, the mice received 2 injections of the analgesic butorphanol (Fort Dodge Animal Health; 1.7 µg/g BW). To control for gonadectomy, gonadally intact WT and SF-1 KO mice were sham operated at P21.

To study the effect of EB exposure during a pubertal time period, SF-1 KO and gonadectomized WT mice were anesthetized with a mixture of ketamine, xylazine and acepromazine and implanted subcutaneously with Silastic capsules [1.02 mm i.d. × 2.16 mm o.d.; Dow Corning, Midland, Mich., USA; 5 mm of tubing filled with EB (Sigma) and cholesterol (Sigma) at a ratio of 1:1] for the period from P25 to P36 as reported previously [58].

At the time of sacrifice, the mice were anesthetized as described above and perfused with 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer (pH = 7.4). All adult, gonadally intact WT females were sacrificed in diestrus, based on vaginal smear cytology. After removal, the brains remained in the same fixative overnight at 4°C and were then stored until immunocytochemical processing in 0.1 M phosphate buffer at 4°C.

Puberty was determined by monitoring vaginal opening in a separate cohort of 10 female WT and SF-1^{+/-} mice. The time of vaginal opening ranged from P34 to P38, with most female mice in the colony presenting with vaginal opening by P36.

Experimental Design

To study the effects of sex chromosomes and gonadal steroids on kisspeptin sexual differentiation in the brain, SF-1 KO mice (M: n = 18; F: n = 19) were compared to gonadally intact (M: n = 19; F: n = 18) or gonadectomized (M: n = 9; F: n = 10) control WT mice at three different stages of development: before puberty at P25, approximately during puberty at P36 and in young adulthood at P60. The effect of EB on kisspeptin brain sexual differentiation was studied in gonadectomized WT mice (M: n = 5; F: n = 6) and SF-1 KO mice (M: n = 6; F: n = 5) that were implanted with EB Silastic capsules from P25 to P36 (during a pubertal period) and sacrificed at P36.

IHC on Floating Brain Sections

Brains were embedded in 5% agarose (Sigma) and sectioned at 50 µm in cold 0.05 M PBS using a vibrating microtome (Integralslice 7550 MM; Campden Instruments, Loughborough, UK). Sections were placed in alternating containers to aid free-floating tissue processing and to help track locations more readily. Before primary antibodies, sections were incubated in 0.1 M glycine (Sigma) and 0.5% sodium borohydride (Sigma) in 0.05 M PBS for 30 min and 15 min at 4°C, respectively. They were then blocked in 5% normal goat serum (Chemicon, Temecula, Calif., USA) containing 0.5% Triton X-100 (Sigma) and 1% H₂O₂ (Merck, Darmstadt, Germany) for 30 min at 4°C. Sections were incubated in rabbit primary antiserum against kisspeptin 10 (gift from Dr. A. Caraty; this antiserum has been used and validated before [16]) diluted 1:30,000 in 0.05 M PBS containing 1% bovine serum albumin (Sigma) and 0.5% Triton X-100 over 3 nights at 4°C with shaking. They were then washed in 0.05 M PBS containing 1% normal goat serum and 0.02% Triton X-100 four times 15 min at room temperature. Biotinylated secondary antibodies (Jackson ImmunoResearch, West Grove, Pa., USA) against primary rabbit antisera were diluted 1:500 in 0.05 M PBS containing 1% normal goat serum and 0.5% Triton X-100. Sections were incubated with secondary anti-

bodies for 2 h, followed by 4 washes (15 min each) in 0.05 M PBS buffer containing 0.02% Triton X-100. Streptavidin-horseradish peroxidase complex (Jackson ImmunoResearch) was diluted 1:2,500 in 0.05 M PBS solution containing 0.5% Triton X-100. Sections were incubated with streptavidin-conjugated horseradish peroxidase for 1 h at room temperature and then washed in Tris-buffered saline (0.05 M Tris-HCl/0.9% NaCl; pH 7.5; Sigma) for 1 h at room temperature. Antigen-antibody complexes were visualized as a black reaction product by incubating sections in 0.025% 3,3'-diaminobenzidine/ammonium nickel(II) sulfate substrate (Sigma) in Tris-buffered saline (pH 7.5) containing 0.02% H₂O₂ for 5 min at room temperature. After mounting on slides, sections were dried and coverslipped using hydrophobic medium (Pertex, Burgdorf, Germany). Immunocytochemical controls were based on omitting the primary antibodies from control sections and on a validation of immunoreactivity with patterns of distribution from a prior report [16].

Data Collection and Analyses

Digital images of the AVPV and Arc were obtained using a Nikon Eclipse 80i microscope with a Nikon DS-Fi1 camera. Kisspeptin-ir cells in the AVPV were counted directly under the microscope in coronal sections considered in order from rostral to caudal on the microscopic slides. Cells were counted on both the left and the right side of the AVPV on two sections that always corresponded to sections approximately 0.14 and 0.26 mm rostral to bregma according to stereotaxic coordinates [59]. Kisspeptin immunoreactivity on both the left and the right side of the Arc was quantified in coronal sections ($\times 100$ magnification; the third ventricle and base of the brain served as reference boundaries) corresponding to sections approximately 1.7 mm caudal to bregma according to stereotaxic coordinates [59] using a custom software (Surfkvad; made by Dr. Marko Kreft, Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana). For the purpose of analysis with Surfkvad, all digital images were taken with the same illumination settings and prepared accordingly using the Adobe Photoshop software package (version 8.0) as follows: digital images were standardized for illumination, which eliminated possible differences in intensity of the background between images, were converted to grayscale and were then subjected to threshold conversion in order to selectively identify immunoreactive elements with the threshold limit set to 50%. Black-and-white images were then analyzed with Surfkvad software, which calculates the percentage of dark area (immunoreactivity) in a boxed area of interest ($940 \times 970 \mu\text{m}$) bounded by the third ventricle medially and the base of the brain.

Statistical Analysis

Statistical analysis was performed using the NCSS software package (NCSS 2007; NCSS, Kaysville, Utah, USA). Statistical differences in the number of kisspeptin-ir cells in the AVPV and the kisspeptin-ir area in the Arc between gonadally intact WT mice, gonadectomized WT mice and SF-1 KO mice were examined by three-factor ANOVA with sex, genotype/gonadal status and age as independent factors. Differences in the number of kisspeptin-ir cells in the AVPV between different stages of development in gonadally intact male and female WT mice were examined in a planned comparison by two-factor ANOVA with sex and age as independent factors. Differences in the kisspeptin-ir area between different developmental stages in gonadally intact WT and SF-1 KO mice were examined in a planned comparison with sex, geno-

Fig. 1. The digital inset image shows representative sections with kisspeptin-ir cells in the AVPV at P60 in WT male (WTM) and WT female (WTF) mice. The graph illustrates the number of kisspeptin-ir cells in the AVPV in gonadally intact WTM and WTF mice, WT/CAS and WT/OVX mice (gonadectomized at P21) and agonadal SF-1 KO male (KOM) and female (KOF) mice in three different developmental stages: at P25 (before puberty), at P36 (during puberty) and at P60 (in adulthood). Numbers of cells are presented as means $\pm$ SEM. * $p < 0.05$ (different from WTM, WT/CAS, WT/OVX, KOM and KOF mice); ^a $p < 0.05$ (different from WTF mice at P25); ^b $p < 0.05$ (different from WTF mice at P36). 3V = Third ventricle; OT = optic tract. Scale bar = 100 μm .

type and age as independent factors. Differences in the kisspeptin-ir area between gonadally intact WT, gonadectomized WT and SF-1 KO mice at P36 only were examined in a planned comparison by three-factor ANOVA with sex, genotype and gonadal status as independent factors. The effect of EB on the number of kisspeptin-ir cells in the AVPV and the kisspeptin-ir area in the Arc was examined in the 36-day-old mice only by three-factor ANOVA with genotype/gonadal status, sex and treatment as independent factors. When applicable, Fisher's least significant difference (LSD) post hoc analyses were used to determine statistical differences between groups. Statistical differences were considered significant at $p < 0.05$. All data are presented as means $\pm$ SEM.

Results

Lack of SF-1 Did Not Result in WT Female Levels of Kisspeptin-ir Cells in the AVPV in the Absence of Gonadal Steroids

As expected, kisspeptin-ir cells in the AVPV were detected in the periventricular area (fig. 1). The number of kisspeptin-ir cells in the AVPV increased with age from

Fig. 2. The graph illustrates the number of kisspeptin-ir cells in the AVPV from 36-day-old, gonadally intact WT male (WTM) and female (WTF) mice (same bars as in fig. 1, included for comparison), WT/CAS and WT/OVX mice (gonadectomized at P21) and SF-1 KO male (KOM) and female (KOF) mice. Gonadectomized mice and agonadal SF-1 KO mice were treated with EB from P25 to P36. Treatment restored the number of kisspeptin-ir cells in WT/OVX mice to the levels seen in WTF mice, while the number of kisspeptin-ir cells in agonadal SF-1 KO mice of both sexes was significantly lower ($p < 0.05$) in comparison to that in WTF mice even after EB treatment. Numbers of cells are presented as means $\pm$ SEM. * $p < 0.05$ (lower than in EB-treated WT/OVX and untreated WTF mice).

P25 to P60 and reached a maximum at P60 [$F(4, 103) = 25.73$; $p < 0.001$, Fisher's LSD; $p < 0.05$] in gonadally intact male and female WT mice in a sex-specific manner [$F(2, 103) = 81.61$; $p < 0.001$, Fisher's LSD; $p < 0.05$]. Furthermore, a planned comparison showed that the number of kisspeptin-ir cells significantly [$F(2, 35) = 17.68$; $p < 0.001$, Fisher's LSD; $p < 0.05$] increased from P25 to P36 and from P36 to P60 in gonadally intact WT females but not males, which led to higher [$F(2, 35) = 17.68$; $p < 0.001$, Fisher's LSD; $p < 0.05$] kisspeptin-ir cell numbers in WT females than in WT males at P25, P36 and P60. Gonadectomy in WT mice before puberty at P21 led to significantly reduced numbers of kisspeptin-ir cells in males (WT/CAS) and females (WT/OVX) in comparison to gonadally intact WT mice [$F(2, 103) = 81.61$; $p < 0.001$, Fisher's LSD; $p < 0.05$]. In gonadectomized WT mice, a sex difference in the number of kisspeptin-ir cells was conserved at P25 ($p < 0.05$, Fisher's LSD), but not at P36 and P60, when kisspeptin-ir cells were not detected in this

area in either sex. In agonadal SF-1 KO mice, the total number of kisspeptin-ir cells was indistinguishable ($p > 0.05$, Fisher's LSD) from that in gonadally intact/gonadectomized WT males regardless of sex at all three ages studied (fig. 1), and it did not differ ($p > 0.05$, Fisher's LSD) from that in WT/OVX females at P60.

Lack of SF-1 Did Not Result in Female Levels of Kisspeptin-ir Cells in the AVPV in the Presence of Gonadal Steroids

To examine the effect of exposure to EB on the number of kisspeptin-ir cells in the AVPV during puberty, gonadectomized WT and agonadal SF-1 KO mice were exposed to EB from P25 to P36. EB treatment increased [$F(1, 55) = 23.29$; $p < 0.001$] the total number of kisspeptin-ir cells in both gonadectomized WT and SF-1 KO mice. A significant interaction [sex $\times$ genotype/gonadal status $\times$ treatment; $F(2, 55) = 4.37$; $p < 0.05$] suggests that EB treatment from P25 to P36 restored the total number of kisspeptin-ir cells to WT female levels only in WT/OVX mice, but surprisingly not in male and female agonadal EB-treated SF-1 KO mice, which had significantly fewer [$F(2, 55) = 20.86$; $p < 0.001$, Fisher's LSD; $p < 0.05$] kisspeptin-ir cells compared to EB-treated WT/OVX and untreated, gonadally intact female WT mice (fig. 2).

Lack of SF-1 Did Not Prevent Sexually Dimorphic Kisspeptin Immunoreactivity in the Arc at the Time of Puberty Onset in the Absence of Gonadal Steroids

The kisspeptin-ir area in the Arc increased with age from P25 to P60, reaching a maximum at P60 [$F(4, 92) = 7.97$; $p < 0.001$, Fisher's LSD; $p < 0.05$], in gonadally intact WT mice in a sex-specific manner [$F(2, 92) = 23.51$; $p < 0.001$, Fisher's LSD; $p < 0.05$], with gonadally intact WT females having a significantly greater immunoreactive area than males at all three ages studied [$F(4, 92) = 3.09$; $p < 0.05$, Fisher's LSD; $p < 0.05$]. Overall, gonadectomy at P21 significantly decreased [$F(2, 92) = 26.87$; $p < 0.001$] the kisspeptin-ir area in comparison to gonadally intact WT mice in a sex-dependent manner, with WT/OVX mice having a significantly greater [$F(2, 92) = 23.51$; $p < 0.001$, Fisher's LSD; $p < 0.05$] kisspeptin-ir area than WT/CAS mice (fig. 3).

The kisspeptin-ir area in the Arc was smaller in male and female SF-1 KO mice than in gonadally intact female WT and WT/OVX mice [$F(2, 92) = 23.51$; $p < 0.001$, Fisher's LSD; $p < 0.05$] at all ages studied (fig. 3), and this was due to the substantial increase observed at P25 in WT/OVX mice, which may reflect increased kisspeptin immunoreactivity after gonadectomy, as reported previous-

Fig. 3. a–d Representative images of kisspeptin immunoreactivity in the Arc at P36 in gonadally intact WT male (WTM; **a**) and female (WTF; **b**) as well as in agonadal SF-1 KO male (KOM; **c**) and female (KOF; **d**) mice. Gonadally intact WTF and agonadal KOF mice had a significantly greater kisspeptin-ir area in the Arc during puberty at P36 than gonadally intact WTM and agonadal KOM mice, respectively. **e** The graph illustrates the kisspeptin-ir area in the Arc in gonadally intact WTM and WTF mice, WT/CAS and WT/OVX mice (gonadectomized at P21) and agonadal KOM and KOF mice in three different developmental stages: at P25 (before

puberty), at P36 (during puberty) and at P60 (in adulthood). The kisspeptin-ir area is presented as the mean $\pm$ SEM. * $p < 0.05$ (different from WTM, WT/CAS, WT/OVX and SF-1 KO mice over all ages studied); *** $p < 0.001$ (higher immunoreactivity in WT than in gonadectomized WT mice); # $p < 0.05$ (different from WT/OVX mice); ^a $p < 0.05$ (different from WTF and WT/OVX mice); ^b $p < 0.05$ (different from WTF, WT/OVX and WT/CAS mice); ^c $p < 0.05$ (higher immunoreactivity than in KOM mice at P36). 3V = Third ventricle; ME = median eminence. Scale bar = 100 μ m.

ly [12]. Later, at P36 and P60, the kisspeptin-ir area in WT/OVX mice was within the range of immunoreactivity found in agonadal SF-1 KO mice. However, female agonadal SF-1 KO mice had a significantly greater immunoreactive area in the Arc than WT/CAS mice [$F(2, 92) = 23.51$; $p < 0.001$, Fisher's LSD; $p < 0.05$] (fig. 3).

Interestingly, the kisspeptin-ir area in gonadally intact WT females and SF-1 KO females reached adult levels already at P36, while in gonadally intact WT males and SF-1 KO males, the immunoreactive area at P36 was smaller ($p < 0.05$; Fisher's LSD) than that at P60 (fig. 3). This suggests a later maturation of immunoreactive kisspeptin levels in gonadally intact WT and SF-1 KO males in comparison to females, and this is likely regulated by gonad-independent factors, as it is conserved in SF-1 KO mice.

Sexually Dimorphic Kisspeptin Immunoreactivity in the Arc Was Eliminated in the Presence of Sex Steroid Hormones in Mice Lacking SF-1

To examine the effect of sex steroid hormones on immunoreactive kisspeptin levels after gonadectomy, WT

mice gonadectomized at P21 and SF-1 KO mice were treated with EB from P25 to P36 and examined at P36. An analysis did not reveal any statistically significant effect of EB treatment on the kisspeptin-ir area, although, interestingly, slight increases in immunoexpression in SF-1 KO males and slight decreases in kisspeptin immunoexpression in SF-1 KO females, respectively, eliminated the sex difference observed in hormonally naïve SF-1 KO mice at P36 (fig. 4). In gonadectomized WT mice, the kisspeptin-ir area remained greater [$F(1, 52) = 44.08$; $p < 0.001$, Fisher's LSD; $p < 0.05$] in females than in males.

Discussion

Neurons expressing the *Kiss1* gene and its receptor have a major role in the regulation of GnRH release in the hypothalamus and in the onset of puberty, as evidenced by studies in mice [2, 9, 60] and humans [1, 3]. In the murine brain, expression of *Kiss1* is sex dependent, and this sex difference is usually considered a result of exposure to

Fig. 4. The graph illustrates the kisspeptin-ir area in the Arc from 36-day-old WT/CAS and WT/OVX mice gonadectomized at P21 and SF-1 KO male (KOM) and female (KOF) mice. All mice were either untreated or treated with EB from P25 to P36. EB treatment from P25 to P36 had no effect on the kisspeptin-ir area in both WT/CAS and WT/OVX mice when compared to untreated gonadectomized WT mice. However, EB treatment did eliminate the sex difference in agonadal SF-1 KO mice at P36 by moderately (nonsignificantly) increasing immunoexpression in males and decreasing immunoexpression in females. The kisspeptin-ir area is presented as the mean $\pm$ SEM. * $p < 0.05$ (higher immunoreactivity in WT/OVX than WT/CAS mice).

sex steroid hormones, especially neonatally in male rats [15], but also prenatally in mice [27]. Previous studies have suggested that the numbers of *Kiss1* mRNA-expressing [61] and kisspeptin-ir neurons [20] reach adult levels by the time of puberty and that EB treatment before/during puberty is sufficient for the full restoration of these levels in the AVPV in WT/OVX mice [20].

In the present study, the kisspeptin-ir neural population in the AVPV was therefore examined in agonadal SF-1 KO mice during postnatal development and after treatment with EB during the pubertal period. If perinatal gonadal steroids are sufficient to cause a masculine pattern of kisspeptin expression in adult mice, then in agonadal SF-1 KO mice (regardless of genetic sex) kisspeptin immunoreactivity should follow a female pattern of expression after treatment with EB. The results of the current study, however, show a diminished number of cells containing immunoreactive kisspeptin in untreated agonadal SF-1 KO mice from P25 to early adulthood. Treat-

ment of WT/OVX mice with EB from P25 to P36 restored the number of kisspeptin-ir cells to the levels found in gonadally intact female WT mice at P36 in the AVPV. In contrast, similar treatment of SF-1 KO mice of both sexes did not produce similar numbers of kisspeptin-ir cells.

An analysis of the significant interaction between genotype/gonadal status, sex and treatment suggests that treatment from P25 until P36 was not sufficient for full induction of immunoreactive kisspeptin in cells in the AVPV in SF-1 KO mice, and that either earlier programming of this response or some other factor(s) besides estradiol is needed for full induction of immunoreactive kisspeptin in the AVPV. Such programming could be caused by earlier exposure to gonadal steroid hormones or some other gonadal factors that are absent in SF-1 KO mice due to early gonadal agenesis. These results are similar in part to results from mice lacking the aromatase enzyme (ARKO mice) [23]. In that study, postnatal or adult treatment with EB induced expression of kisspeptin in the hypothalamus of male and female ARKO mice (that normally express very low levels of kisspeptin), but the number of cells in ARKO mice was lower than in WT mice even after treatment with EB, suggesting an earlier organizational period, probably dependent on ovarian steroid hormones [23]. In agreement, another report [21] indicated that exposure to estradiol during an earlier juvenile period – and not just during puberty, as suggested in some previous studies [20] – is likely needed for full feminization of the kisspeptin system in the AVPV region. Taken together, it seems likely that there are additional factors, perhaps an early exposure to low levels of ovary-derived estrogens between P10 and P15, that are indispensable for the development of *Kiss1* neurons in the AVPV in female mice, and possibly some other female-specific factors [23, 62–64].

In the present study, immunoreactive kisspeptin in Arc fibers was followed from before puberty (P25) to after puberty (P60) in gonadally intact/gonadectomized WT and agonadal SF-1 KO mice. Previous studies have shown that besides gonadal steroid hormones [12–15, 19, 23], *Kiss1* expression at the mRNA and peptide level in the Arc is also regulated by various biological molecules including neuropeptides (dynorphin A [34], neurokinin B [35] and prolactin [37]), neurotransmitters (GABA and glutamate [43, 44]), trophic factors (LRH-1 [42], insulin [38] and mTOR signaling [39]), and metabolic factors (leptin [36], ghrelin [41] and neuropeptide Y [40]). Further, functional changes in *Kiss1* mRNA expression in the Arc associated with puberty control independent of gonadal hormones were reported to depend on the poly-

comb group of transcriptional silencers [33]. The kisspeptin-ir area in adult, gonadally intact WT mice was established through a gradual increase starting from P25 in both male and female WT mice, with females having a greater immunoreactive area than males at all ages studied. The kisspeptin-ir area in the Arc in agonadal SF-1 KO mice did not differ between sexes at P25 and P60, but there was a significant difference between SF-1 KO males and females during puberty at P36. While gonadectomy decreased kisspeptin immunoreactivity in the Arc in both males and females in comparison to gonadally intact WT mice, it did not eliminate the sex differences in the kisspeptin-ir area. Similarly as in intact WT mice, gonadectomized WT females had a significantly greater kisspeptin-ir area than gonadectomized WT males at all ages studied. These results suggest that sexual differentiation of the kisspeptin system in the Arc during postnatal development in mice depends on gonadal hormone-dependent factors. However, our results also suggest that sexual differentiation of the kisspeptin system during puberty differs between males and females, and this depends on additional regulatory mechanisms provided by gonad-independent factors, as has been observed in agonadal SF-1 KO mice.

Functional reactivation of GnRH neurons and consequent activation of the hypothalamic-pituitary-gonadal axis represents a hallmark of the beginning of puberty that occurs earlier in girls than in boys. In spite of dealing with the regulation of gonadal hormone synthesis and the almost complete dependence of *Kiss1* mRNA and kisspeptin expression on gonadal hormones, it is interesting that some changes in gene expression controlling GnRH neuron activity occur independently of circulating gonadal levels. For example, increases in *Kiss1* mRNA expression in the Arc during puberty occur in male mice gonadectomized at P14 [65] and in male and female hypogonadal (*hpg*) mice [32]. Observations in rats that the hypothalamic-pituitary-gonadal axis becomes responsive to kisspeptin-54 at P30 and not earlier [66] further suggest a regulation of GnRH neuron reactivation by intrinsic biological clocks. The current study extends these observations by showing sex-specific increases in the kisspeptin-ir area in the Arc during puberty in agonadal SF-1 KO mice. At the time of puberty, female agonadal SF-1 KO mice had a significantly greater area containing kisspeptin immunoreactivity than male agonadal SF-1 KO mice. The levels of immunoreactive kisspeptin were maximal in female agonadal SF-1 KO mice already at P36, while there was a significant increase between days 36 and 60 in SF-1

KO males, suggesting that males reach the adult pattern of expression later than females, possibly due to later pubertal development. Despite the differences in the total area of kisspeptin immunoreactivity between agonadal SF-1 KO and gonadally intact WT mice, developmental dynamics in the area of kisspeptin immunoreactivity similar to those in agonadal SF-1 KO mice were observed in gonadally intact WT mice. The results of the current study suggest that sexually dimorphic timing in changes in the area of kisspeptin immunoreactivity during puberty is regulated by gonad-independent factors.

Sex differences in kisspeptin expression in the Arc during early postnatal development in mice to some extent depend on gonadal hormones [23]. In the current study, gonadectomy before puberty at P21 decreased but did not eliminate kisspeptin immunoreactivity in male and female WT mice later during puberty (at P36) and in early adulthood (at P60) in a sex-specific manner. However, treatment with EB during puberty from P25 to P36 did not restore the kisspeptin-ir area in gonadectomized WT mice to the levels in gonadally intact WT mice, suggesting that other gonadal hormones (perhaps testosterone in males [13] and progesterone in females) and/or other as yet unknown factors regulate the levels of immunoreactive kisspeptin during puberty in the Arc. This is in contrast to the results of a study in ARKO mice that reported a complete restoration of kisspeptin immunoreactivity in the Arc in adult WT and ARKO mice after 10 days of treatment with estradiol or dihydrotestosterone [23]. However, the mice in that study were left gonadally intact during puberty and gonadectomized only in adulthood, which might have impacted the Arc *Kiss1* circuitry differently than prepubertal gonadectomy. Furthermore, the mice in the present study were not treated with colchicine before sacrifice, which is why the effect of EB on the increased kisspeptin-ir area could be masked by an increased kisspeptin transport and secretion. Kisspeptin expression in the Arc was studied by IHC, which did not allow us to determine whether there were other factors regulating the amount of immunoreactive kisspeptin in fibers. Therefore, the current data (area of kisspeptin immunoreactivity) only provide information about the quantity of immunoreactive kisspeptin in fibers in the Arc at the time of sacrifice.

In conclusion, the results of the current study show that EB treatment from P25 to P36 could not restore the number of kisspeptin-ir cells in the AVPV in agonadal SF-1 KO mice to numbers found in gonadally intact WT

or EB-treated WT/OVX females. This suggests that either earlier programming by sex steroids (before P21) is needed for a normal expression of kisspeptin in the AVPV or other factor(s) besides estradiol, either at puberty or during earlier development, are needed for the capacity to express kisspeptin in the AVPV. Furthermore, results from the Arc analyses suggest that maturation of immunoreactive kisspeptin in the Arc occurs at different ages in males and females, and this sex difference is independent of gonads, since it was also observed in agonadal SF-1 KO mice.

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