Journal of Neuroendocrinology

Journal of Neuroendocrinology, 2012, **24**, 1222–1233

ORIGINAL ARTICLE

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Aromatase Knockout Mice Show Normal Steroid-Induced Activation of Gonadotrophin-Releasing Hormone Neurones and Luteinising Hormone Surges With a Reduced Population of Kisspeptin Neurones in the Rostral Hypothalamus

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Journal of Neuroendocrinology

We recently reported that female aromatase knockout (ArKO) mice show deficits in sexual behaviour and a decreased population of kisspeptin-immunoreactive neurones in the rostral periventricular area of the third ventricle (RP3V), resurrecting the question of whether oestradiol actively contributes to female-typical sexual differentiation. To further address this question, we assessed the capacity of ArKO mice to generate a steroid-induced luteinising hormone (LH) surge. Adult, gonadectomised wild-type (WT) and ArKO mice were given silastic oestradiol implants s.c. and, 1 week later, received s.c. injections of either oestradiol benzoate (EB) followed by progesterone, EB alone, or no additional steroids to activate gonadotrophin-releasing hormone (GnRH) neurones and generate an LH surge. Treatment with EB and progesterone induced significant Fos/GnRH double-labelling and, consequently, an LH surge in female WT and in ArKO mice of both sexes but not in male WT mice. ArKO mice of both sexes had fewer cells expressing Kiss-1 mRNA in the RP3V compared to female WT mice but had more Kiss-1 mRNA-expressing cells compared to WT males, reflecting an incomplete sexual differentiation of this system. To determine the number of cells expressing kisspeptin, the same experimental design was repeated in Experiment 2 with the addition of groups of WT and ArKO mice that were given EB + progesterone and sacrificed 2 h before the expected LH surge. No differences were observed in the number of kisspeptin-immunoreactive cells 2 h before and at the time of the LH surge. The finding that ArKO mice of both sexes have a competent LH surge system suggests that oestradiol has predominantly defeminising actions on the GnRH/LH surge system in males and that the steroid-induced LH surge can occur in females even with a greatly reduced population of kisspeptin neurones in the RP3V.

Key words: aromatase, kisspeptin, sexual differentiation, oestradiol, hypothalamus.

doi: 10.1111/j.1365-2826.2012.02334.x

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The capacity to generate a preovulatory surge of gonadotrophin-releasing hormone (GnRH)/luteinising hormone (LH) in response to gonadal steroids is perhaps the most robust sexually differentiated endocrine response in rodents, with only females being capable of generating regular LH surges. The release of LH from the pituitary is stimulated by the secretion of GnRH from a diffuse population of neurones in the hypothalamus, which is in turn modulated by the actions of circulating oestradiol and progesterone. During most of the oestrous cycle, oestrogens exert a negative-feedback on the hypothalamic-pituitary axis. By contrast, on the afternoon of pro-oestrous, oestrogens and/or progesterone exert a positive-feedback on the hypothalamic-pituitary axis, leading to a massive release of GnRH and, subsequently, LH, and in turn to ovulation, at the same time as facilitating sexual behaviour (1–3). Thus, ovarian oestradiol secreted from metoestrous through early pro-oestrous by the developing follicle stimulates the release of GnRH and sensitises the pituitary gland to become more responsive to hypothalamic GnRH (4). The hypothalamic-pituitary axis in males, on the other hand, does not show positive-feedback in response to oestradiol and males are not able to support ovulation when implanted with ovarian tissue as adults (5).

This sex difference in the GnRH/LH surge system appears to be organised by the perinatal actions of testosterone and/or oestradiol on the hypothalamus (6, 7). In rodents, neonatal castration feminises the ability of males to show the capacity to generate an LH surge, and treatment with testosterone or oestradiol in the neonatal period will prevent the development of the GnRH/LH surge system in females (8–10). In males, testosterone is secreted from the developing testis and is subsequently aromatised in the brain and other tissues to oestradiol by the enzyme aromatase. Prenatal treatment with the aromatase inhibitor, 1,4,6-androstatrien-3,17-dione disrupts LH responses to oestradiol in adult male guinea pigs (11), and affects male-typical sex behaviour in guinea pigs (12), sheep (13) and rats (14–16), indicating that the prenatal aromatisation of testosterone to oestradiol is crucial to the normal development of reproductive behaviours and responses to gonadal hormones in adulthood. Accordingly, perinatal treatment with exogenous oestradiol defeminises the GnRH/LH system in female rats (9, 17, 18). Further evidence for a defeminising role for oestradiol is found in female mice which, as a result of a targeted mutation of the *Afp* gene, lack the foetal circulating oestradiol-binding protein, α-foetoprotein and thus the protection against maternal oestrogens (19). Previous work in our laboratory shows that *Afp* null female mice do not show any LH surges after treatment with oestradiol and progesterone (20), indicating that exposure to oestradiol during the prenatal period defeminises the GnRH surge system. However, the exact mechanism by which oestradiol defeminises the potential to show such steroid-induced LH surges has not been fully identified.

It is possible that another neuropeptide system organised by oestradiol also plays a role in the organization of the GnRH/LH surge system. Recent studies have focused on the role of kisspeptin, a small peptide that appears to play a crucial role in GnRH secretion. Kisspeptin stimulates GnRH secretion via its receptor, GPR54 (now known as Kiss1r), which is present on GnRH neurones (21). Treatment with kisspeptin stimulates LH secretion in many species, including mice (22), rats (23), sheep (21, 24), monkeys (25) and humans (26). Treatment with kisspeptin antibodies or antagonists can block the ability to show an LH surge in female rats (27, 28). In addition, the expression of this peptide is sexually dimorphic in rodents: the number of kisspeptin neurones in the rostral periventricular area of the third ventricle (RP3V), an area comprised of the anteroventral periventricular (AVPV), periventricular preoptic and median preoptic nuclei (29), is much higher in females than in males (30), suggesting that this brain area may play an important role in the sexually dimorphic control of GnRH secretion. Another population of kisspeptin neurones exists in the arcuate nucleus; however, this population is not sexually dimorphic in adulthood and not considered to be implicated in the generation of the LH surge in rodents (31). Kisspeptin neurones express oestradiol receptor (ER)α and progesterone receptors (32–34) and oestradiol treatment increases kisspeptin expression in the RP3V (35), whereas GnRH neurones only appear to contain ERβ, which does not appear to mediate the effects of oestradiol on the generation of the GnRH/LH surge (36–38). These findings suggest that kisspeptin neurones likely modulate the effects of gonadal steroids on GnRH secretion. However, the effects of deletion of the kisspeptin (*Kiss-1*) gene or *Kiss1r* gene on the GnRH/LH surge system do not provide consistent support for the necessity of kisspeptin in the generation of steroid-induced LH surges. One group has found that neither *Kiss-1*, nor *Kiss1r* null mice are capable of generating steroid-induced LH surges (34). By contrast, another group using a different transgenic mouse line has shown that *Kiss1r* null mice are capable of generating a steroid-induced LH surge (39). Furthermore, *Kiss-1r* and *Kiss* null male mice show increased LH secretion in response to central, but not peripheral treatment with NMDA, a potent stimulator of LH secretion (40). Lastly, female mice in which the majority of kisspeptin- or Kiss1r-expressing neurones have been genetically ablated show ovarian follicles in all stages of development and are fertile (41), suggesting that normal reproductive development can occur in the absence of kisspeptin signalling to GnRH neurones. These conflicting results may not only be a result of differences in mouse strains used, but they also illustrate that the role of kisspeptin in the generation of the LH surge is not completely understood.

Although there is a strong defeminising role for oestradiol in the development of the GnRH surge system, recent data obtained in female aromatase knockout (ArKO) mice that cannot synthesise oestradiol from its androgenic precursor as a result of a targeted mutation in the *Cyp19* gene also suggest that oestradiol may be necessary to feminise the kisspeptin system. Female ArKO mice treated with oestradiol and progesterone in adulthood show decreased levels of lordosis behaviour when paired with a sexually active male and have reduced numbers of kisspeptin neurones in the RP3V (42, 43). Oestradiol treatment alone fails to induce ovulation in female ArKO mice (44), indicating a possible link between the lack of ovulation (and presumably steroid-induced LH surges) and the absence of a female-typical population of kisspeptin in the RP3V. Clarkson and Herbison (45) have shown that ovariectomy of female mice at postnatal day (P)15 results in a 70–90% reduction in kisspeptin expression within the RP3V analysed at either P30 or P60, whereas treatment with 17β-oestradiol in P15-ovariectomised mice from P15–30 or P22–30 resulted in a complete restoration of kisspeptin expression in this brain region, indicating that oestradiol can affect the organisation of the kisspeptin system after the perinatal period. Conversely, female rats treated with oestradiol at birth show reduced numbers of kisspeptin-expressing neurones in the rostral hypothalamus (9). Taken together, these results suggest that oestradiol not only affects the organisation of the brain during the prenatal and neonatal period, but also can act during the prepubertal period to organise the kisspeptin system.

Therefore, in the present study, we tested the hypothesis that oestradiol is necessary to feminise the capacity to show preovulatory LH surges by determining whether female ArKO mice are capable of showing steroid-induced LH surges. In addition, we also investigated the potential of ArKO males to show steroid-induced LH surges aiming to confirm the hypothesis that oestradiol defeminises the potential to show preovulatory LH surges in this sex. If oestradiol only defeminises the potential to show an LH surge, we would expect that both ArKO males and females would show an LH surge. If oestradiol is required to feminise the LH surge system, we would expect that neither ArKO males, nor ArKO females would show a steroid-induced surge of LH. Interestingly, ArKO males and

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ArKO females have low numbers of kisspeptin neurones (43); thus, if ArKO mice fail to show a steroid-induced LH surge, this may be a result of this decreased population of kisspeptin neurones in the RP3V. To test the hypothesis that a female-typical number of kisspeptin neurones is necessary for a steroid-induced LH surge, the number of cells expressing Kiss-1 mRNA and kisspeptin protein in the RP3V of ArKO mice was determined at and around the time of the expected LH surge.

Materials and methods

Animals

The present study used aromatase knockout mice with a targeted disruption of exons 1 and 2 of the *Cyp19* gene (46, 47). All breeding and genotyping were carried out at the department GIGA Neurosciences, University of Liège, Belgium. Heterozygous males and females of the CD1 (Swiss-Webster) strain were bred to generate wild-type (WT) and knockout (ArKO) offspring. Genotyping was performed by polymerase chain reaction analysis of tail DNA (48). Food and water were available *ad lib.* and the temperature was maintained at 22 °C. 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 Committee for Animal Use of the University of Liege.

Surgery

Adult (3–6 months) WT and ArKO mice of both sexes were anaesthetised using an s.c. injection of ketamine (80 mg/kg per mouse) and medetomidine (Domitor, Pfizer (Domitor Pfizer Animal Health BV, Capelle a/d Ijssel, the Netherlands; 1 mg/kg per mouse) and the gonads were removed. At the same time as gonadectomy, all mice were implanted s.c. with a silastic capsule (5 mm in length, inner diameter 1 mm, outer diameter 2 mm) containing 1 μg of 17β-oestradiol dissolved in 7.35 μl of sesame oil (SO) to maintain the negative-feedback effects of low levels of oestradiol on circulating gonadotrophins. At the end of surgery, all mice received an s.c. injection of atipamezole (Antisedan, Pfizer; 4 mg/kg per mouse) to antagonise medetomidine-induced effects and accelerate recovery. After surgery, mice were placed in individual cages, and males and females were housed in different rooms under a reversed 12 : 12 h light/dark cycle.

Experiment 1: Determinination of whether ArKO mice can generate a steroid-induced activation of GnRH neurones and a plasma LH surge

Hormone treatment

One week (7 days) after gonadectomy, WT and ArKO mice were divided into three groups: Group 1 mice [oestradiol benzoate (EB) + progesterone (P): WT/male, n = 10; ArKO/male, n = 11; WT/female, n = 9; ArKO/female, n = 8] received an s.c. injection of EB (1 μg/20 g body weight/0.05 ml of SO) 4 h before the onset of the dark period (day 8) and, 24 h later (day 9), an s.c. injection of progesterone (500 μg in 0.05 ml of SO) to induce an LH surge; group 2 mice (EB only: WT/male, n = 11; ArKO/male, n = 11; WT/female, n = 7; ArKO/female n = 8) received only an EB injection (day 8) and nothing on day 9, and group 3 mice (implant only: WT/male, n = 10; ArKO/male, n = 11; WT/female, n = 6; ArKO/female, n = 7) received no additional steroid injections on either day 8 or day 9. At the expected time of the LH surge (within 1 h of the onset of the dark period, day 9 of treatment), mice were anaesthetised with an overdose of ketamine (160 mg/kg/mouse) and medetomidine (2 mg/kg/mouse). Approximately 1 ml of blood was drawn via cardiac puncture for LH assay. Next, all mice were perfused transcardially with saline followed by 4% cold paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 2 h at room temperature (RT). Brains were then placed in a 30% sucrose solution at 4 °C until they sank, and were then frozen on dry ice and stored at −80 °C. Brains were sliced coronally on a cryostat from the rostral telencephalon to the posterior hypothalamus making four sets of 30 μm sections. Sections were saved in cryoprotectant solution and stored at −20 °C for immunohistochemistry for GnRH/Fos and *in situ* hybridisation for Kiss-1 mRNA levels. As a result of technical problems, not all samples used for immunohistochemistry had sufficient tissue remaining for *in situ* hybridisation, and the group numbers used for *in situ* hybridisation were: for EB + P treatment: WT/males, n = 7; ArKO/males, n = 7; WT/females, n = 6; ArKO/females, n = 6; for EB only treatment: WT/males, n = 4; ArKO/males, n = 5; WT/females, n = 6; ArKO/females, n = 6; for implant only treatment: WT/males, n = 5; ArKO/males, n = 6; WT/females, n = 5; ArKO/females, n = 5.

Immunohistochemistry and data analysis for paraformaldehyde-fixed tissue

We previously showed that the steroid-induced LH surge was accompanied by an induction of Fos, the protein product of the immediate early gene, c-Fos, in GnRH neurones (20). Therefore, in the present study, we performed double-labelling immunohistochemistry on the brain sections to determine the co-localisation of Fos- and GnRH-expressing neurones in the preoptic area. Primary antibodies were specific for Fos polyclonal antibody (dilution 1 : 2000; sc-52 rabbit Fos pAB; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and GnRH I [dilution 1 : 1000; GnRH I (FI-92); Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA]. In experiment 1, double-label immunohistochemistry sections were first rinsed in 0.01 M phosphate-buffered saline (PBS) (pH 7.4), then peroxidase activity was blocked in a 1 : 4 methanol : PBS solution with 3% H_2O_2. After peroxidase blocking, sections were rinsed in PBS-0.1% Triton-X 100 (PBST) and then incubated in a solution of 5% normal goat serum (NGS) in PBST. Sections were then incubated overnight in a solution of Fos Ab in NGS (Fos Ab, dilution 1 : 2000) at 4 °C. The next day, sections were rinsed in PBST, and then incubated in polyclonal goat anti-rabbit biotinylated secondary antibody (dilution 1 : 1000 in PBST; Dako, Glostrup, Denmark) for 1 h at RT. Sections were washed in PBST and incubated in the Vectastain Elite ABC Kit (PK6100; Vector Laboratories, Burlingame, CA, USA), then rinsed in PBST. After rinsing in a solution of 0.05 M Tris-HCl, sections were developed with the DAB substrate Kit (SK-4100; Vector Laboratories) to give a black precipitate [3,3′-diaminobenzidine (DAB) +Ni^{2+}]. Sections were then rinsed in MilliQ H_2O (Millipore, Billerica, MA, USA) followed by PBS, re-fixed in 4% paraformaldehyde and the residual peroxidase activity was blocked in 1 : 4 methanol : PBS solution. Sections were rinsed in PBST then incubated in GnRH pAb (dilution 1 : 1000) at 4 °C for 24 h, respectively. Next, secondary antibody and ABC steps were performed as described above, and the tissue was developed using Vector NovaRED substrate Kit (SK-4800; Vector Laboratories) to produce a red precipitate. Sections were then mounted on slides, dried and cover slipped in Eukitt (Sigma, Bornem, Belgium).

For GnRH and Fos/GnRH double-labelled cells, the counting area was located within the preoptic area (interaural 4.42–4.30 mm and Bregma 0.62–0.50 mm), as previously described (49). Both hemispheres of two brain sections (with an interval of 120 μm between sections) were counted.

In situ hybridisation of Kiss mRNA levels: preparation of riboprobe

A plasmid containing mouse Kiss1 riboprobe was generously provided by Dr Alexander Kauffman (Department of Reproductive Medicine and the Centre

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for Chronobiology, University of California, San Diego, La Jolla, CA, USA). The sequence of the riboprobe spans bases 78–486 of the mouse Kiss1 gene (Genbank accession number AF473576). The plasmid was heat-transfected and amplified in competent *Escherichia coli* DH5α cells. The circular plasmid was extracted from these cells and purified using the QIA Filter Plasmid Midi-Kit (Qiagen Inc., Valencia, CA, USA). Plasmids were linearised by incubation with *Hind*III restriction enzyme (Roche Diagnostics GmbH, Mannheim, Germany) and the linearised DNA was purified using QIAprep Spin Miniprep Kit (Qiagen Inc.). The antisense Kiss1 probe was synthesised using T7 transcription enzyme, DIG labelling mix, and RNAase inhibitors (Roche Diagnostics GmbH). The antisense probe was purified in Sephadex G-50 columns (Kit-Illustra microspin G50 columns; GE Healthcare, Milwaukee, WI, USA).

In situ hybridisation

Free-floating brain sections prepared as previously described for immunohistochemistry were used for *in situ* hybridisation. Sections were washed in PBS-diethylpyrocarbonate (DEPC) then briefly post-fixed with 4% paraformaldehyde. Sections were washed again in PBS-DEPC and then treated with 100 mM triethanolamine acetylated with 0.25% acetic acid. Sections were washed with PBS-Tween 0.1% and then pre-hybridised with pre-warmed hybridization buffer for 1 h at 70 °C. Sections were then hybridised with a mix of 4 μl DIG-labelled Kiss1 probe and 250 μ of pre-warmed hybridisation buffer overnight in a 70 °C water bath. Sections were washed in washing buffer (50% formamide, 2×SSC, 0.1% Tween) at 72 °C, washed in buffer B1 (100 mM Tris-HCl pH 7.5, 150 nM NaCl, 0.1% Tween) at RT, then blocked in buffer B2 (10% NGS in buffer B1) for 1 h 15 min at RT. Next, tissue was incubated with anti-DIG coupled to alkaline phosphatase (Anti-DIG-AP Fab fragments; Roche Diagnostics GmbH) diluted 1 : 2000 in B2 overnight at 4 °C. Tissue was washed in B1 then incubated in B3 (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM MgCl2, 0.1% Tween) at RT. Next, tissue was added to 3–400 μl of filtered NBT/BCIP liquid substrate system (Sigma Aldrich, St. Louis, MO, USA) in the dark for 1 h at RT. The tissue was transferred to fresh NBT/BCIP and the reaction was allowed overnight in the dark at 4 °C until the desired signal was reached. Tissue was washed in PBS-Tween to stop the reaction, and was mounted on slides in an aqueous gelatin medium and coverslipped. After allowing the slides to dry, *Kiss1*-mRNA containing cells were counted. The counting areas were limited to the rostral hypothalamus within interaural: 4.06–3.94 mm and Bregma 0.26–0.14 mm according to Franlyn and Paxinos (50). Both hemispheres of two to four brains sections (with an interval of 120 μm between sections) were counted.

Experiment 2: comparison of numbers of kisspeptin-immunoreactive neurones before and after the steroid-induced LH surge in ArKO versus WT mice

In Experiment 1, we observed that ArKO mice of both sexes were clearly capable of showing a steroid-induced LH surge at the same time as having a decreased level of Kiss-1 mRNA expression in the RP3V, which might question the role of kisspeptin in regulating the preovulatory LH surge. Therefore, we conducted a second experiment to determine the expression of kisspeptin protein before and during the steroid-induced surge of LH.

Hormone treatment

A new batch of WT and ArKO mice was gonadectomised and implanted with silastic capsules containing oestradiol as described in Experiment 1. One week (7 days) after gonadectomy, mice were divided into four groups: group 1 mice received a s.c. injection of oestradiol benzoate (EB) (1 μg/20 g body weight/0.05 ml of SO) 4 h before the onset of the dark period (day 8) and, 24 h later (day 9), an s.c. injection of progesterone (500 μg in 0.05 ml of SO) to induce an LH surge. Two animals had plasma LH concentrations that were more than ten-fold lower than the others in the same sex/genotype (WT/female, ArKO/male) and so were removed from the study (resulting group numbers for EB + P: WT/male, n = 4; ArKO/male, n = 3; WT/female, n = 4; ArKO/female, n = 5); group 2 mice received only an EB injection (day 8) and nothing on day 9 (EB only: WT/male, n = 5; ArKO/male, n = 4; WT/female, n = 6; ArKO/female, n = 5) and group 3 mice received no additional steroid injections on either day 8 or day 9 (implant only: WT/male, n = 5; ArKO/female, n = 4; WT/female, n = 5; ArKO/female, n = 5). All mice in groups 1–3 were sacrificed at the expected time of the LH surge (within 1 h of the onset of the dark period, on day 9 of treatment). An additional group received an injection of EB on day 8 and progesterone on day 9, and were sacrificed 2 h before the expected time of the LH surge (EB + P – 2 h: WT/male, n = 6; ArKO/male, n = 4; WT/female, n = 5; ArKO/female, n = 5).

At the time of sacrifice, mice were anaesthetised with an overdose of ketamine (160 mg/kg/mouse) and medetomidine (2 mg/kg/mouse). Approximately 1 ml of blood was drawn via cardiac puncture for LH assay. Next, the brains were removed and fixed in 5% acrolein solution for 2.5 h, and then rinsed 2 × 30 min in PBS at RT. We used acrolein as fixative because we observed only few kisspeptin-immunoreactive (-IR) neurones in ArKO mice when brains were fixed with paraformaldehyde (43). In addition, the type of fixative used for immunohistochemistry can affect staining (51). Brains were then placed in a 30% sucrose solution at 4 °C until they sank, and were then frozen on dry ice and stored at −80 °C until use. Brains were sliced coronally on a cryostat from the rostral telencephalon to the posterior hypothalamus, making four sets of 30-μm sections. Sections were saved in cryoprotectant solution and stored at −20 °C for immunohistochemistry. .

Immunohistochemistry and data analysis for acrolein-fixed tissue

We performed double-labelling immunohistochemistry on the brain sections to determine the co-localization of Fos- and Kisspeptin-IR neurones in the rostral hypothalamus. Immunohistochemical procedures were very similar to those described for Experiment 1, with the exception that all rinses were performed in Tris buffer solution (pH 7.4) and that sections were first incubated in 1% sodium borohydride solution and tissue was developed with the DAB substrate Kit (SK-4100; Vector Laboratories) to give a brown precipitate for kisspeptin immunoreactivity. Primary antibodies were specific for Fos polyclonal antibody (dilution 1 : 2000; sc-52 rabbit Fos pAB, Santa Cruz Biotechnology Inc.), and kisspeptin (dilution 1 : 5000; rabbit pAb donated by Dr A. Caraty, UMR Physiologie de la Reproduction et des Comportements, INRA, Nouzilly France). Sections were incubated in kisspeptin antibody for 72 h at 4 °C. This concentration of kisspeptin antibody was chosen after testing with lower and higher concentrations and found to provide the best quality labelling in our laboratory. For Kisspeptin and Fos/Kisspeptin double-labelled cells, the counting areas were limited to the rostral hypothalamus within interaural: 4.06–3.94 mm and Bregma 0.26–0.14 mm according to Franklin and Paxinos (50). Both hemispheres of three to four brains sections (with an interval of 120 μm between sections) were counted.

LH analysis

Serum LH levels were determined in duplicate in a volume of 100 μl using a double-antibody method and radioimmunoassay kits, kindly supplied by the National Institute of Health (Dr A. F. Parlow, National Institute of Diabetes and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA, USA). Rat LH-I-10 (AFP-11536 B) was labelled with ^{125}I by the chloramine-T method. The hormone concentrations were expressed using the mouse LH reference preparation (AFP-5306A) as standard. The intra-assay and inter-assay coefficients of variations were less than 7% and 10% respectively. The sensitivity of the assay was 0.04 ng/ml.

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Statistical analysis

Two-way ANOVAS with genotype/sex and treatment as factors were carried out for all dependent variables (STATISTICA, version 9; StatSoft, Inc., Tulsa, OK, USA). When appropriate, the ANOVAS were followed by Fisher's least significant difference post-hoc comparisons (for the percentage Fos/GnRH data, the one-tailed test was used, whereas a two-tailed test was used for all other data). $P < 0.05$ was considered statistically significant.

Results

Experiment 1: Determinination of whether ArKO mice can generate a steroid-induced activation of GnRH neurones and a plasma LH surge

Plasma LH levels

Treatment with EB + P induced a LH surge in WT females and ArKO mice of both sexes but not in WT males (Fig. 1A), supporting the hypothesis that oestradiol defeminises the GnRH/LH surge system. The two-way ANOVA indicated a significant effect of genotype/sex ($F_{3,97} = 12.43$, $P < 0.0001$), treatment ($F_{2,97} = 28.58$, $P < 0.0001$) and a significant genotype/sex × treatment interaction ($F_{6,97} = 8.22$, $P < 0.0001$). Post-hoc analysis showed that, in WT female mice, treatment with EB + P resulted in higher plasma concentrations of LH compared to EB treatment ($P < 0.05$) or implant alone ($P < 0.01$). ArKO female mice treated with EB + P also showed significantly higher plasma concentrations of LH than ArKO female mice given EB ($P < 0.0001$) or implant only ($P < 0.0001$), indicating that female ArKO mice are capable of showing steroid-induced LH surges. ArKO males treated with EB + P had significantly higher concentrations of plasma LH than ArKO males treated with EB ($P < 0.0001$) or given an implant only ($P < 0.0001$), and ArKO males treated with EB + P also had significantly higher concentrations of plasma LH than WT males given EB + P ($P < 0.0001$), indicating that ArKO males are also capable of showing steroid-induced LH surges. WT male mice treated with EB + P had significantly lower plasma concentrations of LH than WT male mice given an implant only ($P < 0.05$) but did not differ significantly from WT male mice treated with EB only ($P = 0.78$).

Number of GnRH-IR neurones and percentage of double-labelled Fos/GnRH neurones

In accordance with plasma LH concentrations, subsequent treatment with EB + P induced Fos protein in GnRH neurones in the preoptic area in WT females and ArKO mice of both sexes but not in WT males (Fig. 1B). There were no significant differences between groups in the number of GnRH-IR neurones in the preoptic area (Table 1); however, differences in the percentage of double-labelled Fos/GnRH neurones were found (Fig. 1B). Images of the Fos/GnRH staining are presented in Fig. 2. Two-way ANOVA revealed significant main effects of genotype/sex ($F_{3,97} = 7.49$, $P < 0.001$) and treatment ($F_{2,97} = 18.68$, $P < 0.001$) and a significant genotype/sex × treatment interaction ($F_{6,97} = 3.86$, $P < 0.001$). Post-hoc analysis indicated that WT female mice treated with EB + P had a higher percentage of double-labelled Fos/GnRH neurones than WT female mice treated with EB ($P = 0.05$) or implant only ($P = 0.05$), and also differed significantly from WT males treated with EB + P ($P < 0.05$). ArKO female mice treated with EB + P also showed a significantly higher percentage of double-labelled Fos/GnRH neurones than ArKO female mice given EB ($P < 0.01$) or implant only ($P < 0.01$). ArKO males treated with EB + P had a higher percentage of double-labelled Fos/GnRH neurones than ArKO males treated with EB ($P < 0.0001$) or implant only ($P < 0.0001$), and ArKO males treated with EB + P had a significantly higher percentage of double-labelled Fos/GnRH neurones than WT males treated with EB + P ($P < 0.0001$), indicating that the LH surge produced in WT female mice and ArKO mice of both sexes in response to EB + P is accompanied by a significant co-expression of Fos in GnRH neurones.

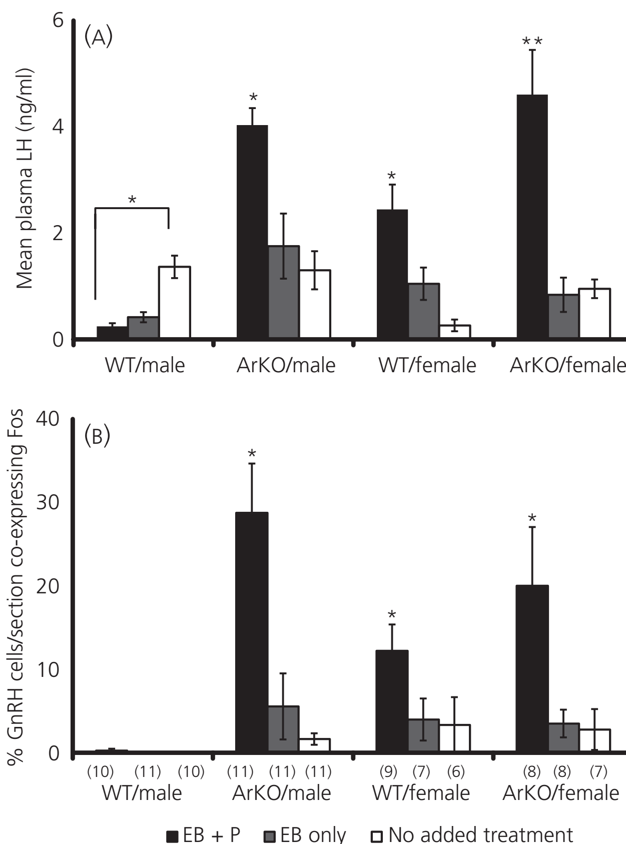

Fig. 1. Capacity of aromatase knockout (ArKO) mice to show a steroid-induced luteinising hormone (LH) surge. (A) Mean ± SEM plasma concentrations of LH (ng/ml) and (B) Mean ± SEM percentage of gonadotrophin-releasing hormone (GnRH) neurones co-expressing Fos/section in the preoptic area in gonadectomised male and female wild-type (WT) and ArKO mice given a silastic implant containing 1 μg of 17β-oestradiol in 7.35 μl of sesame oil for 1 week, then treated with injections of oestradiol benzoate (EB) and progesterone (EB + P), oestradiol only (EB only) or no further injections (no added treatment). *$P < 0.05$. **$P < 0.01$. The number of animals per experimental group is given in parentheses.

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Table 1. Mean ± SEM Number of Gonadotrophin-Releasing Hormone (GnRH) Neurones in the Preoptic Area in Gonadectomised Male and Female Wild-Type (WT) and Aromatase Knockout (ArKO) Mice Given a Silastic Implant Containing 1 μg of 17β-Oestradiol in 7.35 μl of Sesame Oil for 1 Week, Then Treated With Injections of Oestradiol Benzoate (EB) and Progesterone (EB + P), Oestradiol Only (EB only) or No Further Injections (No Added Treatment).

	WT/male		KO/male		WT/female		KO/female	
Treatment	GnRH	n	GnRH	n	GnRH	n	GnRH	n
EB + P	34.6 ± 5.6	10	36.1 ± 3.4	11	26.6 ± 2.6	9	29.5 ± 4.2	8
EB only	29.6 ± 6.2	11	30.2 ± 3.9	11	26.1 ± 3.1	7	28.4 ± 5.3	8
No added treatment	36 ± 3.1	10	27.3 ± 3.8	11	26.3 ± 5.2	6	27.6 ± 6.0	7

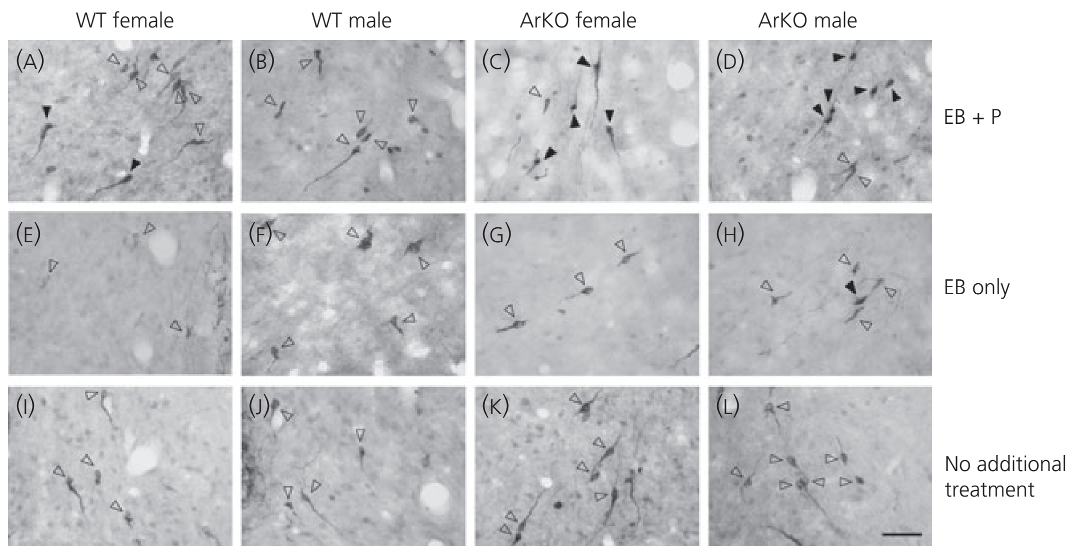

Fig. 2. Representative photomicrographs (× 40) of gonadotrophin-releasing hormone (GnRH) neurones in the preoptic area of gonadectomised male and female wild-type (WT) and aromatase knockout (ArKO) mice given a silastic implant containing 1 μg of oestradiol in 7.35 μl of sesame oil and then treated with injections of oestradiol benzoate and progesterone (EB + P), oestradiol only (EB only) or no further injections (no additional treatment). (A) Female WT mouse treated with EB + P. (B) Male WT mouse treated with EB + P. (C) Female ArKO mouse treated with EB + P.(D) Male ArKO mouse treated with EB + P. (E) Female WT mouse treated with EB only. (F) Male WT mouse treated with EB only. (G) Female ArKO mouse treated with EB only. (H) Male ArKO mouse treated with EB only. (I) Female WT mouse given silastic implant only. (J) Male WT mouse given silastic implant only. (K) Female ArKO mouse given silastic implant only. (L) Male ArKO mouse given silastic implant only. Open arrows indicate GnRH neurones, whereas closed arrows indicated Fos-activated GnRH neurones. Scale bar = 50 μm.

Number of neurones containing Kiss-1 mRNA

ArKO mice were capable of generating a steroid-induced LH surge despite having significantly fewer Kiss-1 expressing cells in the RP3V compared to WT female mice (Fig. 3), which does not support the hypothesis that WT female-typical numbers of kisspeptin neurones in the RP3V are necessary for the generation of the LH surge. Two-way ANOVA of the number of neurones containing Kiss-1 mRNA in the RP3V revealed that there were significant main effects of genotype/sex ($F_{3,56}$ = 150.81, $P < 0.001$) but no significant effect of treatment or genotype/sex × treatment interaction. Post-hoc analysis confirmed that WT female mice had a significantly higher number of neurones containing Kiss-1 mRNA in the RP3V than WT males and ArKO mice of both sexes, regardless of hormonal treatment ($P < 0.01$). WT males had a significantly lower number of neurones containing Kiss-1 mRNA in the RP3V than WT females and ArKO mice of both sexes ($P < 0.01$).

Experiment 2: Comparison of numbers of kisspeptin-IR neurones before and after the steroid-induced LH surge in ArKO versus WT mice

Number of kisspeptin-IR neurones and percentage of double-labelled Fos/kisspeptin neurones in acrolein-fixed tissue

As shown previously (43), WT female mice had a greater number of kisspeptin-IR neurones in the RP3V than WT male mice and ArKO mice of both sexes at the expected time of the LH surge, regardless of hormonal treatment (Fig. 4A). The number of kisspeptin-IR neurones in EB + P treated mice killed 2 h before the expected surge of LH was not significantly different from that at the time of the LH surge. Thus, the low numbers of kisspeptin-IR neurones in ArKO male and female mice showing a steroid-induced surge of LH is not likely the result of a EB + P induced depletion of kisspeptin

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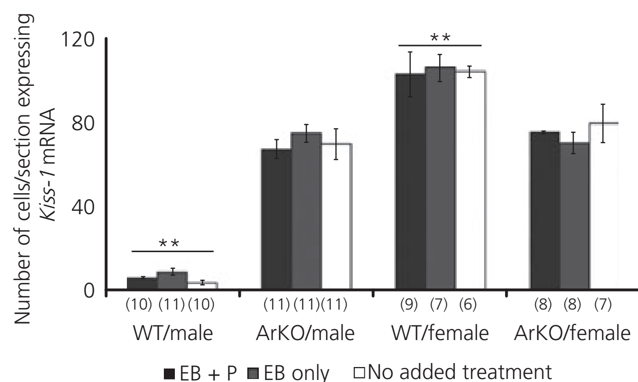

Fig. 3. Mean ± SEM number of *Kiss-1* mRNA-expressing neurones/section in the rostral periventricular area of the third ventricle of gonadectomised male and female wild-type (WT) and aromatase knockout (ArKO) mice given a silastic implant containing 1 μg of 17β-oestradiol in 7.35 μl of sesame oil for 1 week, then treated with injections of oestradiol benzoate and progesterone (EB + P), oestradiol only (EB only) or no further injections (no added treatment). **P < 0.01. The number of animals per experimental group is given in parentheses.

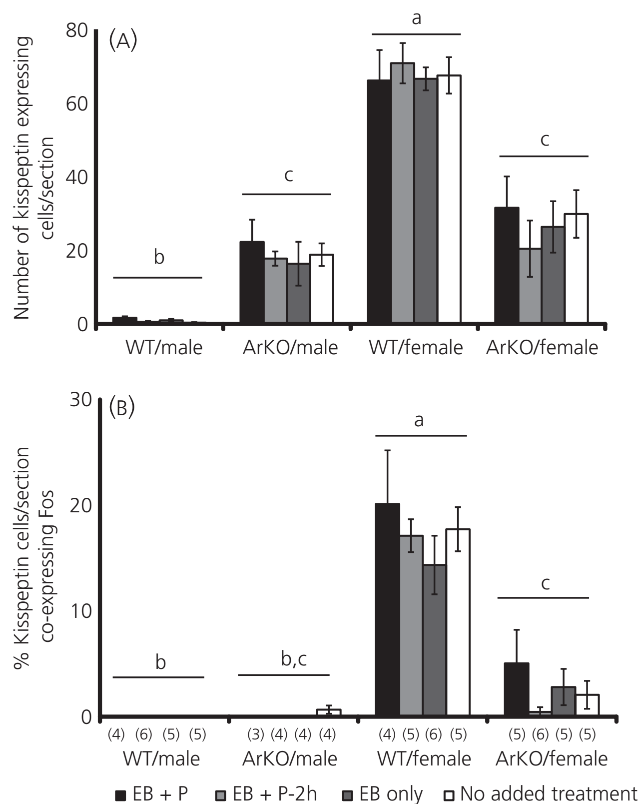

Fig. 4. (A) Mean ± SEM number of kisspeptin-expressing neurones/section and (B) mean ± SEM percentage of kisspeptin-immunoreactive neurones co-expressing Fos-IR/section in the rostral periventricular area of the third ventricle of gonadectomised male and female wild-type (WT) and aromatase knockout (ArKO) mice given a silastic implant containing 1 μg of 17β-oestradiol in 7.35 μl of sesame oil for 1 week, then treated with injections of oestradiol benzoate (EB) and progesterone (P) and sacrificed 2 h before the expected luteinising hormone (LH) surge (EB + P – 2 h), or sacrificed at the expected time of the LH surge after injections of oestradiol benzoate and progesterone (EB + P), oestradiol only (EB only) or no further injections (no added treatment). Groups with different letter notations are significantly different from one another within the same genotype/sex (P < 0.05). The number of animals per experimental group is given in parentheses.

in the RP3V. The two-way ANOVA of the number of kisspeptin-IR neurones in the RP3V revealed significant main effects of genotype/sex ($F_{3,60}$ = 118.29, P < 0.001) but no significant effect of treatment or genotype/sex × treatment interaction. Post-hoc analysis of this effect revealed that WT female mice had a higher number of kisspeptin-IR neurones/section than WT male mice and ArKO mice of both sexes (P < 0.001). WT male mice had a lower number of kisspeptin-IR neurones/section than WT females and ArKO mice of both sexes (P < 0.001).

WT female mice had a higher percentage of double labelled Fos/kisspeptin neurones/section in the RP3V compared to WT male mice and ArKO mice of both sexes (Fig. 4B). Two-way ANOVA of the number of double-labelled Fos/kisspeptin-neurones in the RP3V revealed significant main effects of genotype/sex ($F_{3,60}$ = 95.40, P < 0.001) but no significant effect of treatment or genotype/sex × treatment interaction. Post-hoc analysis of this effect revealed that WT female mice had a higher percentage of double-labelled Fos/kisspeptin neurones than WT males and ArKO mice of both sexes, regardless of treatment (P < 0.001). WT males had a lower percentage of double-labelled Fos/kisspeptin neurones than WT and KO females, regardless of treatment (P < 0.05). Images of Fos/Kisspeptin staining are presented in Fig. 5.

Plasma LH levels

As observed in Experiment 1, treatment with EB + P induced a LH surge in WT females and ArKO mice of both sexes but not in WT males (Fig. 6). As a result of technical problems, two samples could not be analysed (the adjusted group numbers are noted). Two-way ANOVA indicated a significant effect of genotype/sex ($F_{3,58}$ = 10.23, P < 0.001), treatment ($F_{3,58}$ = 10.99, P < 0.001) and a significant genotype/sex × treatment interaction ($F_{9,58}$ = 6.72, P < 0.001). Post-hoc analysis showed that, in WT female mice, treatment with EB + P resulted in higher plasma concentrations of LH compared to implant only (P < 0.05). ArKO female mice treated with EB + P also had significantly higher plasma concentrations of LH than all other groups (P < 0.05), indicating that female ArKO mice are capable of showing steroid-induced LH surges. In addition, the fact that ArKO females treated with EB + P had significantly higher plasma concentrations than ArKO females treated with EB + P and sacrificed 2 h before the expected surge (EB + P – 2 h, P < 0.001) indicates that the LH surge is occurring at the time of sacrifice (4 h after progesterone injection, within 1 h of the onset of the dark period) in these animals. Similarly, ArKO males treated with EB + P had significantly higher concentrations of plasma LH than ArKO males given an implant only (P < 0.001) and ArKO males treated with EB + P and sacrificed 2 h before the expected time of the LH surge (P < 0.0001). As observed in Experiment 1, WT male mice treated with EB + P had significantly lower plasma concentrations of LH

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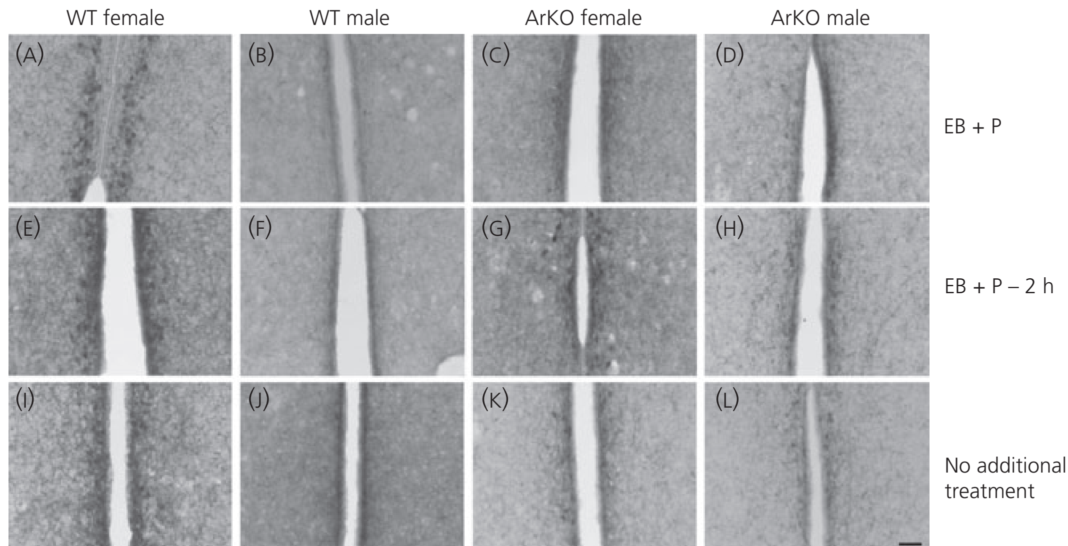

Fig. 5. Representative photomicrographs (× 20) of kisspeptin and Fos/kisspeptin neurones in the rostral periventricular area of the third ventricle of gonadectomised male and female wild-type (WT) and aromatase knockout (ArKO) mice given an silastic implant containing 1 μg of oestradiol in 7.35 μl of sesame oil then treated with injections of oestradiol benzoate and progesterone (EB + P), oestradiol and progesterone then sacrificed 2 h prior to the expected time of the LH surge (EB + P – 2h), or no further injections (no additional treatment). (A) Female WT mouse treated with EB + P. (B) Male WT mouse treated with EB + P. (C) Female ArKO mouse treated with EB + P. (D) Male ArKO mouse treated with EB + P. (E) Female WT mouse treated with EB + P – 2h. (F) Male WT mouse treated with EB + P – 2h. (G) Female ArKO mouse treated with EB + P – 2h. (H) Male ArKO mouse treated with EB + P – 2h. (I) Female WT mouse given silastic implant only. (J) Male WT mouse given silastic implant only. (K) Female ArKO mouse given silastic implant only. (L) Male ArKO mouse given silastic implant only. Scale bar = 50 μm.

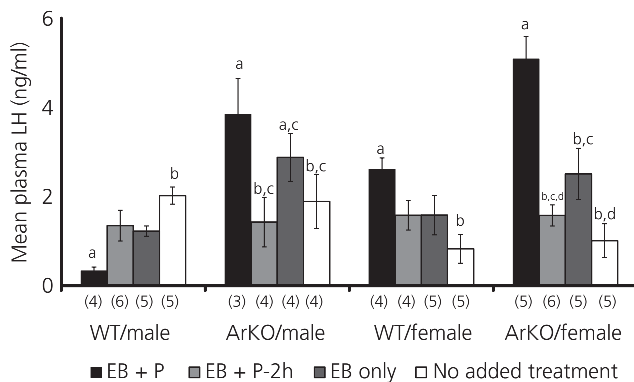

Fig. 6. Capacity of aromatase knockout (ArKO) mice to show a steroid-induced luteinising hormone (LH) surge. Mean ± SEM plasma concentrations of LH (ng/ml) in gonadectomised male and female WT and ArKO mice given a silastic implant containing 1 μg of 17β-oestradiol in 7.35 μl of sesame oil for 1 week, then treated with injections of oestradiol benzoate (EB) and progesterone (P) and sacrificed 2 h before the expected LH surge (EB + P – 2 h) or sacrificed at the expected time of the LH surge after injections of oestradiol and progesterone (EB + P), oestradiol only (EB only) or no further injections (implant only). Groups with different letter notations are significantly different from one another within the same genotype/sex ($P < 0.05$). The number of animals per group is given in parentheses.

than WT male mice given an implant only ($P < 0.05$). It is important to note that there were no significant differences between sex/genotype in the EB + P – 2 h groups, indicating that, 2 h before the expected time of the LH surge, there is no difference in plasma concentrations of LH between sex or genotype. Thus, the timing of the expected LH surge is likely to be accurate in this experiment.

Discussion

The present study shows that both male and female ArKO mice were capable of generating an LH surge in response to adult treatment with EB + P, lending further support to the idea that oestradiol defeminises the capacity to show steroid-induced activation of GnRH neurones and, by consequence, an LH surge in male mice. The finding that female ArKO mice showed LH surges when treated with EB + P in adulthood indicates that this particular female characteristic develops in the absence of any previous developmental action of oestradiol. Development of this female-typical neuroendocrine circuit differs from the organisation of the neural circuits underlying courtship behaviours, which require feminisation by oestradiol during prepubertal development (42, 52). Oestradiol presumably acts prenatally because female mice that lack α-foetoprotein, a foetal circulating oestradiol-binding protein that protects against maternal oestradiol (19), do not show an LH surge after treatment with EB + P (49).

Interestingly, ArKO mice did not have female-typical numbers of kisspeptin-IR or Kiss-1 mRNA expressing neurones in the RP3V, indicating that the steroid-induced LH surge occurred with a reduced population of kisspeptin neurones. Furthermore, kisspeptin neurones in ArKO mice showed reduced Fos co-expression after EB + P treatment compared to WT female mice, raising the

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question of whether this reduced population of kisspeptin neurones in the RP3V of ArKO females, as well as ArKO males, is essential for the generation of the steroid-induced GnRH/LH surge. Kisspeptin has been proposed to be an important player in the cyclic generation of preovulatory LH surges because *Kiss1r* and *Kiss1* null mice do not show an LH surge or Fos/GnRH double-labelling in response to EB + P. However, a different strain of *Kiss1r* null mice retains the capacity to generate a steroid-induced LH surge (39). Similarly, recent work by Mayer and Boehm (41) showed that female mice, in which over 95% of kisspeptin- or Kiss1r-expressing neurones were ablated during embryonic development, show all stages of oestrous cyclicity and were fertile in adulthood. However, when kisspeptin- or Kiss1r-expressing neurones are ablated in adulthood, only females in which Kiss1r-expressing neurones are ablated remained fertile (41). Nevertheless, there is evidence questioning the absolute requirement of the full female-typical complement of kisspeptin neurones in generating GnRH/LH surges.

The ArKO mouse model permits the study of the differentiation of the GnRH surge system and the kisspeptin system in the absence of oestradiol exposure across development. Although female ArKO mice have altered ovarian phenotype and are infertile (53), our results showed that the GnRH/LH surge system was still capable of generating a steroid-induced LH surge in these mice. ArKO mice typically show increased plasma concentrations of gonadotrophins (presumably as a result of the lack of negative-feedback by oestradiol), although treatment with a low dose of oestradiol decreases plasma LH concentrations to normal levels (53, 54). Our observation of low levels of plasma LH in implant-only treated ArKO mice of both sexes is consistent with this finding. ArKO mice are also characterised by higher levels of androgens in adulthood as a result of the absence of negative-feedback actions of oestradiol on the hypothalamic-pituitary-gonadal axis (47). It has been shown that, in male mice, treatment with androgens in adulthood can affect kisspeptin expression in the arcuate nucleus and median eminence, although the Kiss-1 population in the RP3V appears to be less affected (55). Thus, it is more likely that the reduced population of kisspeptin neurones in the RP3V of ArKO mice is a result of the absence of oestradiol during a critical prepubertal period (45) than that it has been defeminised by androgens. If the latter was true, then ArKO males should have had a male-typical population of kisspeptin neurones in the RP3V. Typically, ArKO female mice do not show ovulation in response to oestradiol treatment in adulthood; however, recently, it has been discovered that combined treatment with oestradiol, pregnant mare serum gonadotrophin and human chorionic gondatrophin can indeed induce ovulation in ArKO female mice (56), suggesting that the reproductive system has the capacity to function despite high levels of circulating gonadotrophins.

There is strong evidence that oestradiol plays an important role in the sexual differentiation of the kisspeptin system during both pre- and postnatal development (31). Neonatal treatment with oestradiol defeminises the population of kisspeptin neurones in the RP3V of females (9, 57, 58), whereas neonatal castration prevents the defeminisation of the number of Kiss-1 mRNA-expressing neurones in the RP3V in male rats (9). Although the precise mechanism of action of oestradiol remains to be elucidated, it is possible that the defeminising actions of oestradiol on kisspeptin result from cell death, similar to the oestradiol-induced cell death in tyrosine hydroxylase-IR neurones in the AVPV (59). Although recent evidence has suggested that the development of the sexually dimorphic number of kisspeptin neurones is not dependent on the proapoptotic protein BAX (B-cell lymphoma 2-associated protein X) (60), it is possible that oestradiol could regulate cell death in RP3V kisspeptin neurones via a BAX-independent apoptotic pathway. It should be noted, however, that the number of Kiss-1 mRNA expressing neurones is lower in neonatally castrated male rats compared to female rats (9), suggesting a postnatal contribution of oestradiol to the development of the kisspeptin system in females. Indeed, our results, as well as those obtained previously (30, 45), suggest that oestradiol exerts feminising effects on kisspeptin neuronal development during the prepubertal period. If oestradiol only defeminised the numbers of kisspeptin-expressing neurones in the RP3V, both male and female ArKO mice, which are characterised by the lack of circulating oestradiol throughout development (47), should have had a female-typical population of kisspeptin neurones in the rostral hypothalamus, which is clearly not the case (43). Furthermore, the development of kisspeptin neurones in the RP3V appears to occur between P15 and P30, and the sex differences in this neuronal population do not emerge until after P10 (30, 45), which is well past the perinatal surge of testosterone and oestradiol that is considered to be responsible for the defeminisation of the GnRH/LH surge system in males (61, 62). Taken together, these results show that oestradiol plays a dual role in the sexual differentiation of the kisspeptin population in the RP3V. It has clear defeminising effects when present prenatally (49) and shortly after birth (9, 58, 63) that are normally prevented in the female hypothalamus as a result of the presence of oestradiol-binding α-foetoprotein. In addition, oestradiol has clear feminising effects during the prepubertal period by inducing a female-typical population of kisspeptin neurones in the RP3V (45). Presumably, kisspeptin neurones in the RP3V in males are either not exposed to adequate feminising levels of oestradiol during this particular prepubertal period or become insensitive to such actions during postnatal development and adulthood. Because ArKO mice are deprived of oestradiol during both the prenatal and prepubertal period, their kisspeptin population is neither defeminised, nor feminised, and thus not completely sexually differentiated. Even so, treatment with EB + P induced GnRH neurone activation leading to an LH surge in ArKO mice of both sexes.

In the present study, we observed numbers of kisspeptin-IR neurones and those expressing Kiss-1 mRNA. Consistent with other rodent studies, a clear sex difference was observed, with WT females having higher numbers of Kiss-1 mRNA neurones than WT males (32, 57, 63, 64). By contrast, the numbers of Kiss-1 mRNA expressing cells were much higher than the numbers of kisspeptin-IR neurones for all experimental groups. A similar discrepancy between kisspeptin protein expression and Kiss-1 mRNA content has been reported by Clarkson and Herbison (65), who have shown that kisspeptin cell number or their apposition upon GnRH

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neurones remain constant throughout the oestrous cycle, in stark contrast to the results obtained in other laboratories, which have shown that Kiss-1 mRNA levels are strongly modulated by gonadal hormones and vary over the course of the oestrous cycle (35, 66). The reasons for the discrepancy between kisspeptin protein levels and Kiss1 mRNA content remain unclear. It could reflect differences in sensitivity between *in situ* hybridisation for mRNA and immunohistochemistry for the protein. Nevertheless, it is the kisspeptin protein that interacts with GnRH neurones and thus the essential marker of kisspeptin function (65). This is particularly relevant to the lower numbers of kisspeptin protein containing neurones observed in ArKO mice that were still capable of generating LH surges in response to EB + P treatment. A possible explanation might have been that kisspeptin stores were already depleted at the time of sacrifice, which presumably coincided with the timing of the LH surge in these animals. However, kisspeptin protein expression (reflected by the number of kisspeptin-IR neurones) in mice sacrificed 2 h before the expected time of the LH surge did not differ significantly from those sacrificed at the time of the LH surge. Thus, it is unlikely that the low number of kisspeptin-IR neurones seen in ArKO mice was the result of a depletion of kisspeptin protein in the RP3V before the LH surge. It is also possible that the kisspeptin system, similar to the GnRH system, only requires a minimum number of neurones to permit the induction of the LH surge.

In summary, both male and female ArKO mice showed an activation of hypothalamic GnRH neurones leading to a plasma LH surge in response to adult treatment with EB + P. This result has two implications. First, the GnRH/LH surge control circuit system is normally defeminised in male mice by the early actions of oestradiol. Second, in contrast to its role in feminising the circuits controlling courtship behaviours, oestradiol is not required to feminise the GnRH/LH surge control circuit in female mice. Our observation of reduced numbers of kisspeptin-IR neurones in ArKO female mice compared to WT females further suggests that postnatal oestradiol is normally required to feminise the RP3V kisspeptin system. Our unexpected result that hormone-induced activation of GnRH neurones led to a surge of plasma LH in ArKO mice lacking a female-typical population of RP3V kisspeptin neurones suggests that a reduced number of kisspeptin neurones may be sufficient for a functional GnRH/LH surge system in these mice. It is possible that compensatory mechanisms have allowed the surges to proceed with a partially reduced population of kisspeptin neurones in the RP3V in ArKO mice, or that the female-typical number of RP3V kisspeptin neurones is redundant, just as the number of preoptic GnRH neurones in female rodents far exceeds the number needed to generate preovulatory LH surges (41).

Acknowledgements

The authors thank Arlette Gerard for the execution of the LH assays, as well as Bertrand Thimister and Olivier Brock for technical assistance. We also thank Dr Alexander Kauffman for generously providing the plasmid for Kiss-1. This research was funded by FNRS Mandat d'Impulsion F.4502.07, FRSM grant 3.4571.10 and NIH grant HD 044897. Laura Szymanski is a postdoctoral fellow for the FNRS and Julie Bakker is a research associate of the FNRS.

Received 7 December 2011,
revised 18 April 2012,
accepted 29 April 2012

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