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# Absence of Gonadotropin-Releasing Hormone 1 and Kiss1 Activation in α-Fetoprotein Knockout Mice: Prenatal Estrogens Defeminize the Potential to Show Preovulatory Luteinizing Hormone Surges

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**Sex differences in gonadal function are driven by either cyclical (females) or tonic (males) hypothalamic GnRH1 release and, subsequently, gonadotrophin (LH and FSH) secretion from the pituitary. This sex difference seems to depend on the perinatal actions of gonadal hormones on the hypothalamus. We used α-fetoprotein (AFP) knockout mice ($Afp^{-/-}$) to study the mechanisms by which estrogens affect the sexual differentiation of the GnRH1 system. $Afp^{-/-}$ mice lack the protective actions of AFP against estrogens circulating during embryonic development, leading to infertility probably due to a hypothalamic dysfunction. Therefore, we first determined whether $Afp^{-/-}$ females are capable of showing a steroid-induced preovulatory LH surge by FOS/GnRH1 immunohistochemistry and RIA of plasma LH levels. Because the KISS1/GPR54 system is a key upstream regulator of the GnRH1 system as well as being sexually dimorphic, we also analyzed whether Kisspeptin-10 neurons were activated in $Afp^{-/-}$ mice after treatment with estradiol and progesterone. We found that the GnRH1 and Kisspeptin-10 neuronal systems are defeminized in $Afp^{-/-}$ females because they did not show either steroid-induced LH surges or significant FOS/GnRH1 double labeling. Furthermore, Kisspeptin-10 immunoreactivity and neural activation, measured by the number of double-labeled FOS/Kisspeptin-10 cells, were lower in $Afp^{-/-}$ females, suggesting a down-regulation of GnRH1 function. Thus, the sex difference in the ability to show preovulatory LH surges depends on the prenatal actions of estrogens in the male hypothalamus and, thus, is lost in $Afp^{-/-}$ females because they lack AFP to protect them against the defeminizing effects of estrogens during prenatal development. (*Endocrinology* 149: 2333–2340, 2008)**

GnRH1 IS THE key hormone that provokes the release of LH and FSH from the pituitary gonadotrophs. Moreover, the GnRH1 system presents sex differences in their function, *i.e.* triggering a cyclical gonadal function in females and a distinct tonic gonadal function in males.

Both GnRH1 and gonadotrophin hormones are regulated by steroid hormones, in particular estrogens, acting at the level of the hypothalamus and pituitary, respectively. For most of the estrous cycle, estrogens exert a negative feedback on the hypothalamus-pituitary axis. By contrast, on the afternoon of proestrus, estrogens exert a positive feedback that produces the release of GnRH1 from the median eminence and, subsequently, LH from the pituitary. Both are essential for the induction of ovulation (1, 2). Thus, ovarian estradiol (E2) secreted by the developing follicles from metestrus through early proestrus sensitizes the pituitary to become more responsive to GnRH1 (3). In males this hypothalamic-pituitary positive feedback does not occur, and, therefore, there is no ovulation in castrated males that were implanted with ovarian tissue (4). This sex difference presumably depends on the perinatal actions of testosterone and/or E2 on the hypothalamus (4, 5) because male rats that were treated neonatally with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) showed cyclical gonadal function when grafted with ovarian tissue (4).

We recently showed that female mice carrying a targeted mutation in the α-fetoprotein (AFP) gene, $Afp^{-/-}$, are infertile (6, 7). AFP is a major plasma protein that circulates in high concentrations during embryonic development and plays an important role in the sexual differentiation of the rodent brain due to its estrogen-binding capacity (8). The fertility, as well as sexual behavior, of $Afp^{-/-}$ females could be rescued by blocking estrogen production (and, thus, reducing the excessive levels of estrogens derived from the mother and male siblings) during prenatal development by administration of ATD (6, 9). These results indicate that AFP serves to protect the developing female rodent brain from the defeminizing effects of estrogens during prenatal development and have resolved a long-running debate on the role of this fetal protein in brain sexual differentiation.

$Afp^{-/-}$ females are clearly not hypogonadal because their ovaries are normal size, and they have normal, wild-type levels of circulating E2. Furthermore, after an artificial induction of ovulation with gonadotropins, $Afp^{-/-}$ and wild-

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Abbreviations: Ab, Antibody; ADP, anterodorsal preoptic nucleus; AFP, α-fetoprotein; ArKO, aromatase knockout; ATD, 1,4,6-androstatriene-3,17-dione; AVPe, anteroventral periventricular nucleus; DAB, 3,3′-diaminobenzidine; E2, estradiol; EB, estradiol benzoate; ER, estrogen receptor; GPR54, G protein-coupled receptor 54; NGS, normal goat serum; P, progesterone; pAb, polyclonal antibody; PBST, PBS-0.1% Triton X-100; Pe, periventricular hypothalamic nucleus; SO, sesame oil.

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type females exhibited similar numbers of blastocysts in the uterus, indicating that their normal anovulatory state is not due to an ovarian defect (6, 7). Thus, the abnormal sexual cycle found in $Afp^{-/-}$ females reflects a failure to enter into the proestrus phase of the cycle during which the LH surge occurs. Furthermore, they have decreased, male-like numbers of tyrosine hydroxylase-expressing neurons in the anteroventral periventricular nucleus (AVPe) of the preoptic area, a brain region implicated in the positive feedback actions of E2 on the GnRH1 system (10), and they show a transcriptional down-regulation of several genes included in the GnRH1 pathway, including the *Gnrh1* gene itself and its receptor (6).

Thus, these results indicate that the ability to show steroid-induced preovulatory GnRH1 and LH surges may be defeminized in female $Afp^{-/-}$ mice because they are not protected from prenatal estrogens by AFP. Therefore, in the present study, we first determined whether $Afp^{-/-}$ mice are capable of showing a steroid-induced GnRH1 and LH surge. We used the expression of FOS, an immediate early gene product that serves as a neuronal activation marker (11), to determine whether hypothalamic GnRH1 neurons were activated by hormonal replacement treatments with either E2 or progesterone (P). Because we found that $Afp^{-/-}$ females did not show steroid-induced preovulatory GnRH1 and LH surges, we next determined whether upstream signals from GnRH1, such as Kisspeptin, were also altered in $Afp^{-/-}$ females. Kisspeptin is the peptide product of the *Kiss1* gene and the endogenous agonist of the G protein-coupled receptor 54 (GPR54). Recent evidence suggests that the KISS1/GPR54 system is a key upstream regulator of the GnRH1 system. Mutations or targeted deletions of the *GPR54* gene point to this gene as the key regulator for the initiation of puberty (12). Additional studies have revealed that KISS1/GPR54 signaling may serve an important regulatory function in the neuroendocrine reproductive axis after puberty as well (13, 14). Furthermore, it has been demonstrated that peripheral as well as intracerebroventricular administration of the active Kisspeptin fragment, Kisspeptin-10, stimulates the hypothalamo-pituitary-gonadal axis and increases plasma gonadotrophins in both male and female adult rats (15–19). In fact, Kisspeptin has been the most potent activator of GnRH1 neuron firing yet to be discovered (20), acting through the GPR54 in these neurons. Most importantly, a greater number of Kisspeptin-10-immunoreactive neurons are present in the AVPe and periventricular hypothalamic nucleus (Pe) of female as opposed to male mice and rats (21), supporting the hypothesis that Kisspeptin-10 neurons may play a specific role in stimulating the preovulatory LH surge.

## Materials and Methods

### *Experimental animals*

All breeding and genotyping were performed at the Université Libre de Bruxelles, Institut de Biologie et Médecine Moléculaires, Gosselies, Belgium. In the present studies, $Afp^{-/-}$ of the CD1 strain (*Afp* KO1, allele $Afp^{tm1lbmm}$) were used as experimental subjects (7). At the time of the study, these mice had been backcrossed for at least 20 generations. All mice were housed in groups of the same sex, genotype, and treatment under a reversed light-dark cycle (14-h light, 10-h dark cycle; 2300 h lights on and 1300 h lights off). Food and water were always available *ad libitum*. All experiments were conducted in accordance with the guidelines set forth by the National Institutes of Health Guiding Principles for the Care and Use of Research Animals, and were approved by the Ethical Committees for Animal Use of the University de Liège and of the Université Libre de Bruxelles, Institut de Biologie et Médecine Moléculaires.

### *Surgery and hormone feedback*

In adulthood, all subjects were gonadectomized under general anesthesia using a mixture of ketamine and medetomidine (80 and 1 mg/kg, respectively, ip). Mice were injected with atipamezole (4 mg/kg sc) at the end of the surgery to antagonize the medetomidine-induced effects and accelerating their recovery.

At the time of gonadectomies, males and females were implanted sc with a 5-mm long SILASTIC brand capsule (inner diameter: 1 mm; outer diameter: 2 mm; Dow Corning, Corp., Midland, MI) containing crystalline 17$\beta$-E2 diluted in sesame oil (SO) (1 $\mu$g/20 g body weight). Animals were divided into three groups. Group 1 was treated sequentially with estradiol benzoate (EB) and P. Group 2 was treated only with EB, whereas group 3 received no additional hormone treatments and served as a control. Thus, 1 wk after surgery, a single injection of EB (1 $\mu$g/20 g body weight/0.05 ml SO) was administered sc to each animal in groups 1 and 2 at 0900 h (d 1). Then on d 2, 500 $\mu$g P in 0.05 ml SO was administered to each animal in group 1 at 0900 h, whereas none of the animals from group 2 was injected. The same day at 1300 h, when lights went off, all animals were anesthetized with ketamine/medetomidine, and a blood sample was taken by heart puncture to perform the plasma LH content analysis by RIA, before being perfused with paraformaldehyde. The EB plus P treatment has reliably induced preovulatory LH surges in mice (22, 23). We have used P as additional hormone because it has been demonstrated that in ovariectomized, E2 primed rodents, LH surges comparable to those observed in intact proestrus females were produced by injecting both EB and P (2), and that the activation of the P receptors by P during proestrus was necessary for showing increased LH surges (24).

The total number of animals in this study was: 11 $Afp^{+/+}$ female controls; eight $Afp^{+/+}$ EB plus P-treated females; seven $Afp^{+/+}$ EB-treated females; four $Afp^{-/-}$ female controls; eight $Afp^{-/-}$ EB plus P-treated females; five $Afp^{-/-}$ EB-treated females; five $Afp^{+/+}$ male controls; six $Afp^{+/+}$ EB plus P-treated males; three $Afp^{+/+}$ EB-treated males; four $Afp^{-/-}$ EB plus P-treated males; and six $Afp^{-/-}$ EB-treated males.

### *Immunohistochemistry*

To determine the distribution of FOS, GnRH1, and Kisspeptin-10 in $Afp^{+/+}$ and $Afp^{-/-}$ mice, animals were perfused with 4% paraformaldehyde in 0.1 M PBS. Brains were postfixed for 2 h and then transferred to 30% sucrose until they sank. They were then frozen on dry ice and stored at −80 C until used for immunohistochemistry. Cryostat (Leica CM1510 S; Leica Microsystems GmbH, Wetzlar, Germany) brain sections were cut from the rostral telencephalon to the posterior hypothalamus making three sets of 30-$\mu$m sections.

Primary antibodies were specific to FOS polyclonal antibody (pAb) (1:2,000 sc-52 rabbit c-*fos* Ab; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), GnRH1 pAb (1:10,000 LR-1 rabbit Ab, donated by Dr. R. Benoit, Montreal General Hospital, Montreal, Canada), and Kisspeptin-10 [Kisspeptin-10 pAb; Kisspeptin-10 is a polypeptide derived from the *KiSS-1* product; 1:20,000 rabbit Ab, donated by Dr. A. Caraty (Unité Mixte de Recherche Physiologie de la Reproduction, Institut National de la Recherche Agronomique, Centre National de la Recherche Scientifique, Université de Tours, Nouzilly, France) (25)].

The specificity of the Kisspeptin-10 Ab to detect Kisspeptin has been validated recently (26). In the latter study, a clear Kisspeptin-10 immunoreaction was shown in wild-type animals, but no immunoreactivity was observed in mice lacking a functional *Kiss1* gene. In addition, several experiments have been conducted to test the specificity of this Ab (25). They investigated a possible cross-reactivity with peptides of similar size and/or known to be present in the same hypothalamic structures. No cross-reaction could be observed with any of the eight hypothalamic peptides tested, including GnRH1, galanin, neuropeptide Y, specificity protein, $\alpha$-MSH, somatostatin, CRH, and prolactin releasing peptide. Moreover, we have tested the specificity of the Ab by preabsorbing for 2 h at room temperature 1 $\mu$g Ab with 2 $\mu$g of the polypeptide YN-

WNSFGLRY-NH2 corresponding to amino acid residues 43–52 of mouse metastin (Kisspeptin-10) immunogen used for raising this Ab. This control has been done in adjacent sections and shows no labeling with the preabsorbed Ab, as can be seen in Fig. 1H.

For the double-labeling immunohistochemistry, sections were first washed in 0.1 M PBS (pH 7.4), then the peroxidase activity was blocked in 1:4 methanol-PBS solution with 30% $H_2O_2$. Sections were permeabilized in PBS-0.1% Triton X-100 (PBST) and then saturated in 5% normal goat serum (NGS) in NGS-PBST. Immediately after this step, sections were incubated in diluted FOS Ab overnight (1 $\mu$g/ml NGS-PBST). On the following day, sections were washed in PBST and incubated in a goat antirabbit biotinylated secondary Ab [product reference no. B0432 (Dako Corp., Carpinteria, CA); 0.75 $\mu$g/ml PBST]. Sections were then washed in PBST and incubated in the Vectastain Elite ABC Kit (product reference no. PK6100; Vector Laboratories, Burlingame, CA). After development with the 3,3′-diaminobenzidine (DAB) Substrate Kit (SK-4100; Vector Laboratories) in a black precipitate (DAB plus $Ni^{2+}$), sections were washed thoroughly in PBS, refixed in 4% paraformaldehyde, and the residual peroxidase activity was blocked in 1:4 methanol-PBS solution with 30% $H_2O_2$. Sections were then permeabilized and blocked in 5% NGS-PBST and incubated in the correspondent diluted primary Ab from 24 h (GnRH1 Ab) to 72 h (Kisspeptin-10 Ab). Similar secondary Ab and ABC incubation steps were then performed. The developing reaction used in this step was a DAB brown precipitate using the same kit. Once the immunohistochemical reactions were completed, sections were mounted in Eukitt (Sigma-Aldrich, St. Louis, MO) after being air-dried. Photomicrograph bars represent 25 $\mu$m in Fig. 2, 250 $\mu$m in Fig. 1, and 200 $\mu$m in Fig. 3.

The majority of GnRH neurons expressing c-*fos* were located within the preoptic area, more specifically between the region comprising the AVPe and the organum vasculosum laminae terminalis (interaural 4.42–4.30 mm and bregma 0.62–0.50 mm approximately according to Ref. 27). Double-labeled FOS/GnRH neurons were only rarely detected in the more rostral areas, such as the medial septum, and, therefore, were not included in our analysis. For the Kisspeptin-10, the counting areas were limited to the anteromedial part of the Pe, where Kisspeptin-10 neurons were found to be activated, *i.e.* showing FOS/Kisspeptin-10 double labeling. Finally, we also analyzed any differences in Kisspeptin-10 immunoreactivity between different genotypes and treatments (EB plus P *vs.* control) to determine whether $Afp^{-/-}$ females were defeminized in comparison with $Afp^{+/+}$ females. Therefore, we analyzed the area occupied by immunoreactive Kisspeptin-10 fibers that are found in the anterior part of anterior commissure, at the anterodorsal preoptic nucleus (ADP). Kisspeptin-10 expression was analyzed by Scion Image (National Institutes of Health, Bethesda, MD).

### *RIA of plasma LH levels*

Serum LH levels were determined in a volume of 100 $\mu$l using a double Ab method and a RIA kit (mLHRia), kindly supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 (AFP-11536B) was labeled with $^{125}I$ by the chloramine-T method, and the hormone concentration was expressed using the mouse LH reference preparation (AFP-5306A) as standard. Intraassay and interassay coefficients were less than 7 and 10%, respectively. The sensitivity of the assay was 4 pg/100 $\mu$l.

### *Statistics*

All data were analyzed using either one variable or repeated measures ANOVA. When appropriate, all ANOVAs were followed by *post hoc* comparisons with the Bonferroni test. Only significant ($P < 0.05$) effects detected are mentioned in detail in *Results*.

## Results

### *Absence of a steroid-induced LH surge in $Afp^{-/-}$ mice*

$Afp^{-/-}$ female mice did not show a preovulatory LH surge in response to treatment with EB plus P, whereas $Afp^{+/+}$ females clearly did when treated with EB plus P (Fig. 4A). In addition, neither $Afp^{+/+}$ nor $Afp^{-/-}$ males showed any significant increases in plasma LH levels after treatment with EB plus P, thereby further demonstrating the existence of clear sex differences in the ability of mice to show steroid-induced LH surges. Thus, $Afp^{-/-}$ females are clearly defeminized with regard to their ability to show preovulatory LH surges, confirming that their anovulatory state is due to the absence of appropriate signaling from the hypothalamus (6, 7).

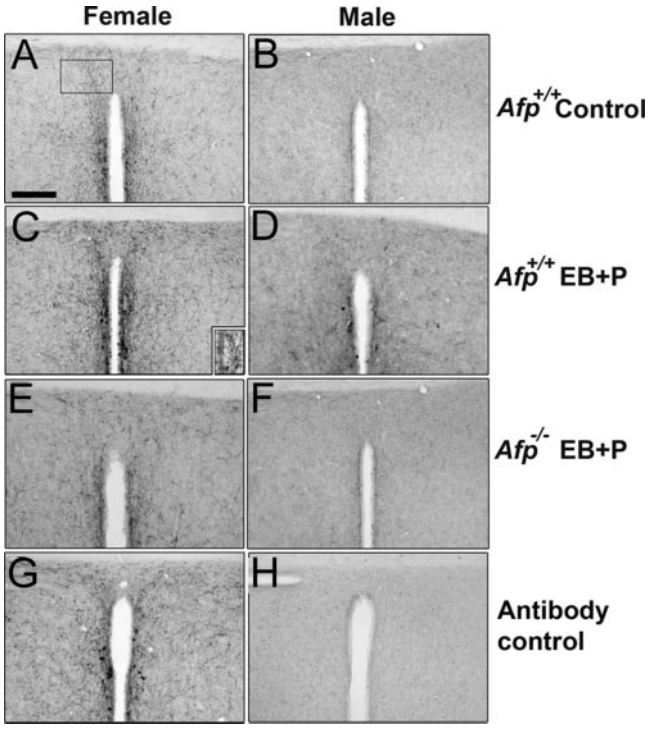


FIG. 1. Kisspeptin-10 distribution in the $Afp^{-/-}$ brain. All photomicrographs depict detailed photomicrographs of Kisspeptin-10 immunoreactivity in the ADP and Pe. A, An untreated $Afp^{+/+}$ female. B, An untreated $Afp^{+/+}$ male. C, A EB plus P-treated $Afp^{+/+}$ female. D, A EB plus P-treated $Afp^{+/+}$ male. E, A EB plus P-treated $Afp^{-/-}$ female. F, A EB plus P-treated $Afp^{-/-}$ male. G, A positive immunoreaction using the anti-Kisspeptin-10 Ab. H, A specific negative control after having incubated an adjacent section with the YNWNSFGLRY-NH2 preabsorbed Ab. *Scale bar* in A represents 250 $\mu$m and is applicable to all photomicrographs. *Labeled areas* in A represent the area of counting the immunoreactive fibers. In C we represent a detailed photomicrograph of a group of Kisspeptin-10 neurons with a round shape and short neurites, found on the ventral part of the Pe.

ANOVA on plasma LH levels showed significant effects of sex ($F_{1,39} = 29.48$; $P < 0.001$), genotype ($F_{1,39} = 33.17$; $P < 0.001$), treatment ($F_{1,39} = 8.90$; $P = 0.0018$), and a significant triple sex X genotype X treatment interaction ($F_{1,39} = 13.55$; $P < 0.001$). *Post hoc* analysis of the significant interaction showed that plasma LH levels were significantly higher in treated $Afp^{+/+}$ females compared with treated $Afp^{-/-}$ females as well as with $Afp^{+/+}$ and $Afp^{-/-}$ males. Furthermore, plasma LH levels were also higher in nontreated $Afp^{+/+}$ females compared with all other groups ($Afp^{-/-}$ females, $Afp^{+/+}$ and $Afp^{-/-}$ males) (Fig. 4A).

Accordingly, female $Afp^{-/-}$ mice, as well as $Afp^{+/+}$ and $Afp^{-/-}$ males, did not show any significant FOS activation in hypothalamic GnRH1 neurons, whereas in $Afp^{+/+}$ females, approximately 50% of GnRH1 neurons showed FOS expression after EB plus P treatment (Figs. 2 and 4B). ANOVA on

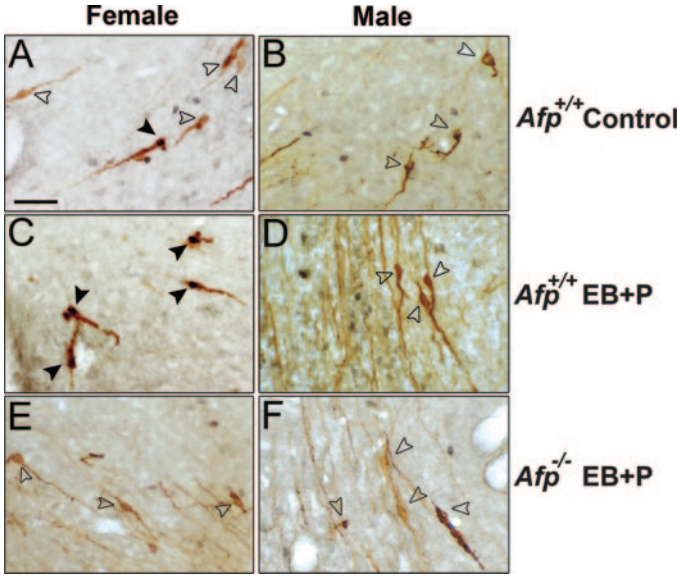


FIG. 2. Qualitative analysis of FOS activation in GnRH1 cells present in the AVPe. A, A detailed photomicrograph of FOS activation in GnRH1 neurons of an untreated $Afp^{+/+}$ female. B, A detailed photomicrograph of the absence of FOS activation in the GnRH1 neurons of an untreated $Afp^{+/+}$ male. C, A detailed photomicrograph of FOS activation in GnRH1 neurons of a treated $Afp^{+/+}$ female. D, A detailed photomicrograph of the absence of FOS activation in GnRH1 neurons of a treated $Afp^{+/+}$ male. E, A detailed photomicrograph of the absence of FOS activation in GnRH1 neurons of a treated $Afp^{-/-}$ female. F, A detailed photomicrograph of the absence of FOS activation in GnRH1 neurons of a treated $Afp^{-/-}$ male. In all pictures GnRH1 immunoreactive neurons are stained with DAB (*brown* precipitate) and FOS with DAB plus nickel (*black* precipitate). *Empty arrowheads* show nonactivated FOS/GnRH1 neurons. *Black arrowheads* show activated FOS/GnRH1 neurons. *Scale bar* in A represents 25 $\mu$m and is applicable to all photomicrographs.

the number of FOS/GnRH1 double-labeled neurons showed a significant effect of sex ($F_{1,38} = 45.51$; $P < 0.001$), genotype ($F_{1,38} = 48.72$; $P < 0.001$), treatment ($F_{1,38} = 28.51$; $P < 0.001$), and a significant triple sex X genotype X treatment interaction ($F_{1,38} = 28.13$; $P < 0.001$). *Post hoc* analysis of the significant interaction showed that only $Afp^{+/+}$ females treated with EB plus P showed a significant induction of FOS in GnRH1 neurons. The mean percentages (±SEM) of FOS double-labeled/GnRH1 neurons of every group studied are presented in Table 1. This percentage was calculated by dividing the number of FOS double-labeled/GnRH1 neurons by the total number of GnRH1 neurons analyzed in the region of interest and then multiplied by 100. The statistical analysis of the numbers of GnRH1 immunoreactive neurons did not show any significant differences between genotypes or hormone treatments as well as no significant interaction between genotypes or hormone treatments (raw data are included in Table 2).

### *Down-regulation of Kisspeptin-10 in $Afp^{-/-}$ females*

The area of Kisspeptin-10 immunoreactivity was clearly defeminized in $Afp^{-/-}$ females, *i.e.* they showed male-typical Kisspeptin-10 immunoreactivity in the ADP and Pe (Figs. 1 and 4C). Furthermore, no significant FOS double labeling could be discerned in Pe Kisspeptin-10 neurons in $Afp^{-/-}$ females (2.6% of Kisspeptin-10 neurons were double labeled for FOS after EB+P treatment) or in $Afp^{+/+}$ and $Afp^{-/-}$ males (no double-labeled FOS/Kisspeptin-10 neurons were found), whereas $Afp^{+/+}$ females showed a significant induction of FOS in Kisspeptin-10-immunoreactive neurons (41.6% of Kisspeptin-10 neurons were double labeled with FOS after EB plus P treatment; Fig. 4C).

This was confirmed by statistical analysis with ANOVA demonstrating clear sex differences in the area occupied by Kisspeptin-10 immunoreactive fibers in the ADP with females showing higher levels of immunoreactivity than males ($F_{1,99} = 125.80$; $P < 0.0001$). Moreover, Kisspeptin-10 immunoreactivity present in the same area showed significant differences between $Afp^{+/+}$ and $Afp^{-/-}$ mice ($F_{1,99} = 117.19$; $P < 0.0001$), and between control *vs.* EB plus P treated ($F_{1,99} = 44.82$; $P < 0.0001$). In addition, we observed a significant triple sex X genotype X treatment interaction ($F_{1,99} = 4.03$; $P < 0.05$). The *post hoc* analysis showed that $Afp^{+/+}$ females treated with EB plus P showed higher levels of Kisspeptin-10 immunoreactivity compared with control $Afp^{+/+}$ females, whereas EB plus P-treated $Afp^{-/-}$ females showed even lower levels than the control $Afp^{+/+}$ females. *Post hoc* analyses on the male data also showed significant differences between control and treated $Afp^{+/+}$ males, but levels in males were always significantly lower than those found in $Afp^{+/+}$ females.

Quantitative analysis of FOS/Kisspeptin-10 double-labeling results showed that only EB plus P-treated $Afp^{+/+}$ females showed double-labeled neurons in the Pe (Fig. 3). Thus, in $Afp^{+/+}$ females around 40% of Kisspeptin-10 immunoreactive neurons in the Pe showed FOS immunoreactivity, whereas female $Afp^{-/-}$ mice, as well as $Afp^{+/+}$ and $Afp^{-/-}$ males, did not show any significant FOS activation in Pe Kisspeptin-10 immunoreactive neurons (Fig. 4D). This was confirmed by ANOVA on the number of FOS/Kisspeptin-10 double-labeled neurons showing a significant effect of sex ($F_{1,27} = 28.24$; $P < 0.001$), genotype ($F_{1,27} = 26.06$; $P < 0.001$), and treatment ($F_{1,27} = 28.24$; $P < 0.001$), and a significant triple sex X genotype X treatment interaction ($F_{1,27} = 34.17$; $P < 0.001$). The mean percentages (±SEM) of FOS double-labeled /Kisspeptin-10 neurons of every group stud-

**TABLE 1.** Mean percentages (±SEMs) of FOS double-labeled GnRH1 and Kisspeptin-10 (Kp10) neurons analyzed per animal group after different treatments in wild-type and knockout subjects

| Analysis treatment | Female $Afp^{+/+}$ | | | Female $Afp^{-/-}$ | | | Male $Afp^{+/+}$ | | | Male $Afp^{-/-}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FOS/Kp10 | FOS/GnRH1 | No. | FOS/Kp10 | FOS/GnRH1 | No. | FOS/Kp10 | FOS/GnRH1 | No. | FOS/Kp10 | FOS/GnRH1 | No. |
| Control | 0 | 6.3 ± 2.2 | 11 | 0 | 0.8 ± 0.4 | 4 | 1.81 ± 3.6 | 0.2 ± 0.1 | 5 | | | |
| EB | 37.5 ± 7.9 | 31.8 ± 9.5 | 7 | 1.8 ± 1.81 | 0 | 5 | 0 | 0 | 3 | 0 | 0 | 4 |
| EB + P | 41.6 ± 12.5 | 47.1 ± 4.8 | 8 | 2.6 ± 5.02 | 0 | 8 | 0 | 0.1 ± 0.1 | 6 | 0 | 0.1 ± 0.6 | 6 |

Analysis refers to sex, genotype, and immunochemical analysis (FOS/Kp10 and FOS/GnRH1). Treatment refers to: Control, EB, and EB+P.

**TABLE 2.** Kisspeptin-10 (Kp10) and GnRH1 neurons detected in each genotype (male-female; $-/-$ *vs.* $+/+$) after control or EB plus P treatments

| Analysis treatment | Female $Afp^{+/+}$ | | | Female $Afp^{-/-}$ | | | Male $Afp^{+/+}$ | | | Male $Afp^{-/-}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Kp10 | GnRH1 | No. | Kp10 | GnRH1 | No. | Kp10 | GnRH1 | No. | Kp10 | GnRH1 | No. |
| Control | 59.1 (650) | 52.2 (574) | 11 | 12.8 (51) | 55.0 (220) | 4 | 4.0 (20) | 53.6 (268) | 5 | | | |
| EB + P | 64.2 (514) | 48.9 (391) | 8 | 11.6 (93) | 63.9 (511) | 8 | 5.3 (32) | 54.3 (326) | 6 | 5.5 (33) | 49.2 (295) | 6 |

The values represent the average cell number per mouse (total number of cells in the brain sections of study) and the number (No.) of animals studied. Analysis refers to sex, genotype, and immunohistochemical analysis (Kp10 and GnRH1 neurons). Treatment refers to: Control and EB+P.

ied are presented in Table 1. This percentage was calculated by dividing the number of FOS double-labeled/Kisspeptin-10 neurons by the total number of Kisspeptin-10 neurons analyzed in the area of interest and then multiplied by 100.

### *Effect of estrogens on the activation of the Kisspeptin-10 and GnRH1 system*

To study the role of estrogen alone in the activation of the GnRH1 and Kisspeptin-10 systems, we have treated an additional group of $Afp^{+/+}$ and $Afp^{-/-}$ males and females (group 2) with a single injection of EB 24 h before the LH, GnRH1, and Kisspeptin-10 analysis. The results clearly showed that treatment with E2 is sufficient to induce an activation of the GnRH1 system as measured by the percentage of FOS/GnRH1 double labeling (31.82 ± 9.46%; Fig. 5B) as well as the Kisspeptin-10 system (percentage of FOS/Kisspeptin-10 double labeling: 37.53 ± 6.71%; Fig. 5C) in $Afp^{+/+}$ females compared with all the other groups. These results are very similar to what has been observed in animals treated with EB plus P. By contrast, treatment with EB alone did not induce an LH surge in $Afp^{+/+}$ females (0.47 ± 0.21 ng/ml; Fig. 5A). LH levels were very similar in all four groups. The mean percentages (±SEM) of FOS double-labeled/GnRH1 neurons and FOS double-labeled/Kisspeptin-10 neurons are presented in Table 1.

## Discussion

In the present study, we showed that the infertility of $Afp^{-/-}$ females is linked to the absence of appropriate GnRH1 signaling and subsequent pituitary LH secretion that is required to induce ovulation. Thus, female $Afp^{-/-}$ mice cannot show preovulatory LH surges in response to estrogen and P treatment. Interestingly, the absence of positive feedback actions of estrogen on the GnRH1 neuronal system might be due to the absence of adequate Kisspeptin-10 signaling because the amount of Kisspeptin-10 immunoreactivity and activation was severely diminished in $Afp^{-/-}$ females. After treatment with estrogen and P, $Afp^{-/-}$ females actually showed male-typical levels of Kisspeptin-10 immunoreactivity (Fig. 1). A recent study in *Kiss1*-null mice supports this hypothesis because it was shown that *Kiss1*-null mice did not become sexually mature, whereas females did not have estrous cycles and had persistently low levels of plasma LH (26). Thus, whereas estrogens clearly activated Kisspeptin-10 neurons in $Afp^{+/+}$ females, confirming previous studies (28, 29) indicating that Pe Kisspeptin-10 neurons are an important upstream signal in GnRH1 signaling, this activation was completely absent in $Afp^{-/-}$ females as well as in males (either $Afp^{+/+}$ or $Afp^{-/-}$). These results suggest that aspects of GnRH1 functioning have been defeminized in $Afp^{-/-}$ females, presumably because they are no longer protected from estrogens during prenatal life due to the absence of AFP (5, 7). Thus, sex differences in the ability to show preovulatory LH surges most likely result from the perinatal actions in the male of estrogens on the Kiss1 and GnRH1 neuronal systems.

De Mees *et al.* (6) have recently shown that *GnRH1* expression is down-regulated in $Afp^{-/-}$ females. Interestingly, prenatal treatment of $Afp^{-/-}$ female mice with the aromatase inhibitor, ATD, rescued their fertility and restored an almost normal level of expression of different pituitary factors found in fertile heterozygous females (6). However, there was evidence indicating that AFP has a dose-dependent effect because it was observed that the expression level of the GnRH1 receptor of heterozygous females was intermediate between values of those of $Afp^{-/-}$ and $Afp^{+/+}$ females. Thus, the absence of any significant steroid-induced activation of the GnRH1 system in $Afp^{-/-}$ females may be explained by either the lack of adequate Kisspetin-10 signaling to the GnRH1 system (see below), initiation of an autocrine control of GnRH1 neurons by ultrashort feedback mechanisms (30), or even the release of pulsatile suboptimal levels of GnRH1 that could elicit an inadequate pituitary response at the level of the GnRH1r expression.

In $Afp^{+/+}$ females the estrogen-induced LH peak is preceded by activation of FOS/GnRH1 expressing cells in the AVPe but also by the presence of activated FOS/Kisspeptin-10 neurons in the same periventricular area. Our results support the idea that Kisspeptin-10 signaling in the preoptic region is regulated by sex steroids and thereby mediating the positive feedback action of estrogen on GnRH1 neurons. Kisspeptin signaling through its receptor (GPR54) might synchronize GnRH1 release into the median eminence and ultimately tuning the hypothalamic control of reproduction. Our data also suggest that in the presence of estrogens, Kisspeptin-10 neurons in the Pe may stimulate GnRH1 neurons present in the same adjacent area, thereby favoring the preovulatory LH surge. This hypothesis is supported by the previous observation of an inhibition of the proestrus LH surge and estrous cyclicity by centrally administered metastin (Kisspeptin) antibodies in the female rat (31). Furthermore, it has already been shown that GnRH1 neurons within the preoptic area do not express estrogen receptor (ER)$\alpha$, but only ER$\beta$, and that ovulation is driven by positive feedback actions of estrogens upon ER$\alpha$-expressing neurons projecting to GnRH neurons existing in the Pe and median preoptic nucleus in addition to the AVPe (22). The mechanism by which Kisspeptin is up-regulated by estrogens likely in-

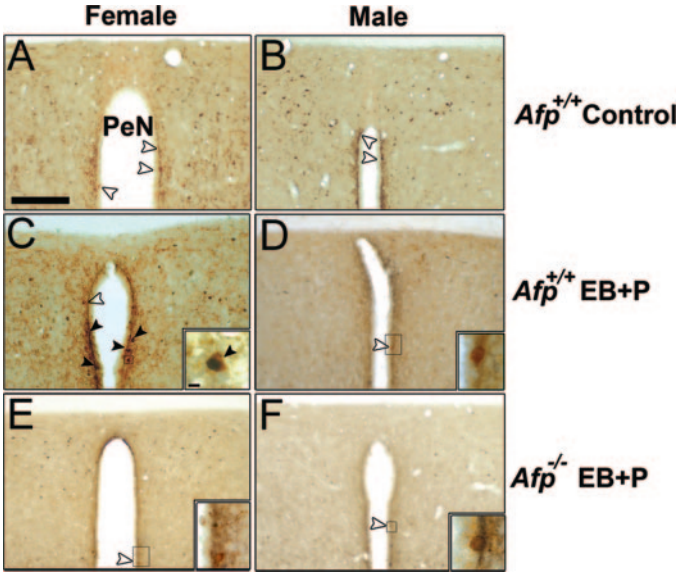


FIG. 3. Analysis of FOS activation in Kisspeptin-10 neurons present in the Pe. A, A detailed photomicrograph of the absence of FOS activation in Kisspeptin-10 neurons of an untreated $Afp^{+/+}$ female. B, A detailed photomicrograph of the absence of FOS activation in the Kisspeptin-10 neurons of an untreated $Afp^{+/+}$ male. C, A detailed photomicrograph of FOS activation in the Kisspeptin-10 neurons of a treated $Afp^{+/+}$ female. A photomicrograph of a double-labeled neuron is represented in the *right-bottom corner of this panel*. D, A detailed photomicrograph of the absence of FOS activation in Kisspeptin-10 neurons of a treated $Afp^{+/+}$ male. A photomicrograph of an unlabeled neuron is represented in the *right-bottom corner of this panel*. E, A detailed photomicrograph of the absence of FOS activation in Kisspeptin-10 neurons of a treated $Afp^{-/-}$ female. A photomicrograph of an unlabeled neuron is represented in the *right-bottom corner of this panel*. F, A detailed photomicrograph of the absence of FOS activation in Kisspeptin-10 neurons of a treated $Afp^{-/-}$ male. A photomicrograph of an unlabeled neuron is represented in the *right-bottom corner of this panel*. In all pictures Kisspeptin-10 immunoreactive neurons are stained with DAB (*brown* precipitate) and FOS with DAB plus nickel (*black* precipitate). *Empty arrowheads* show non-activated FOS/Kisspeptin-10 neurons. *Black arrowheads* show activated FOS/Kisspeptin-10 expressing neurons. *Scale bar* in A represents 200 $\mu$m, and it is applicable to all photomicrographs. The detailed photomicrograph of the selected area in the *right corner* represents 25 $\mu$m except in E, where it represents 50 $\mu$m.

volves the activation of the ER$\alpha$ present in Kisspeptin neurons in the periventricular preoptic region (19, 25, 32, 33). In fact, recent evidence suggests that only Kiss1 neurons expressing the ER$\alpha$ in this area are stimulated by E2, indicating that they may participate in the positive feedback regulation of GnRH1 secretion (33), whereas Kiss1 neurons in the arcuate nucleus are inhibited by E2 and, thus, may play a role in the negative feedback regulation of GnRH1 secretion. Our results suggest that there is an activation of hypothalamic Kisspeptin neurons by estrogen that facilitates GnRH1 release, thereby leading to a preovulatory LH surge. Finally, although there is no clear evidence of sex differences in the GnRH1 neural population, it has been clearly established that Kisspeptin neurons and their innervation are sexually differentiated in both the rat and mouse (21, 34). In fact, Kauffman *et al.* (21) showed that a single injection with testosterone on the day of birth decreased *Kiss1* mRNA levels to male typical levels in the AVPe of female rats, suggesting a defeminizing role of testosterone on the Kiss1 system in female

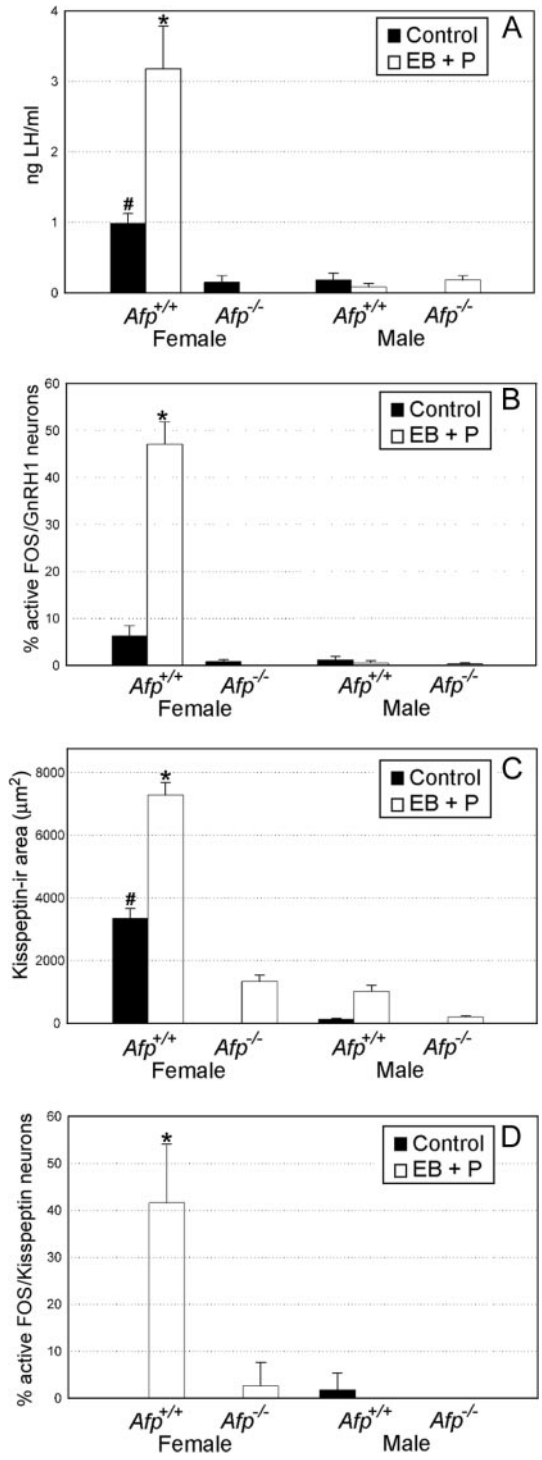


FIG. 4. Quantitative analysis of the ability to show a steroid-induced preovulatory LH surge. A, Plasma LH levels in ng/ml (mean ± SEM). B, The percentage of FOS-activated GnRH1 cells (mean ± SEM) in the AVPe. C, The area occupied by Kisspeptin-10 immunoreactive fibers (mean ± SEM) present in the ADP nucleus. D, The percentage of FOS-activated Kisspeptin-10 cells (mean ± SEM) in the Pe. All experiments were conducted in $Afp^{-/-}$ and $Afp^{+/+}$ mice of both sexes. Control treatments consisted of a sc SILASTIC brand capsule containing crystalline 17$\beta$-E2 (1 wk before hormonal induction). EB plus P treatments consisted of a sc SILASTIC brand capsule containing crystalline 17$\beta$-E2 and sequential treatment with EB (d 1) and P (d 2). *, $P < 0.05$ compared with all other groups. #, $P < 0.05$ compared with all other groups.

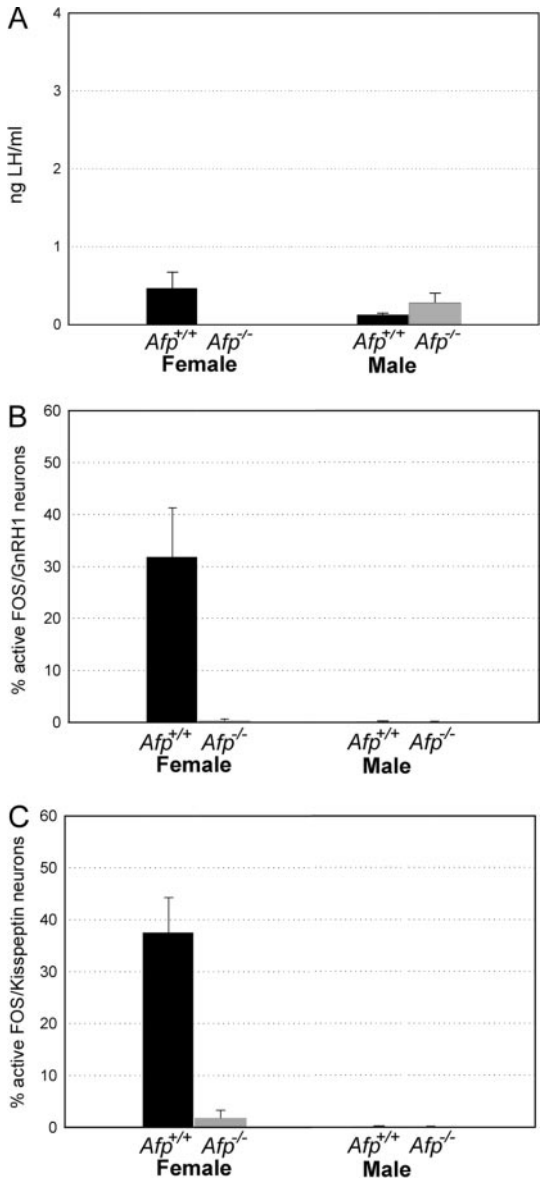


FIG. 5. Quantitative analysis of the ability to show an EB-induced preovulatory LH surge. A, Plasma LH levels in ng/ml (mean ± SEM). B, The percentage of FOS-activated GnRH1 cells (mean ± SEM) in the AVPe. C, The percentage of FOS-activated Kisspeptin-10 cells (mean ± SEM) in the Pe. All experiments were conducted in $Afp^{-/-}$ and $Afp^{+/+}$ mice of both sexes. EB treatments consisted of a sc SILASTIC brand capsule containing crystalline 17$\beta$-E2 and sequential treatment with EB (d 1) and terminating the animals on d 2.

rats. In the present study, we showed that $Afp^{-/-}$ females are defeminized so that they have fewer Kisspeptin-10 neurons as well as fewer Kisspeptin-10 immunoreactive fibers in the preoptic region, and the Kisspeptin-10 neurons present in the Pe lose their ability to be activated by estrogens, suggesting a role for fetal estrogens (in contrast to neonatal androgen) in the sexual differentiation of the Kiss1 system. By contrast, it is unclear whether the Kisspeptin-10 population in the arcuate nucleus is affected in $Afp^{-/-}$ females because no significant Kisspeptin-10 immunoreactivity was observed in this nucleus, either in control (implanted with a low dose of E2), EB, or EB plus P primed animals. These observations are in agreement with previous studies conducted by Smith *et al.* (33) who found that E2 treatment inhibited Kiss1 expression in the mouse arcuate nucleus. However, neither a sex difference nor an influence of neonatal androgen treatment on Kiss1 expression in the arcuate nucleus was observed in the study by Kauffman *et al.* (21), suggesting that the Kiss1 system in the arcuate nucleus is not organized perinatally by sex steroid hormones.

Although $Afp^{-/-}$ males are fertile and seem to have a normal male-typical phenotype, the possibility cannot be excluded that some aspects of brain sexual differentiation have been affected in these males. Presumably, $Afp^{-/-}$ males are overexposed to estrogens prenatally because they are exposed to estrogens derived from neural aromatization of testosterone as normal males are, but they are also exposed to estrogens coming from external sources such as their mother or their brothers because they lack AFP. To date, no clear effects of overexposure to estrogens have been reported for the development of the male brain other than that transgenic male mice expressing human aromatase and, thus, having an increased estrogen to androgen ratio show a multitude of structural and functional alterations in the reproductive organs such as cryptorchidism, Leydig cell hyperplasia, disrupted spermatogenesis, and infertility (20). However, no data are available for the brain for these transgenic mice. In the present study, we determined whether aspects of hypothalamic functioning, such as the Kisspeptin-10 and GnRH1 system, were hypermasculinized in $Afp^{-/-}$ males. However, $Afp^{-/-}$ males did not show any significant differences at the level of either Kisspeptin-10 content, FOS/Kisspeptin-10 activation, FOS/GnRH1 activation, or LH secretion compared with $Afp^{+/+}$ males, suggesting no particular protective role for AFP in the development of the male brain.

In summary, the present study shows that sex differences in the ability to show preovulatory LH surges and, thus, cyclical gonadal function may reflect the perinatal actions of estrogens on the hypothalamic Kiss1 and GnRH1 neuronal systems. This ability is normally absent in males due to prenatal estrogens coming from aromatization of testicular testosterone; in $Afp^{-/-}$ females it is lost due to the absence of AFP, and as a consequence, females are no longer protected against estrogens circulating during prenatal development. The use of the $Afp^{-/-}$ model should further unravel the sex differences of the Kiss1 and GnRH1 systems and their differential sexual functions in vertebrates. The $Afp^{-/-}$ model makes a better model to analyze the role of fetal estrogens in brain sexual differentiation because it is a natural model for prenatal estrogen exposure in females. So there are no particular toxic effects as has been shown when diethylstilbestrol has been used to expose fetuses to estrogens for instance (for example, see Ref. 35). Finally, the role of prenatal estrogens in the sexual differentiation of the GnRH1 and Kiss1 system should be confirmed by analyzing the ability to show steroid-induced LH surges and, accordingly, significant FOS activation of these systems in male aromatase knockout (ArKO) mice. ArKO mice carry a mutation in the aromatase gene and, thus, are incapable of converting androgens to estrogens. Thus, if estrogens are the critical hormone in de-

feminizing the GnRH1 and Kiss1 systems, then ArKO males should not be defeminized.

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