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THE TWO KISSPEPTIN NEURONAL POPULATIONS ARE DIFFERENTIALLY ORGANIZED AND ACTIVATED BY ESTRADIOL IN MICE

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In rodents, kisspeptin expressing neurons are localized in two hypothalamic brain nuclei [anteroventral periventricular nucleus/periventricular nucleus continuum (AVPv/PeN) and arcuate nucleus (ARC)] and modulated by sex steroids. By using wild-type (WT) and aromatase knockout mice (ArKO, which cannot convert testosterone into estradiol) and immunohistochemistry, we observed that WT females showed a continuous increase in kisspeptin peptide expression in the ARC across postnatal ages (P5 to P25), whereas WT males did not show any expression before P25. Kisspeptin peptide expression was also present in ArKO females but did not increase over this early postnatal period, suggesting that kisspeptin peptide expression in the ARC is organized by estradiol-dependent and –independent mechanisms. We also compared kisspeptin peptide expression between groups of adult male and female mice which were left gonadally intact or gonadectomized and treated or not with estradiol (E_2) or dihydrotestosterone (DHT). In the ARC, kisspeptin peptide expression decreased after gonadectomy but was completely rescued by either E_2 or DHT treatment in each sex/genotype. However, kisspeptin peptide expression was lower in ArKO compared to WT subjects. In the AVPv/PeN, ArKO females showed a male-typical kisspeptin peptide expression, and adult E_2 treatment partially restored kisspeptin peptide expression. Finally, we showed that, after E_2 treatment of WT and ArKO mice between either P5 and P15 or P15 and P25, AVPv/PeN kisspeptin peptide expression could be still masculinized at P5, but was feminized from P15 onwards. In conclusion, the two kisspeptin neuronal populations (AVPv/PeN *versus* ARC) seem to be differentially organized and activated by E_2.

Kisspeptin is the processed peptide product of the *Kiss1* gene and the endogenous agonist for the Gpr54 receptor. Over the last eight years, the role of kisspeptin in reproductive maturation and function has been further refined by including its participation in the sexual differentiation of the brain (1, 2), puberty onset (3), feedback regulation of gonadotropin secretion (4), neuroendocrine control of ovulation (5), metabolic modulation of fertility (6), as well as the environmental (photoperiod) control of reproduction in seasonal species (7). Furthermore, all these studies have highlighted a conserved and prominent population of kisspeptin expressing neurons in the arcuate nucleus (ARC) (1, 4, 8) and a second population in the anteroventral periventricular nucleus/periventricular nucleus continuum (AVPv/PeN (9-11)) in rodents. There is ample evidence showing that the kisspeptin neuronal population in the AVPv/PeN is sexually dimorphic in rodents (8, 9) with adult females having more kisspeptin expressing neurons than adult males.

In recent years, many signals have been identified as putative modulators of the hypothalamic kisspeptin system. Among them, the prominent role of sex steroids (estrogens, androgens and progestins) as being important regulators of kisspeptin expression has been described in different species and at various life stages such as during embryonic and peripubertal periods (2, 3, 12-14) and in

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adulthood (10, 11). Indeed, kisspeptin expressing neurons have been proven sensitive to both the early organizing effects as well as the acute regulatory actions of sex steroids; in addition, the regulatory effects of sex steroids are population specific since the ARC and the AVPv/PeN populations of kisspeptin expressing neurons have been shown to respond to sex steroids in a completely opposite manner (10, 11). In adult mice, *Kiss1* mRNA in the AVPv/PeN is robustly increased by estradiol (E_2), and strongly decreased by gonadectomy, in female as well as in male rodents (2, 8, 10, 11). Testosterone (T) can also increase kisspeptin expression in the AVPv/PeN of mice, but non-aromatizable androgens, such as dihydrotestosterone (DHT) have no effect, suggesting that the stimulatory effects of T are due to aromatization to E_2 (11). Furthermore, almost all kisspeptin expressing neurons express estrogen receptor alpha (ERα) providing evidence of a direct pathway by which E_2 could stimulate these neurons and thus participate in the positive-feedback actions of E_2 to generate the preovulatory surge of gonadotrophins (15). In contrast to the AVPv/PeN population, the kisspeptin neuronal population as measured by the number of *Kiss1* mRNA expressing neurons in the ARC is strongly decreased by E_2, and robustly increased by gonadectomy, in both sexes (10, 11). T can also decrease the total amount of *Kiss1* mRNA in the ARC, and unlike the AVPv/PeN, some of these effects can be mediated via androgen receptors since DHT is also able to decrease *Kiss1* mRNA levels (11). Based on the ability of E_2, T and DHT to inhibit kisspeptin expression in the ARC, it was proposed that the ARC kisspeptin population might be important in the negative-feedback actions of E_2 on gonadotropin secretion. During the last few years, studies focused essentially on the characterization of the development of the rodent AVPv/PeN and ARC kisspeptin systems (for review, see (16)). It has been shown that during development, females had significantly more *Kiss1* mRNA and kisspeptin immunoreactivity (Kisspeptin-ir) in the AVPv/PeN than males, and that this sex difference was organized by gonadal hormones (1, 2, 13, 17-20). Unlike the AVPv/PeN, *Kiss1* mRNA and Kisspeptin-ir can be detected in the rodent ARC prenatally (21) and at birth (22-24) in both sexes. Initial mRNA data obtained in the rat suggested that *Kiss1* mRNA expression in the ARC was not sexually dimorphic and appeared to be insensitive to the effects of early exposure to sex steroids (2); however, more recent studies, using in situ hybridization and immunohistochemistry, showed that the ARC kisspeptin population is likely to be sexually dimorphic as well, with higher numbers in female rats after birth (23, 24) but not during embryonic life (21).

With regard to all these studies, most observations on the regulation of neuronal kisspeptin populations were derived from in situ hybridization experiments. However, showing that *Kiss1* mRNA is present and specifically regulated in a specific nucleus does not mean that the derived protein will be also secreted and will follow the same regulation pattern (25-27). We thus studied here by immunohistochemical studies the expression and the regulation by sex steroids (E_2, T and DHT) of kisspeptin expressing neurons in the AVPv/PeN and the ARC during early postnatal development and in adulthood. We also used the aromatase knockout (ArKO) mouse model, which carries a targeted mutation in the aromatase gene and as a result cannot convert T into E_2, to determine the potential organizational role of E_2 on the development of the two different neuronal kisspeptin populations. These transgenic mice still have functional estrogen receptors and thus allow us to determine the potential organizational and/or activational role of E_2 on kisspeptin expressing neurons following an exogenous treatment with E_2 during the early postnatal period and/or in adulthood. In a previous study (28), we showed that an estrogenic treatment during an early postnatal (P5-P15) or a prepubertal (P15-P25) period respectively defeminized or feminized the expression of different reproductive behaviors in WT and ArKO female mice. Since the kisspeptin system is strongly related to reproduction and modulated by E_2, we determined in the present study whether these estrogenic treatments also affected the organization of the kisspeptin system itself. Clarkson and colleagues (3) previously showed that replacement of E_2 in P15-ovariectomized mice from P15-30 or P22-30 resulted in a complete restoration of kisspeptin peptide expression in the AVPv/PeN of P30 mice suggesting that E_2 was essential for the prepubertal expression of kisspeptin in the AVPv/PeN.

Materials and Methods

Animals

ArKO mice were generated by targeted disruption of exons 1 and 2 of the *Cyp 19* gene (29). Heterozygous males and females of the C57BL/6J strain were bred to generate wild-type (WT), heterozygous, and homozygous-null (ArKO) offspring at the GIGA Neurosciences, University of Liège, Belgium. Food ("phytoestrogen-free" mouse chow D10001 AIN-76A, Brogaarden, Denmark) and water were available to mice ad libitum. All animals were housed under a 12h light: 12h dark cycle (lights on between 8:00 and 20:00h).

All experiments were conducted in accordance with the guidelines set forth by the National Institutes of Health "Guide for the Care and Use of Research Animals, Eight Edition", and were approved by the Ethical Committee for Animal Use of the University of Liege.

Experiment 1: Kisspeptin peptide expression during early postnatal development

To obtain brain tissues at early postnatal ages, pregnant females were checked daily for parturition towards the end of pregnancy. The day of birth was designated as P0 and all subjects were left gonadally intact. Brain tissues were collected on P5, P10, P15, P20 and P25. Postnatal mice were decapitated whereupon brains were rapidly removed from the skull and immediately immersion-fixed in 5% acrolein in 0.1 M phosphate-buffer-saline (PBS; pH 7.6) for 2.5h, rinsed twice in PBS for 30min and then cryoprotected in 30% sucrose and when sunken, frozen on dry ice and stored at –80°C until being processed for kisspeptin immunohistochemistry. To determine the sex and the genotype of the pup, the tail was kept and stored at –20°C for later PCR analysis of DNA. For each age, six animals were used per sex/genotype condition.

Experiment 2: Kisspeptin peptide expression in adulthood

Wild-type and ArKO mice were weaned at 21 d and group-housed until 3 mo of age. Subjects were then either left gonadally intact (Int) or gonadectomized (Ovx - Gdx) and received (during 10 d) s.c. in the neck at the time of surgery a 5-mm-long Silastic capsule (inner diameter: 1.57 mm; outer diameter: 2.41 mm) containing either crystalline 17β-estradiol (diluted 1:1 with cholesterol; Ovx-Gdx/E_2 10d) or crystalline DHT (Ovx-Gdx/DHT) (supplemental Figure 1). Gonadectomy was performed as previously described (30). Groups of gonadectomized subjects were also left either three or six weeks (Gdx 3w; Ovx 3w versus Ovx 6w) undisturbed before brain collection (see supplemental Figure 1). The dose of E_2 (E8875, Sigma) was based on a previous study (for details, see (31)) showing that this treatment leads to proestrous levels of E_2. The dose of DHT has been previously shown to produce significant androgenic actions at the target tissues, e.g., spermatogenesis in gonadotropin-deficient mice (32). In previous experiments (17, 33), we observed that adult treatment with a low dose of E_2 during a short period only led to a slight increase of AVPv/PeN kisspeptin population in ArKO females whereas this specific neuronal population was completely absent in nontreated adult ArKO female mice (3). In order to determine the short term versus the long term effects of an E_2 treatment on neural kisspeptin expression in adulthood, we added a group of ovariectomized females (WT and ArKO) implanted with an E_2 capsule during six weeks (Ovx/E_2 6w). Male subjects were not exposed to E_2 for more than 10 d (Gdx/E_2 10d) since their health deteriorates rapidly when exposed to such high E_2 concentrations.

Experiment 3: Kisspeptin peptide expression following early postnatal or prepubertal hormonal treatment

A first cohort of WT females was injected s.c. between the early postnatal ages of P5-P15 with either estradiol-benzoate (EB, E8515, Sigma) (0.5 μg every 2 d) or sesame oil (S3547, Sigma). This specific EB treatment led to a defeminization of sexual behaviors in WT female mice (28). Females were then ovariectomized in adulthood and treated with EB (1 μg every day) two weeks prior to sacrifice (see supplemental Figure 1). A second cohort of WT and ArKO mice of both sexes was injected s.c. between the prepubertal ages of P15-P25 with either EB (0.05 μg every day) or sesame oil. This speficic EB treatment led to a feminization of sexual behaviors in ArKO female mice (28). Animals were gonadectomized in adulthood and then treated with EB (1 μg every day) two weeks prior to sacrifice. Brains were collected as described above. The number of animals used for each group in each experiment is summarized in supplemental Table 1.

Immunohistochemistry

Brain sections (30 μm thick) were cut on a Leica CM3050S cryostat. Forebrains were cut coronally from the rostral telencephalon to the posterior hypothalamus. Sections were saved in four different series, placed in antifreeze solution, and stored at –20°C for later immunohistochemistry.

Every effort was made to include an equal number of brains from all experimental conditions in each immunohistochemistry run. Kisspeptin immunohistochemistry was carried out on free-floating sections (for a detailed protocol, see (33)). We used a rabbit polyclonal antibody (48h incubation - 1/5000 in TBST-NGS 5%; Anti-Kisspeptin antibody, AB9754, Chemicon, Millipore) against the decapeptide Kisspeptin-10 (derived from the *Kiss-1* gene product). The final reaction used 3,3′diaminobenzidine tetrahydrochloride (DAB Kit, Vector Laboratory). Sections were then washed, mounted onto slides, dried overnight, left in xylene (Sigma) for 15min and coverslipped using Eukit (Fluka, Steinheim, Germany).

Data analysis

An experimenter, who was blind to the treatment of subjects, counted the relative amount of kisspeptin-ir in the ARC. We analyzed kisspeptin-ir in three different parts of the ARC, i.e., the rostral, the middle and the caudal parts. Since we did not observe any statistical difference in kisspeptin-ir expression between the three parts, we only presented here the results concerning the middle part (Figure 45, interaural 2.10 mm, bregma 1.70 mm, according to (34)). Brains sections were digitized through a video camera and then made binary for image quantification (for a detailed protocol, see (35)). Briefly, the total amount of kisspeptin-ir in the ARC was measured in one entire field (objective 20X – 720 × 480 pixels – area 303372 μm^2) placed in a standardized manner based on predefined anatomical landmarks in the section (respectively the top of the third ventricle and the bottom of the anterior commissure, and right or left lateral wall of the third ventricle and the top of the median eminence), and determined by measuring the area (μm^2) covered by "thresholded" pixels [those pixels with a gray level higher than a defined threshold density (specific immunoreactive staining)]. "Threshold" was determined as a constant function of the background optical density defined as the mean optical density three to five times the standard deviation higher than the mean background density. The mean background density was measured in a region devoid of kisspeptin-ir, immediately lateral to the analyzed region containing kisspeptin-ir. We measured the relative amount of kisspeptin-ir (fibers + cell bodies) in both sides of the section and grouped into a "total amount of kisspeptin-ir" in the ARC.

Kisspeptin-ir cells bodies were also counted bilaterally in four adjacent brain sections (with an interval of 120 μm between them) delimiting the anteroventral periventricular nucleus (AVPv) and the periventricular nucleus continuum (PeN) (40X

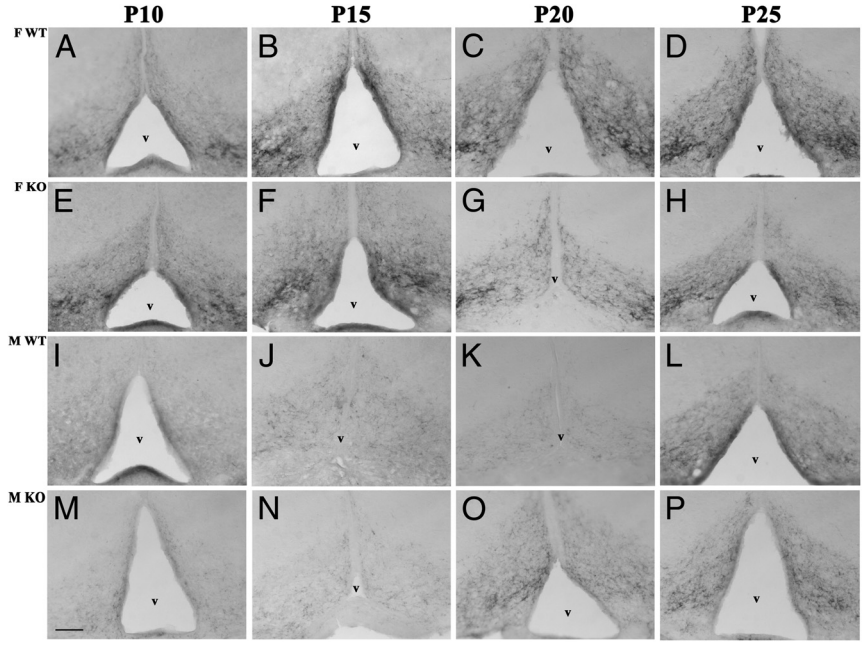

Figure 1. Representative photomicrographs show coronal sections of the arcuate nucleus (ARC) with kisspeptin immunoreactivity in a WT female (panels A-D), an ArKO female (panels E-H), a WT male (panels I-L) and an ArKO male (panels M-P) across different postnatal ages [P10 (panels A,E,I,M) – P15 (panels B,F,J,N) – P20 (panels C,G,K,O) – P25 (panels O,H,L,P)]. V = third ventricle. Scale bar = 100 μm.

objective). Cell counts for both sides of each section were summed to provide a total number of kisspeptin expressing neurons comprised in the AVPv/PeN per experimental condition.

Statistics

The total amount of kisspeptin-ir in the ARC or the total number of kisspeptin-ir cells in the AVPv/PeN was analyzed using one-, two- or three-way analysis of variance (ANOVA) (ANOVA – Statistica 10.0) with total amount of kisspeptin-ir or total number of kisspeptin-ir cells as the dependent measure and age (5), sex (2), genotype (2) and/or hormonal treatment (2, 4 or 6) as independent factors. When appropriate, all ANOVAs were followed by Fisher Least Significant Difference (LSD) post hoc comparisons. Only significant ($P < .05$) effects are presented below. Sex differences were analyzed using Student *t test* ($P < .05$).

Results

Experiment 1: Kisspeptin peptide expression during early postnatal development

In the ARC, we observed a clear sex difference in kisspeptin peptide expression: WT females showed a significant increase in kisspeptin peptide expression across all postnatal ages, whereas WT males did not show any significant kisspeptin peptide expression before P25 (Figure 1). Interestingly, kisspeptin peptide expression did not increase in ArKO females over this early postnatal period as observed for WT females, whereas ArKO males had very similar kisspeptin peptide expression as WT males.

ANOVA on the relative amount of kisspeptin-ir in the ARC showed a significant interaction between age, sex and genotype ($F(4, 92) = 3.81$; $P = .007$). Post hoc analysis of this interaction indicated a strong sex difference with WT females showing significantly more kisspeptin-ir than WT males from P15 to P25 (Figure 2). Post hoc analysis also indicated a significant genotype effect with WT females showing significantly more kisspeptin-ir than ArKO females at P20 and P25, whereas kisspeptin peptide expression was significantly higher in ArKO males at P20 compared to WT males, but this difference disappeared at P25.

In the AVPv/PeN, we found the same pattern of kisspeptin peptide expression during early postnatal development than Clarkson and colleagues (1, 3) previously described. Kisspeptin-ir cells first appeared around P15-P20 in WT females whereas they were first detected at P25 in WT males; a significant sex difference was observed at P25, with WT females having more kisspeptin-ir cells than WT males (data not shown). No kisspeptin-ir cells were detected in ArKO males and females between P5 and P25.

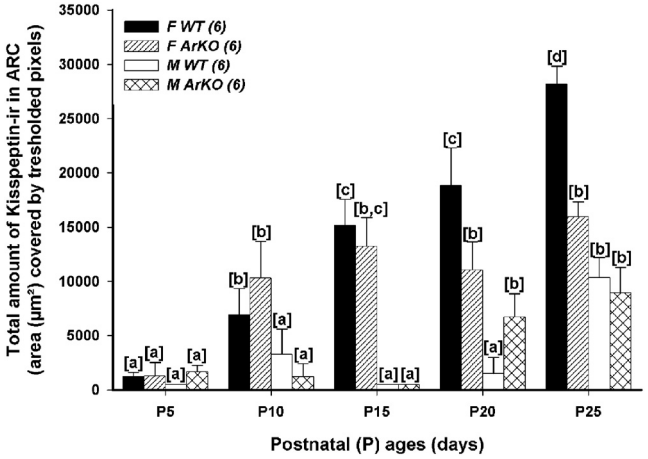

Figure 2. The total amount (mean ± S.E.M) of kisspeptin-immunoreactivity (-ir) in the arcuate nucleus (ARC) across the postnatal period in male and female wild-type (WT) and aromatase knockout (ArKO) mice. For each postnatal age, means with different superscript letters are significantly different from each other by post hoc comparisons ($P < .05$). The number of subjects in each group is given in parentheses.

Experiment 2: Kisspeptin peptide expression in adulthood

In the ARC, in contrast with the early postnatal period, no difference was observed in kisspeptin-ir between intact WT males and females. However, ArKO mice of both sexes had lower kisspeptin-ir in this brain region. In contrast with previous results on *Kiss1* mRNA levels (for review, see (10, 11, 36)) gonadectomy decreased significantly kisspeptin peptide expression in the ARC of both sexes, with the exception of ArKO females, whereas treatment with either E_2 (10 d) or DHT increased kisspeptin peptide expression up to levels observed in WT intact animals. However, E_2 treatment (6 wk) totally suppressed kisspeptin peptide expression in ovariectomized WT and ArKO females.

ANOVA on kisspeptin-ir in the ARC showed a significant interaction between hormonal treatment and genotype in females ($F(5, 42) = 3.79$; $P = .006$) and a significant effect of hormonal treatment in males ($F(3, 30) = 15.18$; $P < .001$). Post hoc analysis revealed that WT females showed significantly more kisspeptin-ir compared to ArKO females (Figure 3). However, when treated with E_2 (10 d) or DHT ArKO females showed WT-like levels of kisspeptin-ir. In WT and ArKO males, post hoc analysis revealed that castrated animals showed significantly lower levels of kisspeptin-ir, but treatment with E_2 or DHT restored it to WT-like levels. A *T* test showed that intact females had the same level of kisspeptin-ir than intact males in the ARC ($t_{(10)} = 1.82$; $P = .10$).

In the AVPv/PeN, intact ArKO mice of both sexes showed a "male-typical" expression of kisspeptin in the AVPV/PeN. Short-term (3 wk) ovariectomy had no effect on kisspeptin peptide expression in the AVPV/PeN whereas long-term (6 wk) ovariectomy led to a very strong decrease of the number of kisspeptin expressing neurons (Figure 4). In males, gonadectomy had no effect regardless of the genotype. Short-term treatment (10 d) with E_2, but not with DHT, increased kisspeptin peptide expression in the AVPV/PeN of ovariectomized WT females and ArKO mice of both sexes, but not in gonadectomized WT males, compared to intact subjects. However, kisspeptin peptide expression remained lower in ArKO mice of both sexes compared to WT females. When E_2 treatment was prolonged (6 wk), kisspeptin peptide expression was still slightly increased in the AVPv/PeN of ovariectomized ArKO, but not WT, females. This was confirmed by ANOVA showing a significant interaction between hormonal treatment and genotype in females ($F(5, 43) = 3.70$; $P = .007$) and in males ($F(3, 34) = 28.32$; $P < .001$) (Figure 5). Finally, we observed the well-known sex difference in adult kisspeptin peptide expression, with intact WT females showing more kisspeptin-ir cells compared to intact WT males ($t_{(11)} = 5.54$; $P < .001$).

Experiment 3: Kisspeptin peptide expression following early postnatal and prepubertal hormonal treatment

Treatment of WT females with EB over an early postnatal period (P5-P15) defeminized the AVPV/PeN kisspeptin population in adulthood, but did not significantly affect the ARC kisspeptin population (Figure 6). ANOVA on the number of kisspeptin-ir cells in the AVPv/PeN revealed a significant effect of treatment ($F(1, 21) = 41.33$; $P < .001$) showing that EB-treated females expressed fewer kisspeptin-ir cells compared to oil-treated females. ANOVA on the total amount of kisspeptin-ir in the ARC showed no significant effect of treatment ($F(1, 20) = 2.24$; $P = .16$).

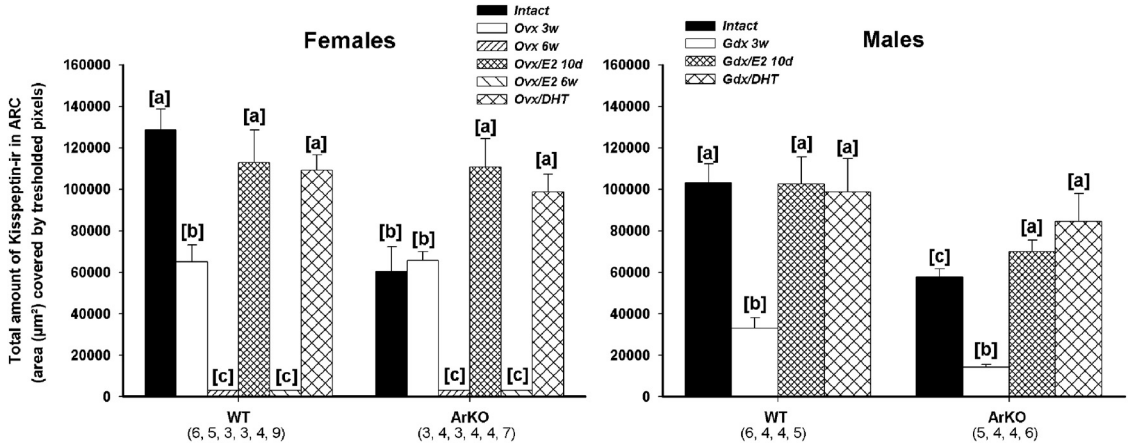

Figure 3. The total amount (mean ± S.E.M.) of kisspeptin-immunoreactivity (ir) in the arcuate nucleus (ARC) following gonadectomy [3 wk (3w) versus 6 wk (6w)] and hormonal treatment with E_2 [10 d (10d) versus 6 wk (6w)] or DHT in adulthood in female (left panel) and male (right panel) wild-type (WT) and aromatase knockout (ArKO) mice. Means with different superscript letters are significantly different from each other by post hoc comparisons ($P < .05$). The number of subjects in each group is given in parentheses.

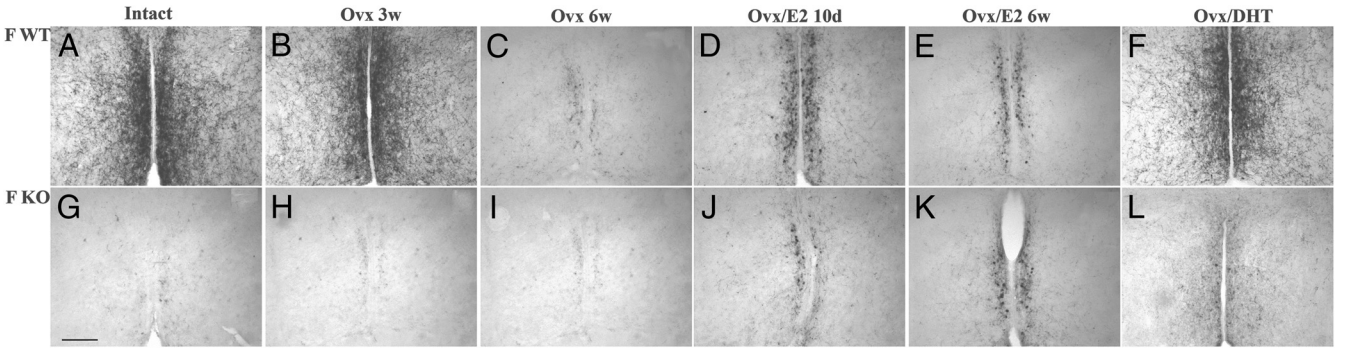

Figure 4. Representative photomicrographs show coronal sections of anteroventral periventricular nucleus/periventricular nucleus continuum (AVPv/PeN) with kisspeptin-immunoreactive cells in a WT female (panels A-F) and an ArKO female (panels G-L) under different hormonal treatment [gonadally intact (panels A,G) – ovariectomized 3 wk prior sacrifice (panels B,H) – ovariectomized 6 wk prior sacrifice (panels C,I) – ovariectomized and treated with E_2 capsule during ten days prior sacrifice (panels D,J) – ovariectomized and treated with E_2 capsule during 6 wk prior sacrifice (panels E,K) – ovariectomized and treated with DHT capsule ten days prior sacrifice (panels F,L)]. Scale bar = 100 μm.

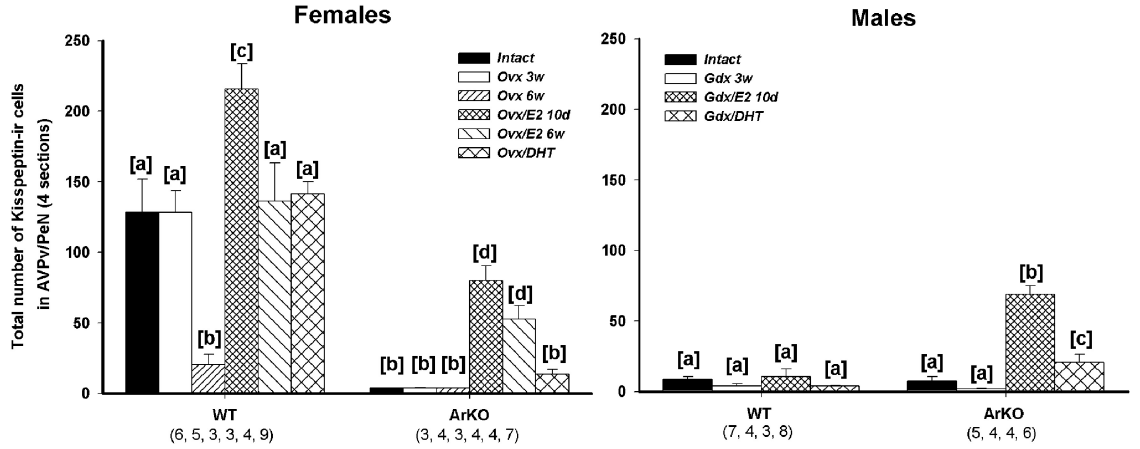

Figure 5. The total number (mean ± S.E.M.) of kisspeptin-expressing cells in the anteroventral periventricular/rostral periventricular nucleus (AVPv/PeN) following gonadectomy [3 wk (3w) versus 6 wk (6w)] and hormonal treatment with E_2 [10 d (10d) versus 6 wk (6w)] or DHT in adulthood in female (left panel) and male (right panel) wild-type (WT) and aromatase knockout (ArKO) mice. Means with different superscript letters are significantly different from each other by post hoc comparisons ($P < .05$). The number of subjects in each group is given in parentheses.

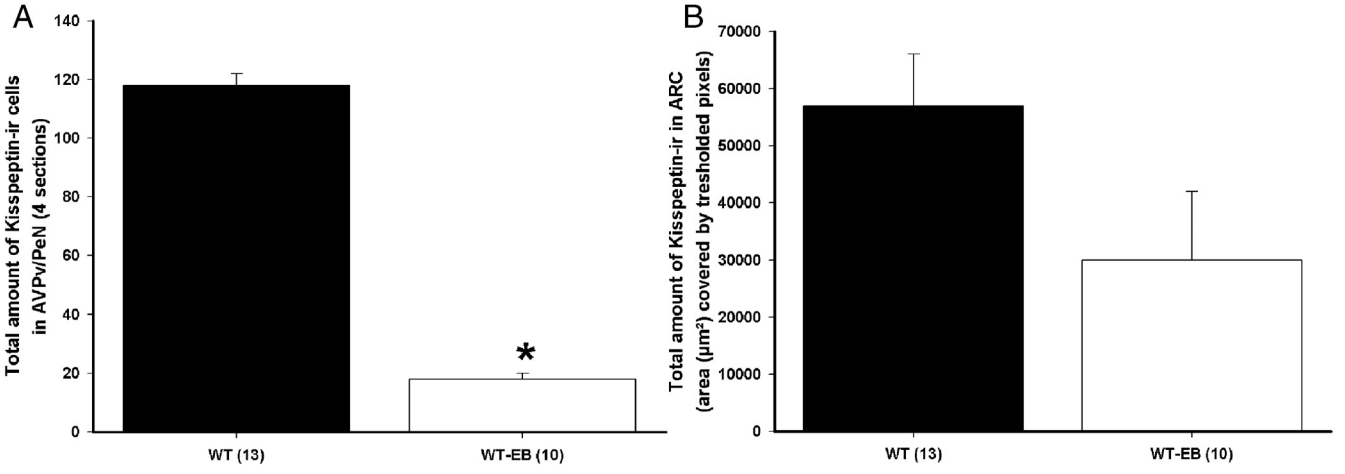

Figure 6. (A) The total number (mean ± S.E.M) of kisspeptin-ir cells in the AVPv/PeN and (B) the total amount (mean ± S.E.M) of kisspeptin-ir in the ARC in adult WT female mice treated with EB 0.5 μg from P5 to P15 (every 2 d). All females were ovariectomized in adulthood and treated with EB 1 μg two weeks prior sacrifice. The number of animals in each group is given in parentheses.

EB treatment from P15 to P25 partially restored the AVPv/PeN kisspeptin population in ArKO females whereas EB given over this prepubertal period had no defeminizing effects on the kisspeptin system in WT females (Figure 7). This prepubertal estrogenic treatment also increased the number of kisspeptin-ir cells in ArKO males.

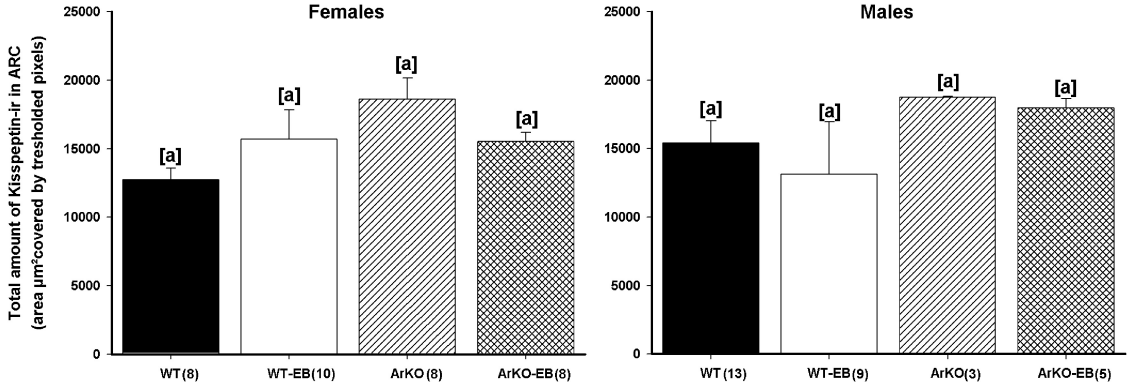

Figure 7. The total number (mean ± S.E.M) of kisspeptin-ir cells in the AVPv/PeN in adult WT and ArKO female and male mice treated with EB 0.05 μg from P15 to P25 (every day). All animals were gonadectomized in adulthood and treated with EB 1 μg two weeks prior sacrifice. Means with different superscript letters are significantly different from each other by post hoc comparisons ($P < .05$). The number of animals in each group is given in parentheses.

ANOVA on the number of kisspeptin-ir cells showed a significant interaction between sex, genotype and treatment ($F(1, 56) = 6.97$; $P = .011$). Post hoc analysis confirmed the well-known sex difference in adult kisspeptin peptide expression with WT females showing more kisspeptin-ir cells than WT males. EB-treated WT females and males showed the same number of kisspeptin-ir cells compared to oil-treated conspecifics. Finally, EB-treated ArKO females and males showed more kisspeptin-ir cells than oil-treated conspecifics, but still fewer compared to WT females. In the ARC, EB treatment had no effect regardless of sex or genotype (Figure 8). This was confirmed by ANOVA on the total amount of kisspeptin-ir in the ARC showing no significant interaction between sex, genotype and hormonal treatment ($F(1, 55) = 1.10$; $P = .30$).

Discussion

By using immunohistochemical methods, we determined kisspeptin peptide expression in two specific brain nuclei (AVPv/PeN versus ARC) throughout early postnatal development and in adulthood in WT and ArKO mice of both sexes. The ArKO mouse model allowed us to distinguish between the organizational and activational effects of E_2 on kisspeptin expression. We observed that kisspeptin peptide expression in the ARC versus the AVPv/PeN were differentially organized and activated by E_2. We also found some discrepancies in the pattern of the expression of kisspeptin protein compared to what was previously reported for the expression of *Kiss1* mRNA in these brain regions.

Effects of sex steroids on the ARC kisspeptin system during early postnatal development

For the first time in mice, we quantified the expression of kisspeptin protein in the ARC and showed that it was sexually dimorphic during the early postnatal period, i.e., from P10 to P25, with females showing more kisspeptin-ir (cell bodies plus fibers) than males. This sex difference also persisted until P30 (data not shown), whereas it was completely abolished in adulthood. In rats, a sex difference in

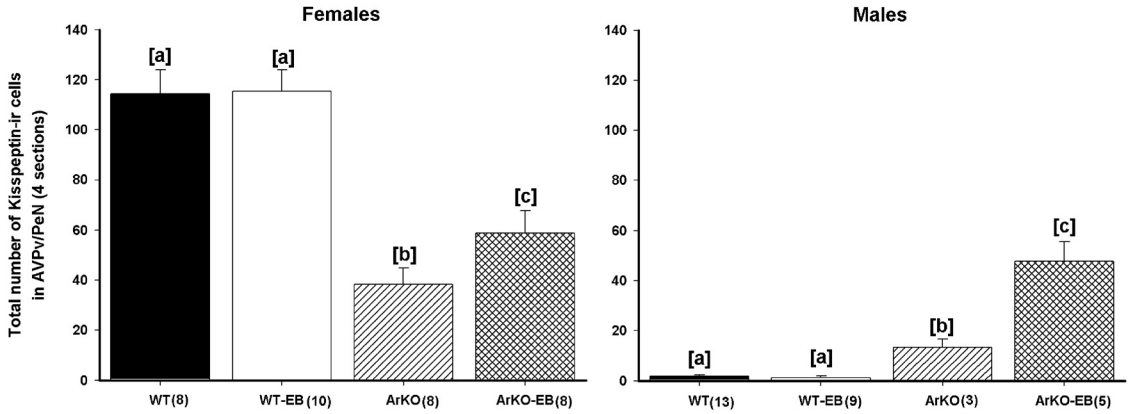

Figure 8. The total amount (mean ± S.E.M) of kisspeptin-ir in the ARC in adult female and male WT and ArKO mice treated with EB 0.05 μg from P15 to P25 (every day). All animals were gonadectomized in adulthood and treated with EB 1 μg two weeks prior sacrifice. No significant difference was found between all groups. The number of animals in each group is given in parentheses.

kisspeptin protein expression was also recently observed during the neonatal period, i.e., as early as P7 (23, 24, 37); however, this sex difference persisted throughout postnatal development, puberty and adulthood. In mice, Clarkson and colleagues (1, 3) detected kisspeptin-ir fibers at different postnatal ages (P10 – P25 – P31 – P61) in the ARC in WT males and females, but did not show a quantification of this expression. More recently, another study showed that the kisspeptin-ir increased qualitatively in the ARC in both WT female and male mice during development, peaking at P45 (38). However, they claimed that kisspeptin staining showed no appreciable differences between male and female ARC, although they did not quantify it. In the present study, we showed an increase in kisspeptin protein expression in the female ARC from P10 to P25 (even to P30, data not shown). The appearance of kisspeptin cells and fibers in WT females between P5 and P10 could coincide with the first sex steroid secretion by the ovaries (39); however, ArKO females expressed similar kisspeptin-ir levels at P10 than WT females although they do not produce E_2 due to their targeted mutation in the aromatase gene. These data thus suggest that the expression of kisspeptin during early postnatal period in the ARC is organized by E_2-dependent and –independent mechanisms. This hypothesis is also reinforced by the fact that WT and ArKO males showed the same significant increase in kisspeptin protein expression at P25. By using the hypogonadal (*hpg*) mouse model which carries a large deletion in the *Gnrh1* gene resulting in a failure to synthesize GnRH and thus in very low levels of gonadal steroid hormones, Gill and colleagues (38) also showed that at the ages of normal pubertal maturation kisspeptin-ir was clearly detected in *hpg* males and females despite the absence of any appreciable exposure to sex steroids. Furthermore, the hormone-independent increase of ARC kisspeptin protein expression in ArKO and *hpg* mice is also consistent with previous studies showing that central mechanisms regulating the onset of puberty were not gonad dependent (40-42). According to *Kiss1* mRNA studies, ARC kisspeptin expressing neurons were identified from embryonic day 12 in mice (43, 44), but did not show any developmental changes across the postnatal period (45) and were present at very low levels at the prepubertal period, i.e., from P16 to P18 (46). Furthermore, no sex difference was ever observed regarding *Kiss1* mRNA levels in the ARC between male and female mice. All these data do not follow the same pattern as what we observed in the present study. However, some reports concerning unrelated changes between *Kiss1* mRNA and kisspeptin-ir levels already exist (25-27, 38) and notably suggest post-transcriptional regulations of the *Kiss1* gene. For example, kisspeptin-ir cells increased in the ARC in the female rat between diestrus and proestrus phases whereas *Kiss1* mRNA levels showed opposite fluctuations (26). Finally, the sex difference in kisspeptin protein expression observed in the present study could also result either from a combination of lower *Kiss1* transcription, or a lower kisspeptin synthesis and/or higher peptide release in males compared to females. In support of this last hypothesis, future studies should focus on kisspeptin-ir levels in the ARC in colchicine-treated mice.

Effects of sex steroids on the AVPv/PeN kisspeptin system during early postnatal development

As previously described (1, 3), we found no kisspeptin protein expression before P15 in the AVPv/PeN and the number of kisspeptin expressing cells increased at P25 in females (data not shown). A strong sex difference was already established between P15 and P25 whereas ArKO males and females showed no kisspeptin expressing cells across all postnatal ages. Our data thus confirm that the major period of appearance of kisspeptin protein expression in the AVPv/PeN occurs over a 15 d time period between P15 and P30, and that reproductive hormones are most likely required for the expression of kisspeptin during early postnatal development in both females and males to establish the sexually dimorphic pattern observed in the AVPv/PeN as already well studied (1, 3, 16, 38).

Interestingly, there seems to be some paradox when comparing the first appearance of kisspeptin peptide expression in the AVPv/PeN versus the ARC. Previous study (47) showed that projections from neurons in the AVPv/PeN to the ARC are well-established before birth, but kisspeptin protein or mRNA is not expressed before birth. By contrast, kisspeptin mRNA is already expressed at E12 in the ARC (43, 44) although its protein probably not before P5, whereas the projections from the ARC to the AVPv are not fully mature before P18 (48, 49). Since kisspeptin has been proposed to play a predominant role in regulating the pubertal and adult reproductive axis, the presence of kisspeptin in the ARC during early development remains unknown. Does it play a specific role in establishing the projections from the AVPv to the ARC? Obviously more research is needed on the ontogeny of kisspeptin in the AVPv/PeN versus the ARC.

Effects of sex steroids on the ARC kisspeptin system in adulthood

In contrast with the early postnatal period, sex differences in kisspeptin protein expression were abolished in the adult ARC. This is in accordance with previous results on *Kiss1* mRNA expression (36). However, we showed that gonadectomy decreased kisspeptin peptide expression in the ARC of both sexes whereas E_2 or DHT short-

term treatment (10 d) restored kisspeptin peptide expression in gonadectomized males and females. This reflects opposite effects compared to the modulation of *Kiss1* mRNA levels by sex steroids in the ARC in adulthood (10, 11, 36). This discrepancy could be explained by the fact that: 1/ the source of kisspeptin fibers revealed by the ICC is still not identified, as they could originate from the ARC but also from the AVPv/PeN (47) and/or even from the MeA (48); 2/ a reduced fiber staining could represent either increased kisspeptin secretion/release or increased storage/decreased transport. It is thus difficult to interpret what the changes in fiber levels reflect in terms of kisspeptin function.

We also observed that a long-term (6 wk) E_2 treatment totally suppressed kisspeptin peptide expression in ovariectomized WT and ArKO females whereas a short-term (10 d) E_2 treatment restored WT-like levels of kisspeptin peptide expression in these animals. After 6 wk of E_2 exposition, the HGP axis might have been overstimulated resulting in an increase of the turnover of the kisspeptin peptide secretion, which could thus be not detected by using immunohistochemistry.

Finally, we showed that DHT treatment had the same effects as E_2 treatment, which is in accordance to previous findings showing that DHT, as T and E_2, decreased *Kiss1* mRNA levels in the ARC (11). However, it is important to note that a metabolite of DHT, such as 5alpha-androstane,3beta,17beta-diol (3beta-Diol) can act as an estrogenic compound by activating the ERβ pathway (49, 50). Thus, the stimulatory effects of DHT on kisspeptin protein expression in the ARC might not imply the androgen receptor pathway (as previously suggested by (11)), but only the ERβ pathway.

Effects of sex steroids on the AVPv/PeN kisspeptin system in adulthood

As observed in the early postnatal period (data not shown), a sex difference in kisspeptin peptide expression was also present in adulthood, with intact WT females showing more kisspeptin expressing cells compared to intact WT males. This fits with the theory that the AVPv/PeN kisspeptin population is required for the regulation of the preovulatory surge of gonadotropins observed in females (3, 51). We also showed that long-term gonadectomy strongly decreased kisspeptin peptide expression in WT females whereas E_2 treatment, but not DHT, increased the number of kisspeptin expressing cells. This is completely in accordance with previous results on *Kiss1* mRNA expression (10, 11, 36). Intact ArKO mice of both sexes showed a low, "male-typical" expression of kisspeptin in the AVPV/PeN suggesting thus that perinatal E_2 exposure is required for the full development of the AVPv/PeN kisspeptin population. This hypothesis was reinforced by the fact that E_2 treatment increased kisspeptin protein expression in adult ovariectomized ArKO females, but failed to restore it to WT-like levels. More surprisingly, adult gonadectomized ArKO males treated with E_2 also showed an increase in the number of kisspeptin expressing cells whereas gonadectomized WT males did not. This could mean that the AVPv/PeN kisspeptin neuronal population in ArKO males was not completely defeminized (and as a result could be still stimulated following exogenous E_2 treatment) due to an absence of E_2 exposure during the perinatal period.

Critical period for development of the two different kisspeptin populations

In the present study, we showed that the ARC kisspeptin population was not affected in adulthood following EB treatment either between P5-P15 or P15-P25 in either sex or genotype. This suggests that the ARC kisspeptin population is regulated by sex steroids or others factors during another critical period such as puberty or the perinatal period. Recent data in rats showed that kisspeptin-ir cells were first detected in the embryonic ARC at E14.5 and increased until E18.5; then the number of kisspeptin-ir cells strongly decreased until birth (21). In mice, ARC *Kiss1* mRNA levels were detected from E12 (43, 44), but future studies have to be conducted to determine the spatio-temporal appearance of kisspeptin protein expression in mouse embryos of both sexes.

In the AVPv/PeN, treatment of WT females with EB over the early postnatal period (P5-P15) defeminized the kisspeptin neuronal population in adulthood whereas the same treatment administered over a prepubertal period (P15 to P25) had not such an effect. However, this particular prepubertal EB treatment partially restored the AVPv/PeN kisspeptin population in adulthood in ArKO females. Our data thus suggest that the AVPv/PeN kisspeptin population could be still defeminized/masculinized at P5 and perhaps until P10 and is feminized from P15 onwards, which is in accordance with previous experiences regarding to the expression of sexual behaviors (28). Prepubertal treatment with EBalso increased the number of kisspeptin-ir cells in ArKO males, but not in WT males suggesting that these neurons still kept the capacity to respond to E_2 and thus be feminized. Taken together, these observations suggest that the increase in T (and conversely E_2) occurring in male rodents during the perinatal period is responsible for the dampening of AVPv/PeN kisspeptin expressing neurons whereas the complete functional feminization of the AVPv/PeN kisspeptin population is not a merely passive process as was previously suggested (8, 13) but requires the active contribution of some estrogenic

inputs. In mice, Clarkson and colleagues (3) showed that replacement of E_2 in P15-ovariectomized females from P15-P30 or P22-P30 resulted in a complete restoration of kisspeptin peptide expression in AVPv/PeN; however, they used a higher dose of E_2 (Silastic capsules) and killed their animals directly after the E_2 treatment, i.e., at P30. In adult rats, a strong decrease of AVPv/PeN kisspeptin-ir fibers was observed following exposure to EB or phytoestrogens (52), ERα (PPT; (53)) or ERβ (DPN; (54)) agonists from P0 to P3. However, a decrease in ARC kisspeptin-ir fibers was also observed after EB exposure from P0 to P3, suggesting species-specific differences compared to our study.

Conclusions

By using immunohistochemical studies, we showed here that the two kisspeptin neuronal populations, i.e., AVPv/PeN and ARC are very plastic systems which are modulated by sex steroids from birth to adulthood. We observed for the first time a clear sex difference in the development of the ARC kisspeptin neuronal population with females showing more kisspeptin-ir from P5 to P30 compared to males. Furthermore, we demonstrated that the ARC kisspeptin system seems to be organized by E_2-dependent and –independent mechanisms since ArKO animals showed significant levels of kisspeptin-ir. In adulthood, sex steroids, i.e., E_2 and DHT, differentially activate the two kisspeptin neuronal populations. Finally, we showed that the AVPv/PeN kisspeptin system could be still masculinized at postnatal day 5, and was feminized from postnatal day 15 onwards showing that E_2 can have both masculinizing and feminizing effects on the kisspeptin system depending on when during development it is present.

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