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ORIGINAL ARTICLE

Delayed pubertal onset and prepubertal *Kiss1* expression in female mice lacking central *oestrogen receptor beta*

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Abstract

Ovarian oestradiol is essential for pubertal maturation and adult physiology of the female reproductive axis. It acts at central and peripheral sites through two main oestrogen receptors (ER) α and β. Here we investigate the role of ERβ on central effects of oestradiol, by generating a mouse line specifically lacking the *ERβ* gene in neuronal and glial cells. Central *ERβ* deletion delays the age at vaginal opening and first oestrous and reduces uterine weight without affecting body growth. Analysis of factors necessary for pubertal progression shows reduced levels of *Kiss1* transcripts at postnatal (P) day 25 in the preoptic area, but not in the mediobasal hypothalamus (MBH) of mutant females. In agreement with these data, the number of kisspeptin-immunoreactive neurons was decreased by 57–72% in the three subdivisions of the rostral periventricular area of the third ventricle (RP3V), whereas the density of kisspeptin-immunoreactive fibres was unchanged in the arcuate nucleus of mutant mice. These alterations do not involve changes in *ERα* mRNAs in the preoptic area and protein levels in the RP3V. The number and distribution of GnRH-immunoreactive cells were unaffected, but gonadotropin-releasing hormone (GnRH) transcript levels were higher in the P25 preoptic area of mutants. At adulthood, mutant females have normal oestrous cyclicity, kisspeptin system and exhibit unaltered sexual behaviour. They display, however, reduced ovary weight and increased anxiety-related behaviour during the follicular phase. This argues for the specific involvement of central ERβ in the regulation of pubertal onset in female reproduction, possibly through prepubertal induction of kisspeptin expression in the RP3V.

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Introduction

Pubertal maturation is triggered by the central activation of pulsatile GnRH secretion, which stimulates pituitary gonadotropin secretion necessary for sexual maturation. This process is timely regulated by genetic, nutritional and environmental factors, with 50–80% of the variance in the age of menarche attributed to genetic effects (1). Major concerns are currently raised about the progressive decrease in age of pubertal onset in girls around the world (2). The factors triggering precocious puberty are not known, but a growing body of evidence suspects a potential involvement of environmental chemicals, which can interfere with sex steroid functions (3–5). Indeed, ovarian oestradiol plays a key role in controlling female pubertal maturation. However, its molecular mechanisms are not completely defined. Human analyses of polymorphic variants encoding the oestrogen receptor α (ERα) suggest either positive or negative association with the age of menarche in girls (6–10). Studies conducted on the ERβ suggest that receptor genotypes may contribute to genetic variability of the age at menarche (9). Moreover, combined ERα and ERβ polymorphisms seem to further influence this event, suggesting a potential interaction between the two receptors in the regulation of menarcheal age (9,11).

Rodent studies highlighted the role of ERα in reproductive functions including pubertal maturation. At the central level, conditional inactivation of *ERα* gene resulted in arrested pubertal onset and infertility, indicating the importance of this signalling pathway in oestradiol effects (12–14). In this context, the role of ERβ still needs to be clarified. Genetic models with ubiquitous deletion limit the understanding of the central contribution of ERβ (15–18). Only one recent study used conditional mutagenesis to target *ERβ* ablation within forebrain neurons, or specifically in GnRH neurons (12). The data showed that ERβ at these sites was neither necessary nor sufficient for oestrous cyclicity and oestradiol negative feedback in adult females. Whether or not ERβ located in other central sites participates in these processes or in pubertal maturation remains to be determined. First, ERβ was detected in both neuronal and glial cells and localized, although to a lesser extent level than ERα, in many regions of the brain known to play a crucial role in central control of fertility (19,20). Second, oestradiol controls both the timing of pubertal maturation and the increase of kisspeptin expression during puberty (13,21,22). Kisspeptin is a key neuropeptide encoded by the *Kiss1* gene, involved in the regulation of GnRH secretion (23–25). Compelling evidence supports its role in the regulation of the hypothalamus-pituitary-gonad axis both at puberty and during adulthood (26,27).

The current study was undertaken to determine the effects of early deletion of central *ERβ* on female pubertal onset and adult reproduction. For this purpose, we generated a mouse model lacking *ERβ* throughout the nervous system by using exon 3-floxed mice (15) and transgenic animals where Cre recombinase was expressed in both neuronal and glial cells (28). The age of pubertal maturation and expression levels of molecular factors known to participate in the central regulation of puberty were assessed in this mouse line. The adult reproductive phenotype was studied by analysing oestrous cyclicity, hormonal levels and fertility. Sexual behaviour was assessed together with general behaviours including locomotor activity and anxiety-related behaviour known to be modulated by oestradiol (29,30).

Results

Selective inactivation of central *ERβ* gene

The mouse line lacking the central *ERβ* gene was obtained by crossing females in which exon 3 of *ERβ* was flanked by loxP sites (15) with males expressing the Cre recombinase under the control of the nestin promotor (Nes) and a neural enhancer (28). To confirm that the *ERβ* deletion was restricted to the nervous system, polymerase chain reaction (PCR) of DNA extracted from the brain, ovary and tail was carried out using primers that flank exon 3 of *ERβ* (15). As shown in Figure 1A, a small amplicon of 250 bp indicating Cre-mediated excision of exon 3, was found in the brain, but not the ovary, of mutant females ($ERβ^{NesCre}$ carrying the NesCre transgene) in comparison to control mice ($ERβ^{fl/fl}$) where a signal of 850 bp was seen. The selective central deletion of *ERβ* was further confirmed by Reverse transcription-PCR (RT-PCR) where the presence of a 177 bp-amplified fragment revealed the presence of ERβ mRNAs (Fig. 1B). This fragment was seen in the ovary of $ERβ^{fl/fl}$ and $ERβ^{NesCre}$ females and in the brain of $ERβ^{fl/fl}$ females. The signal was reduced by 98% in the brain of $ERβ^{NesCre}$ mice.

Delayed puberty of $ERβ^{NesCre}$ females

Pubertal maturation was analysed by measuring the time of vaginal opening and first oestrus. Data illustrated in Figure 2A show that vaginal opening for $ERβ^{NesCre}$ females was approximately 1.7 days later than for their control littermates (26.5 ± 0.7 versus 28.2 ± 0.9) and first oestrus was 6 days later (43.6 ± 0.9 versus

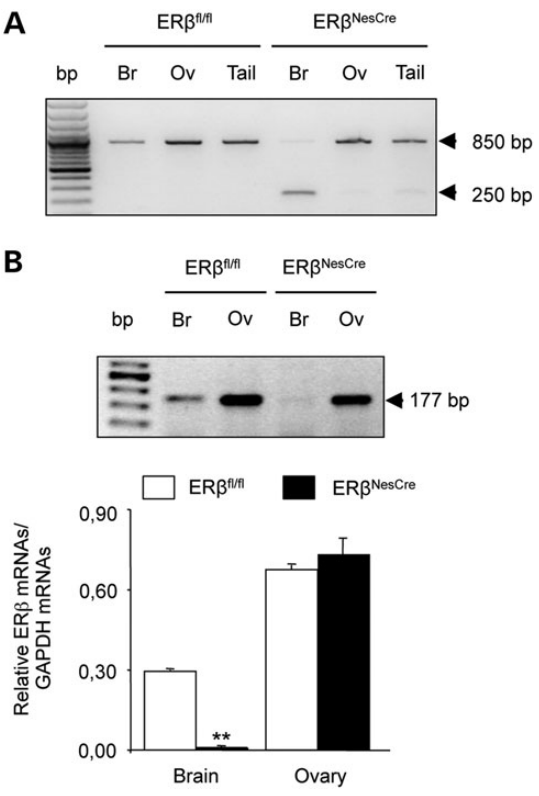

Figure 1. (**A**) PCR analysis showing the presence of the floxed *ERβ* allele (850 bp) in the brain (Br), ovary (Ov) and tail of control mice ($ERβ^{fl/fl}$), and ovary and tail of mutant females ($ERβ^{NesCre}$). The small amplicon of 250 base pair (bp) indicating Cre-mediated excision of exon 3 was present in the brain of mutant females. DNA size markers at 100 bp increments are shown in the left column. (**B**) RT-PCR of total RNAs obtained from the brain and ovary. Up: Representative gel showing the presence of the 177 bp-amplified fragment. DNA size markers at 50 bp increments are shown in the left column. Down: Quantitative data normalized to GAPDH from 3 to 4 females per genotype show a decrease of –98% in ERβ expression in the brain of mutant females. $^{**}P < 0.001$ versus control mice.

37.3 ± 1.2). The time from vaginal opening to first oestrus is also significantly longer in mutants than in controls (15.5 ± 1.5 versus 10.8 ± 1.3, $P < 0.05$). A detailed analysis of the cumulative percentage of females exhibiting first oestrous shows a rightward shift in the age of first oestrous in mutant females (Fig. 2B). The first oestrus occurred in 50% of control females at postnatal day (P) 37 versus P43 in mutant females. This pubertal phenotype was specifically linked to *ERβ* gene disruption in the nervous system since the NesCre transgene alone, in the presence of wild-type *ERβ* alleles, had no effects on pubertal onset (Supplementary Material, Fig. S1A and B).

We then analysed the effects of ERβ^{NesCre} invalidation on the weight of uterus, an oestrogen-responsive tissue usually used to indirectly monitor circulating oestradiol levels. Two-way ANOVA showed an effect of age ($F_{(4,59)} = 33.9$, $P < 0.0001$) and genotype ($F_{(1,59)} = 20.1$, $P < 0.0001$). Post-hoc analyses revealed a significant reduction in uterine weight at P30 and P35 in mutant females (Fig. 2C). Analysis of ovary weight indicated an effect of age ($F_{(4,58)} = 22.53$, $P < 0.0001$) but not of genotype ($F_{(1,58)} = 0.54$, $P > 0.05$), with post-hoc analyses showing a significant difference between controls and mutants at adulthood but not at postnatal stages (Fig. 2D).

The delayed pubertal maturation and uterine growth were not due to an effect of ERβ^{NesCre} on body weight (bw) since comparison between control and mutant females showed an effect of age ($F_{(3.63)} = 464.8$; $P < 0.0001$) but not of genotype ($F_{(1.63)} = 2.6$; $P > 0.05$) (Fig. 2E). Analysis of body composition in live animals showed comparable percentage of fat mass and bone mineral density in control and mutant females (Supplementary Material, Fig. S2A and B).

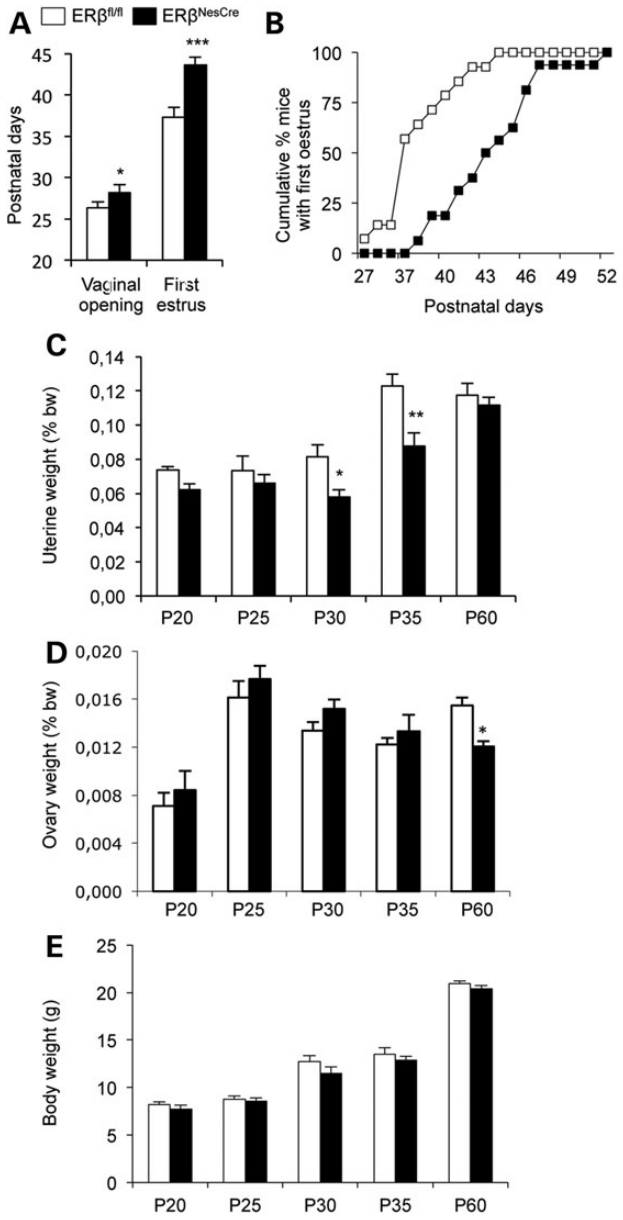

Figure 2. Delayed pubertal onset in ERβ^{NesCre} female mice. (**A**) Age at vaginal opening and first oestrus in ERβ$^{fl/fl}$ and ERβ^{NesCre} females ($n = 14$–16 females per genotype). *$P < 0.05$ and ***$P < 0.001$ versus ERβ$^{fl/fl}$ mice. (**B**) Cumulative percentage of mice over time (days) with first oestrus is given for each genotype: ERβ$^{fl/fl}$—open squares; ERβ^{NesCre}—closed squares. (**C**) Uterine weight expressed as percentage of bw in female mice ($n = 6$–14 females per age and per genotype). *$P < 0.05$ and **$P < 0.01$ versus corresponding ERβ$^{fl/fl}$ mice. (**D**) Ovary weight expressed as percentage of bw in female mice ($n = 6$–14 females per age and per genotype). *$P < 0.05$ versus corresponding ERβ$^{fl/fl}$ mice. (**E**) Body weight of female mice ($n = 6$–14 females per age and per genotype) at postnatal days (P) 20, 25, 30, 35 and 60.

Kiss1 expression is selectively delayed in the postnatal preoptic area of *ERβ*^{NesCre} females

We investigated by quantitative RT-PCR whether the ERβ^{NesCre} mutation affected *Kiss1* expression. Kisspeptin neurons are located in two hypothalamic regions, namely the preoptic area and the MBH. Transcript levels normalized to glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) show that *Kiss1* expression increases progressively in the preoptic area of control females (5-fold at P25 and 8-fold at P30 versus P20; Fig. 3A).

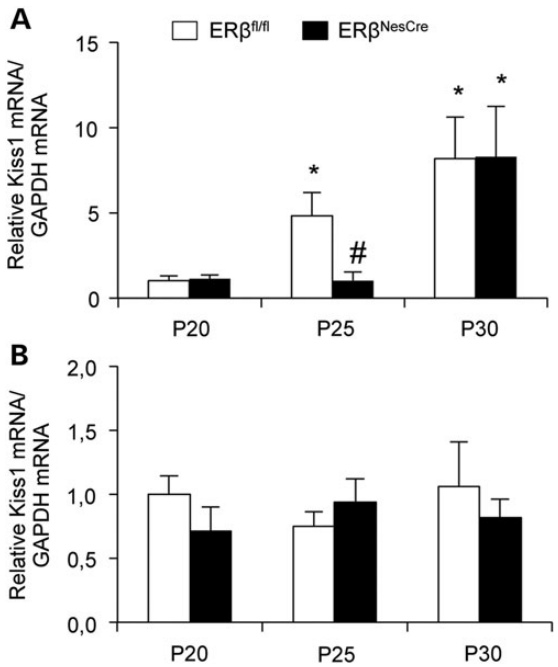

Figure 3. qRT-PCR analysis of *Kiss1* expression. (**A** and **B**) *Kiss1* gene expression normalized to *GAPDH* at postnatal (P) days 20, 25 and 30 in the preoptic area (A) and the MBH (B) for ERβ$^{fl/fl}$ and ERβ^{NesCre} mice ($n = 5$–11 females per age and per genotype). *$P < 0.05$ compared to ERβ$^{fl/fl}$ females at P20. #$P < 0.05$ compared to ERβ$^{fl/fl}$ females at P25.

In contrast, in mutant females, *Kiss1* expression is unchanged at P25 compared with P20 and then shows a sharp increase by P30 (8-fold increase compared with P20) to reach a similar level to that of controls (Fig. 3A). A significant reduction is, indeed, seen at P25 between the two genotypes ($P < 0.05$). In the MBH, there was no effect of age or genotype on *Kiss1* expression (Fig. 3B). In this brain area, we also analysed the expression of *tachykinin precursor 2* (*TAC2*), which encodes neurokinin B, another major regulator of GnRH neurons involved in pubertal maturation and co-expressed with *Kiss1* in cells of the arcuate (ARC) nucleus (31–33). Data did not show any effect of the ERβ^{NesCre} mutation on *Tac2* mRNA levels at P25 (Supplementary Material, Fig. S3A). All these results were confirmed by using a second endogenous reference gene, *polymerase (RNA) II (DNA directed) polypeptide A* (*Polr2a*), as is shown in Supplementary Material, Figure S3B–D.

The levels of *GnRH* transcripts were also analysed in the P25 preoptic area. Data show significantly ($P < 0.01$) increased *GnRH* mRNAs levels normalized to GAPDH in mutant females (2.75 ± 0.32 versus 1.00 ± 0.19 in controls).

Juvenile reduction of kisspeptin-immunoreactivity in the rostral periventricular area of the third ventricle (RP3V) of ERβ^{NesCre} females

Within the murine preoptic area, kisspeptin neurons are located along a periventricular area that crosses the anteroventral periventricular (AVPV), rostral (rPeN) and caudal (cPeN) preoptic periventricular nuclei; a region commonly referred to as the RP3V (34). Immuno-histochemical analyses detected kisspeptin-immunoreactive cells in these three subdivisions of the RP3V at P25 in both control and mutant females (Fig. 4A). However, the number of kisspeptin-immunoreactive cells is significantly reduced in mutant females in comparison to controls (−72%, −64% and −57% in the AVPV, rPeN and cPeN, respectively; Fig. 4C). Within the murine MBH, kisspeptin neurons are concentrated in the ARC nucleus (Fig. 4B). Here, the mean density of kisspeptin-immunoreactivity does not differ significantly between controls and mutants in either the anterior or the posterior areas (Fig. 4D).

Dual labelling of GnRH shows unchanged numbers of GnRH-immunoreactive soma within the medial septum (MS), diagonal band of broca (DBB) and rostral preoptic area (rPOA) of mutant females (Fig. 5A and C). The mean density of fibre immunoreactivity in three levels of the median eminence (medial, caudal, rostral) is also comparable between the two genotypes (Fig. 5B and D).

Analysis of *ERα* expression at P25

Previous studies reported that ERβ modulates *ERα* gene expression (35). Since ERα in kisspeptin cells is involved in pubertal onset (13,36), we therefore investigated whether central *ERβ* deletion indirectly delayed pubertal maturation and kisspeptin expression by affecting *ERα* expression.

In the preoptic area, the analysis of *ERα* expression at P25 showed unchanged mRNA levels in mutant females compared

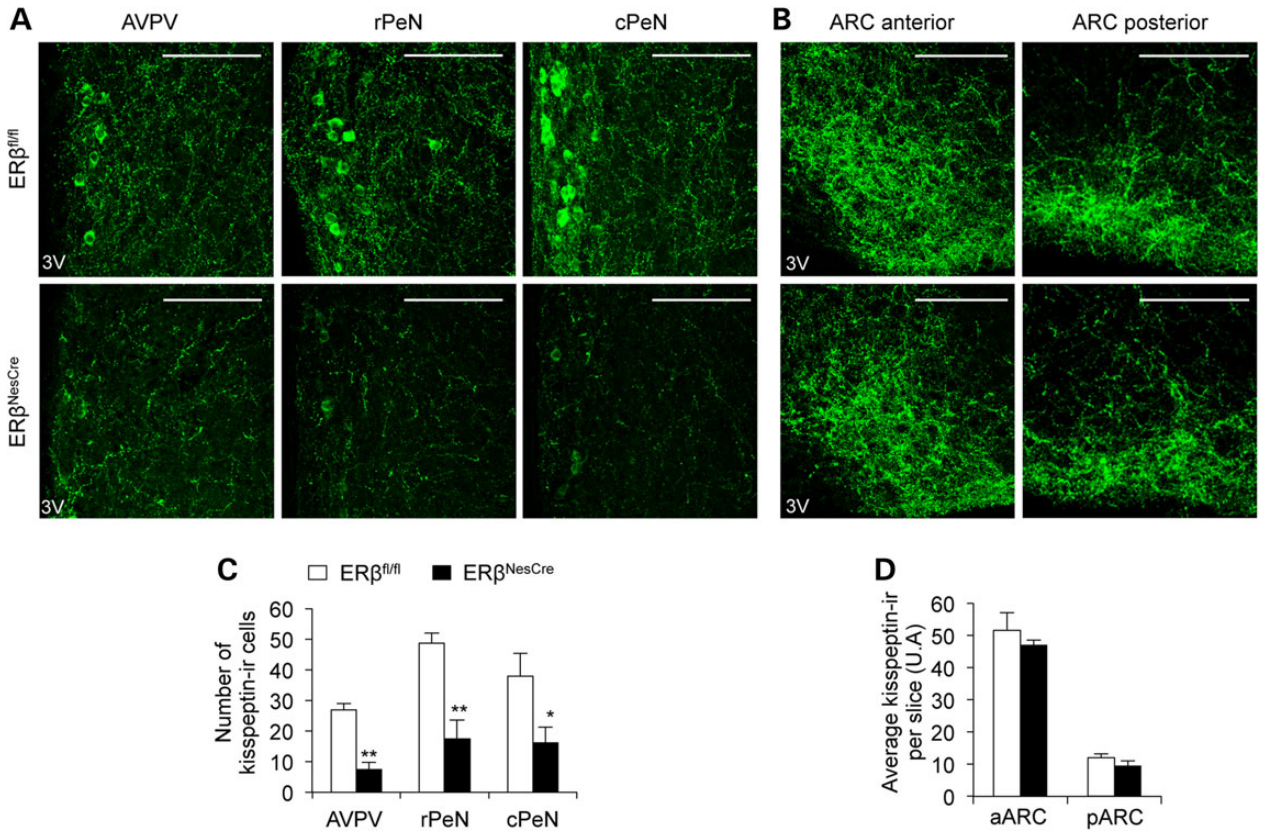

Figure 4. Kisspeptin-immunoreactivity at P25. (**A** and **B**) Representative kisspeptin-immunoreactivity in the AVPV area, the rostral (rPeN) and caudal (cPen) periventricular nuclei of the RP3V (A) and in the anterior and posterior ARC nucleus (B) of ERβ$^{fl/fl}$ and ERβ^{NesCre} mice. Scale bar = 100 μm. (**C** and **D**) Quantification of the number of kisspeptin-immunoreactive (-ir) cells in the three RP3V subdivisions (C) and average kisspeptin-immunoreactivity in the anterior (aARC) and posterior (pARC) parts of the ARC nucleus (D) of ERβ$^{fl/fl}$ and ERβ^{NesCre} mice (n = 6–7 females per genotype), $^{*}P < 0.05$ and $^{**}P < 0.01$ versus controls for the corresponding area.

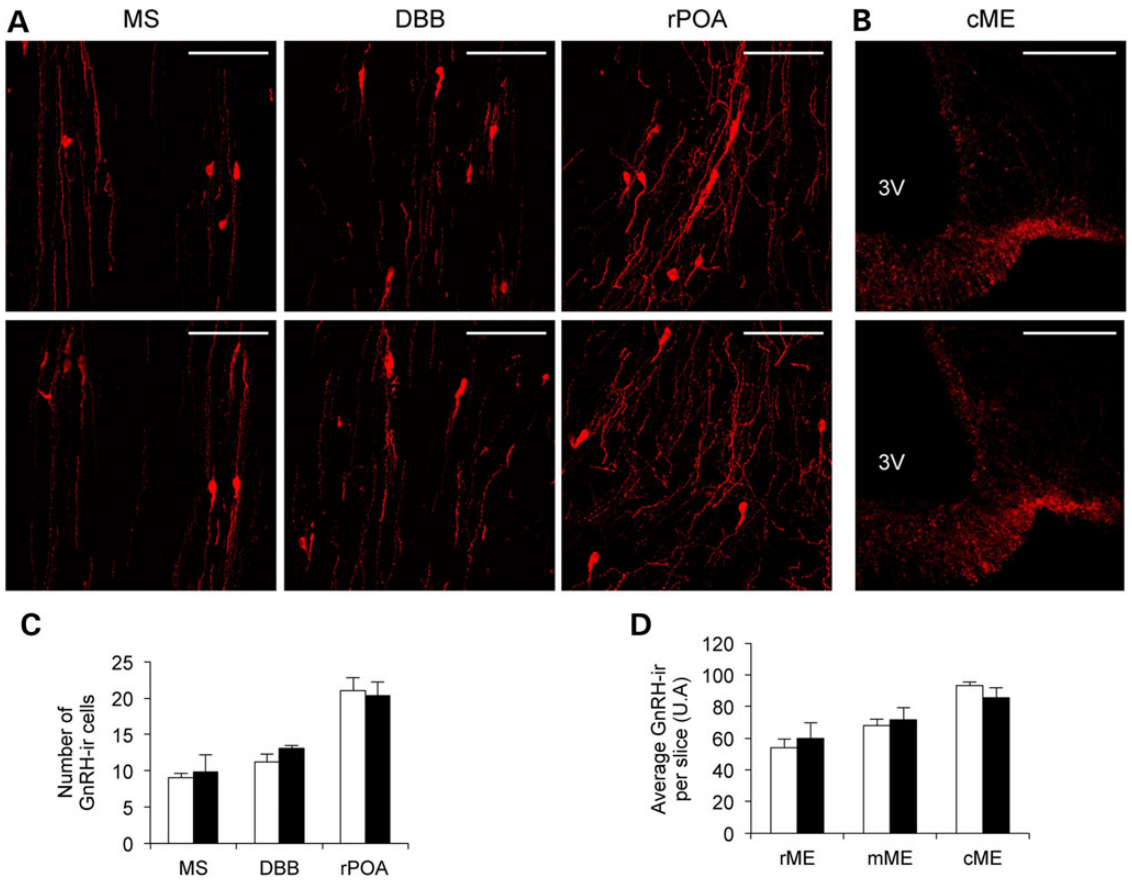

Figure 5. GnRH cell number and distribution, and fibre immunoreactivity at P25. (**A** and **B**) Representative GnRH-immunoreactive cells in the MS, DBB and rPOA (A) and fibre immunoreactivity in the caudal median eminence (B) of $ERβ^{fl/fl}$ and $ERβ^{NesCre}$ mice. Scale bar = 100 μm. (**C** and **D**) Quantification of the number of GnRH-immunoreactive (-ir) cells in the MS, DBB and rPOA (C) and mean density of GnRH fibre immunoreactivity in the rostral (rME), medial (mME) and caudal (cME) areas of the median eminence (D). Values are means ± S.E.M. of 4–6 females per genotype.

with their control littermates (Fig. 6A). In the MBH, a significant elevation (1.5-fold) is observed for *ERα* mRNA levels in mutant females (Fig. 6B). These results were confirmed using the *Polr2a* endogenous reference gene (Supplementary Material, Fig. S3E and F).

To determine whether a similar increase occurred at the ERα protein level, we performed an immuno-histochemical analysis of kisspeptin and ERα in different sub-regions of the RP3V and ARC nucleus. ERα is abundant in the RP3V at P25 (Fig. 6C). The total number of ERα-immunoreactive cells is comparable between the two genotypes in the three RP3V subdivisions (Fig. 6D), whereas the number of neurons co-expressing kisspeptin and ERα proteins is significantly reduced in mutant females compared with their control littermates (7.4 ± 2.4 versus 25.3 ± 2.0 in the RP3V, 17.3 ± 6.0 versus 48.4 ± 3.1 in the rPeN and 16.1 ± 5.1 versus 37.6 ± 7.5 in the cPeN). Since 94–100% of kisspeptin neurons co-express ERα regardless of genotype (Fig. 6E), the reduced number of cells co-expressing kisspeptin and ERα may be attributed to decreased kisspeptin-immunoreactivity rather than to changes in the amounts of ERα protein.

In the ARC nucleus, quantification of the total number of ERα-immunoreactive cells and mean density of ERα-immunoreactivity showed no differences between the two genotypes in either the anterior or the posterior regions at P25 (Supplemental Material, Fig. S4A–F). Similar results were found when other sub-regions of the MBH, such as the ventromedial hypothalamus, were analysed (data not shown).

Adult $ERβ^{NesCre}$ females exhibit normal reproduction and sexual behaviour but display altered anxiety-like behaviour

Examination of 2–3 months old females showed that the average length of the oestrous cycle (4.77 ± 0.12 and 4.91 ± 0.14 days in control and mutant mice, respectively) and the mean length of each stage are comparable between $ERβ^{fl/fl}$ and $ERβ^{NesCre}$ mice (Fig. 7A). Levels of oestradiol were 23.4 ± 2.1 pg/ml in controls and 24.7 ± 2.3 pg/ml in mutants at the dioestrus stage. Analysis of circulating levels of LH in intact and ovariectomized (OVX) mice (Fig. 7B) showed an effect of ovariectomy ($F_{(1.14)} = 17.90$, $P < 0.001$) but not of genotype ($F_{(1.14)} = 0.17$, $P > 0.05$). In correlation with these data, no differences were found between controls and mutants concerning the number of kisspeptin-immunoreactive neurons in the RP3V, the density of kisspeptin-immunoreactivity in the ARC nucleus or the number of GnRH-positive cells within the rPeN preoptic area (Supplementary Material, Fig. S5A–E).

In behavioural tests, females were primarily screened for locomotor activity. No differences were shown between the two genotypes (data not shown). In the O-maze, there was an effect of both oestrous stage ($F_{(1.62)} = 12.19$, $P < 0.001$) and genotype ($F_{(1.62)} = 8.96$, $P < 0.01$) on the time spent in the open arms (Fig. 7C), with a significant interaction between both parameters ($P < 0.001$). Similar effects of oestrous stage ($F_{(1.61)} = 10.88$, $P < 0.01$) and genotype ($F_{(1.61)} = 9.46$, $P < 0.01$) were observed for the number of entries in the open arms (Fig. 7D). During the follicular phase

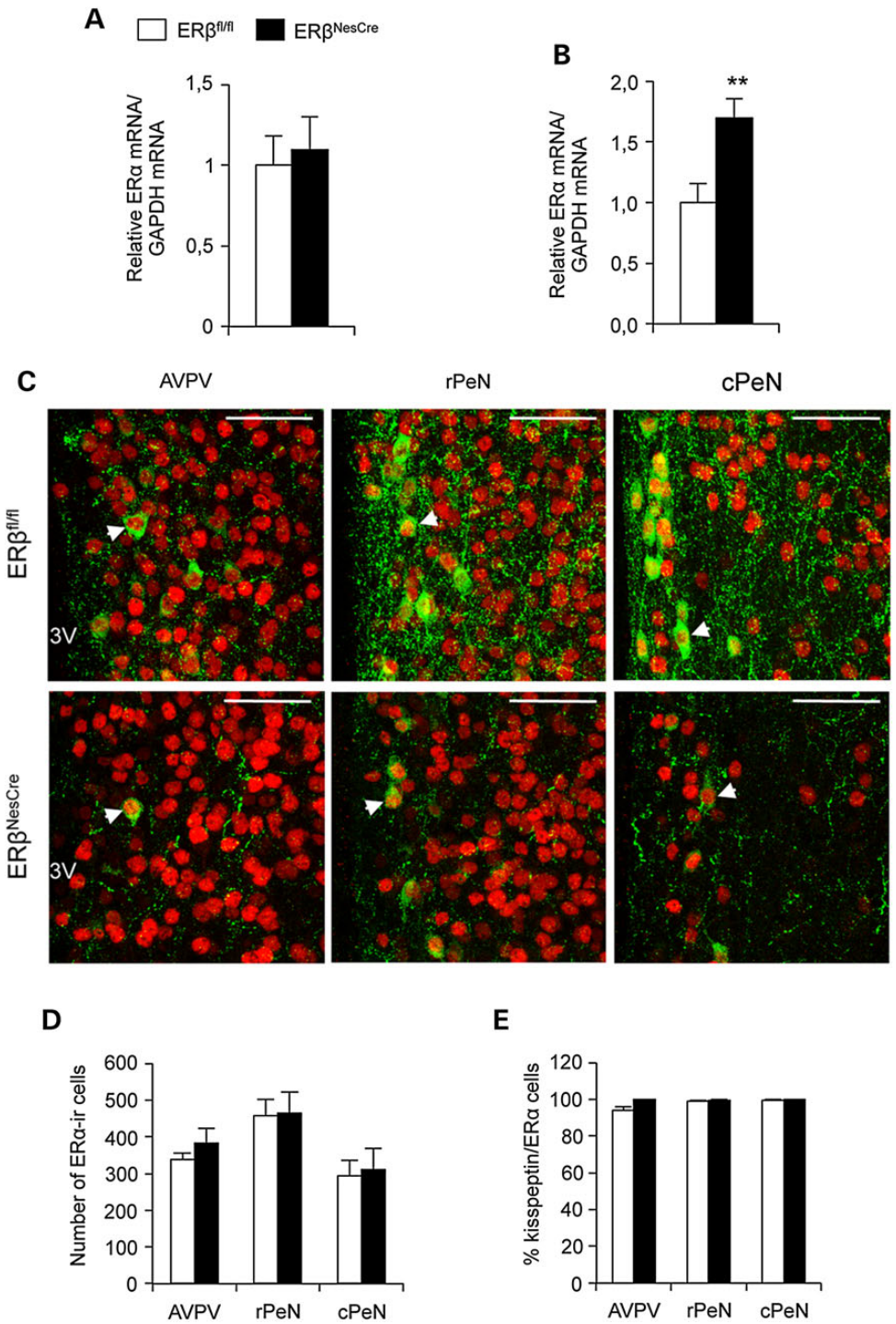

Figure 6. Analysis of *ERα* mRNAs expression and immunoreactivity at P25. (**A** and **B**) *ERα* expression normalized to *GAPDH* in the preoptic area (A) and the MBH (B) of $ERβ^{fl/fl}$ and $ERβ^{NesCre}$ females ($n = 6–13$ females per genotype). $^{**}P < 0.01$ versus $ERβ^{fl/fl}$ females. (**C**) Representative immuno-labelling of kisspeptin (green) and ERα cells (red) within the AVPV, rPeN and cPeN. White arrows show typical kisspeptin and ERα co-labelled cells. Scale bar = 50 μm. (**D** and **E**) Number of ERα-immunoreactive (-ir) cells (D) and percentage of cells co-labelled for kisspeptin and ERα (E) in the AVPV, rPeN and cPeN areas ($n = 6–7$ females per genotype for each region).

(pro-oestrous, oestrous), mutant females spent less time in the open arms compared with their control littermates, thereby revealing an increased anxiety level at this period.

Analysis of the lordosis posture in OVX females primed with oestradiol/progesterone showed a significant effect of experience ($F_{(1.15)} = 20.057$, $P < 0.001$) but no effect of genotype ($F_{(1.15)} = 0.36$, $P > 0.05$) on the lordosis quotient (Fig. 7E). In olfactory preference tests, there was a significant effect of stimulus ($P < 0.05$) but not of genotype, with all females displaying a preference for an intact male over a receptive female (Fig. 7F). In 4-months continuous mating, control and mutant females produced offspring with comparable litter size (3.7 ± 0.3 and 4.0 ± 0.6,

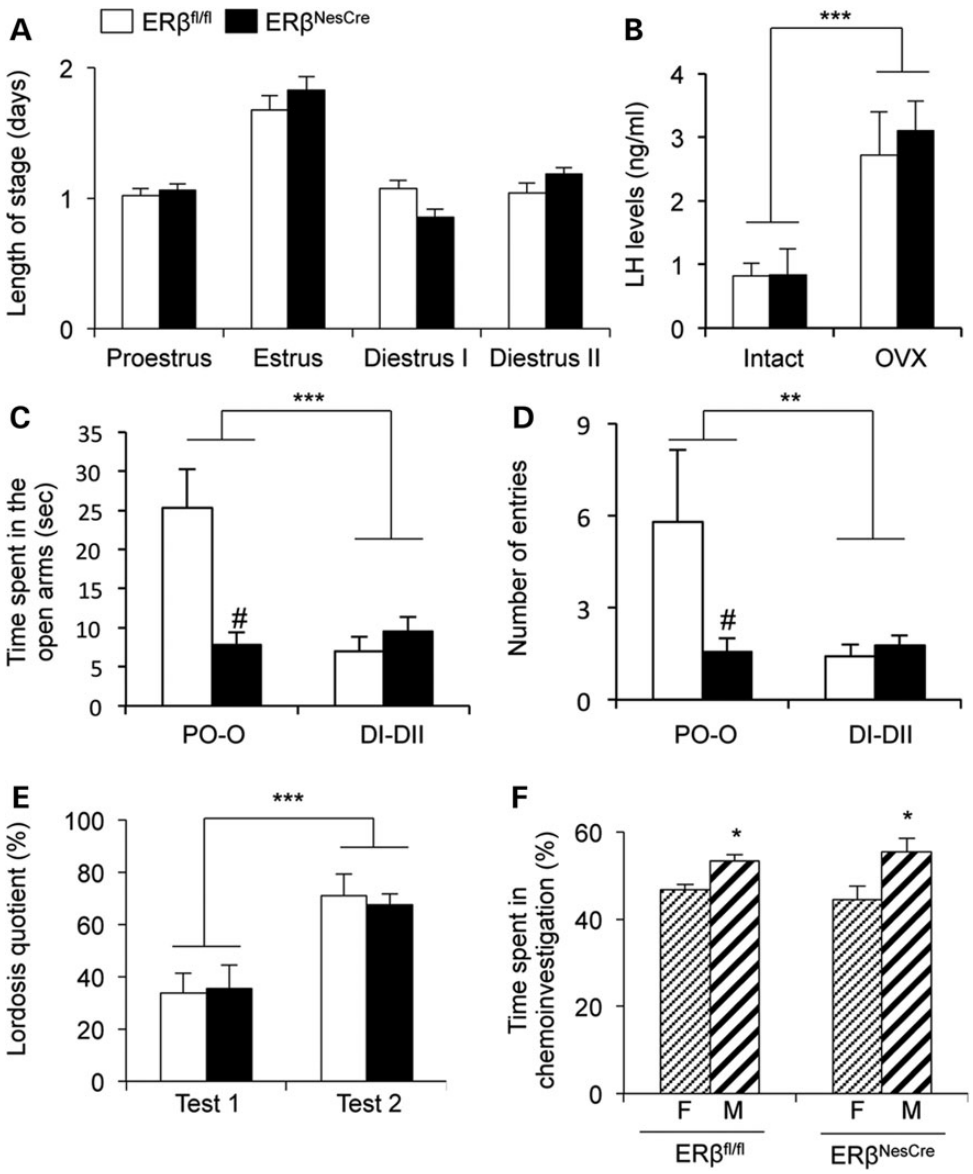

Figure 7. Oestrous cyclicity, hormone levels and behaviours in adult females. (**A**) The average number of days for each oestrus cycle stage ($n = 13–14$ females per genotype). (**B**) Levels of circulating oestradiol in intact and OVX mice ($n = 5$ females per genotype and treatment). ***$P < 0.001$ versus intact females. (**C** and **D**) Time spent in the open arms (C) and number of entries (D) of the O-maze in females at the prooestrous-oestrous (PO-O) or the dioestrous (DI-DII) stage ($n = 10–26$ females per genotype and stage). ***$P < 0.001$, **$P < 0.001$, versus the PO-O stage. $^{\#}P < 0.01$ versus $ER\beta^{fl/fl}$ females at the same oestrous stage. (**E**) Lordosis quotient of females ($n = 9–11$ per genotype) in response to mounts of stud males in Tests 1 and 2. ***$P < 0.001$ versus Test 1. (**F**) Percentage of time spent chemoinvestigating male (M) or female (F) stimuli ($n = 8–10$ females per genotype). *$P < 0.05$ versus female stimulus.

respectively) and total number of pups (29.7 ± 4.3 and 28.7 ± 2.9, respectively).

Discussion

Central *ERβ* deletion in female mice delayed pubertal onset assessed by vaginal opening, first oestrous and uterine growth. This phenotype was associated with normal body growth of mutant females and body fat composition, indicating that the metabolic processes widely known to trigger pubertal onset were not implicated here. As a first approach to investigate the downstream molecular changes that occurred in the brain following central *ERβ* deletion, we focussed on the analysis of *Kiss1*, a gene necessary for pubertal progression (26,27).

In the preoptic area of control female mice, the expression of *Kiss1* mRNA started to increase before vaginal opening or the first oestrous. This finding extends previous observations made in rats (37,38). In mutant mice, *Kiss1* expression also increased between P20 and P30 in the preoptic area, but with a significant delay compared with controls. At P25, *Kiss1* mRNAs levels were significantly reduced compared with control mice. This transient reduction in *Kiss1* transcripts also translated as changes at the protein level since the number of kisspeptin-immunoreactive neurons was greatly reduced in the three RP3V subdivisions of mutant females at P25. The delayed vaginal opening, first oestrous and uterine growth observed in mutant females were preceded by a delayed increase in *Kiss1* expression in the RP3V. It is therefore possible that central *ERβ* deletion delayed female

puberty by altering *Kiss1* expression in the RP3V. The importance of *Kiss1* expression in this specific brain area for pubertal onset is supported by a recent study showing that siRNA-mediated knockdown of *Kiss1* in the AVPV, but not in the ARC nucleus, delays vaginal opening and first oestrous (39).

In the MBH of control females, another site of *Kiss1* up-regulation during the peri-pubertal period in several species (37,40,41), we were unable to detect any changes in *Kiss1* transcript levels between P25 and P30. Whether this is the reflection of a major species difference in the anatomical site of *Kiss1* regulation at the onset of puberty, or of an insufficient methodological sensitivity specific to the mouse model, requires further clarification (42,43). In mutant females, *Kiss1* mRNAs levels in the MBH and fibre immunoreactivity in both the anterior and posterior regions of the ARC nucleus were unaltered. *Tac2* expression was also unchanged in this nucleus. Altogether, these data suggest that central ERβ mediates the timely up-regulation of *Kiss1* expression by oestradiol in the prepubertal RP3V specifically, and that this increase is necessary for puberty onset.

ERβ has been shown to modulate *ERα* expression in both an enhancing and a suppressing fashion (35). Data showed no changes in ERα mRNA levels in the preoptic area or immunoreactivity in the RP3V of mutant females, indicating that the observed phenotype was not due to changes in *ERα* expression in this brain area. In the MBH of P25 mutant mice, where *Kiss1* and *Tac2* expression were unaffected compared with control mice, a significant increase in *ERα* transcripts was detected. The number of ERα-immunoreactive cells and mean grey value of ERα immunoreactivity were, however, unchanged between control and mutant females in the major sites of ERα-expression within the MBH (ARC, VMH). Alterations in mRNA levels generally precede those of proteins; it is therefore possible that enhancement of ERα protein amount occurs later in the prepubertal MBH of mutant females although the physiological meaning of such changes remain to be clarified.

The two nuclear ERα and ERβ exhibit different ligand-binding specificity and transcriptional activities and appear to act in an opposite way in the regulation of target genes in several *in vitro* and *in vivo* models (44–48). Whether ERα and ERβ, which are co-expressed in a significant proportion of *Kiss1*-expressing cells (49), act in a similar fashion in the RP3V needs further investigation. Nevertheless, it is interesting to note that *ERα* deletion in kisspeptin cells advanced (13,36), whereas the present central *ERβ* deletion delayed pubertal maturation. A possible explanation could be that central *ERβ* deletion, by altering the ratio of ER expression levels, promoted ERα signalling and consequently resulted in delayed puberty onset. Therefore, the pubertal phenotype induced by the central deletion of *ERβ* could be rather related to changes in the 'Ying Yang' relationship between ERα and ERβ, which in turn may affect the timing of kisspeptin increase in this brain area.

We analysed the effects of central *ERβ* invalidation on GnRH cell number, distribution and expression. Quantification of the number of GnRH cell bodies in the MS, DBB and preoptic area showed comparable results between control and mutant mice. This indicates that the early deletion of central *ERβ* does not interfere with GnRH cell number or distribution. Quantification of GnRH mRNA showed significantly higher levels in mutant females at P25. This may result from a direct effect of *ERβ* deletion on GnRH neurons. Previous studies reported the presence of *ERβ* transcripts and protein in rat GnRH neurons (50,51). In mice, immuno-histochemical data should be cautiously interpreted due to the lack of antibodies specific enough against this receptor (52), but *in vitro* studies reported negative regulation of GnRH expression by an ERβ agonist in mouse hypothalamic GT1-7 cells (53). Changes in GnRH expression may also be due to reduced kisspeptin expression, which by decreasing GnRH liberation, could result in the accumulation of GnRH mRNAs in cell bodies. Analysis of GnRH fibre immunoreactivity by confocal microscopy in the median eminence did not depict any difference between the two genotypes, suggesting either a delayed increase in GnRH peptide amount or rather a post-transcriptional adjustment of GnRH peptide levels. Female mice lacking *ERβ* in GnRH cells, recently generated by Herbison's laboratory (12), have been characterized at adulthood but not yet during the postnatal/prepubertal period. Further characterization of these mice during development will allow an interesting comparison with our model and may help clarifying the cellular pathways used by ERβ in the prepubertal regulation of GnRH expression. Whatever the mechanism underlying increased GnRH mRNA levels, it did not rescue the pubertal delay induced by $ERβ^{NesCre}$ mutation. This suggests that the alteration of kisspeptin system might be the primary factor responsible for this phenotype in $ERβ^{NesCre}$ mice.

Central *ERβ* deletion did not alter reproductive functions of adult females as shown by normal oestrous cyclicity, oestradiol and LH levels. This finding extends a recent study, which shows that neuronal ablation of *ERβ* in the forebrain results in normal oestrous cyclicity of adult females (12). However, while this work reported a minor failure in the negative feedback exerted by oestradiol on LH secretion, we found a comparable enhancement in LH levels after ovariectomy in mutant and control females. We also found that sexual behaviour, assessed in OVX and hormonally-primed females, was unaffected in naïve and sexually experienced mutants. The discrepancy with the global *ERβ* deletion, which results in both impaired sexual behaviour (54) and infertility (15), suggests that the primary sites of ERβ action in these processes at adulthood might be the ovary. Indeed, a very recent study using ovary transplantation from wild-type to *ERβ*-null mice and *vice versa* showed that ERβ within the ovary is required in normal LH surge and fertility (55). Disruption of ovarian *ERβ*, by altering postnatal synthesis and liberation of oestradiol, can also indirectly interfere with the expression of sexual behaviour since the feminization of the neural circuitry underlying this behaviour is under the control of postnatal/prepubertal oestradiol (56). Nevertheless, as our analyses were performed on young adult female mice, one cannot exclude later effects of the early deletion of central *ERβ* on the reproductive capacity at advanced age. For instance, ovary weight, while unaffected at postnatal stages, was significantly decreased by 22% in adult mutant females.

The altered anxiety state during the follicular phase of the oestrous cycle is reminiscent to that previously reported in global *ERβ* knockout mice (30). The present study further emphasizes the importance of central ERβ in the anxiolytic effect induced by oestradiol during this period. The downstream molecular pathways may involve the serotoninergic system and tryptophan hydroxylase expression in the dorsal raphe nucleus (57). Whether these anxiety disorders take their origin during the prepubertal/pubertal period needs further investigation.

In summary, we provide here the first genetic evidence that central ERβ is involved in the regulation of postnatal *Kiss1* expression in the RP3V and in the timing of pubertal maturation in female mice. The suppression of this signalling pathway delays, but does not inhibit, *Kiss1* expression. We suggest that a tight control of pubertal timing by oestradiol requires both ERβ and ERα, which may function in opposite ways. Postnatal oestradiol might first exert, through ERα, an inhibitory tone on kisspeptin

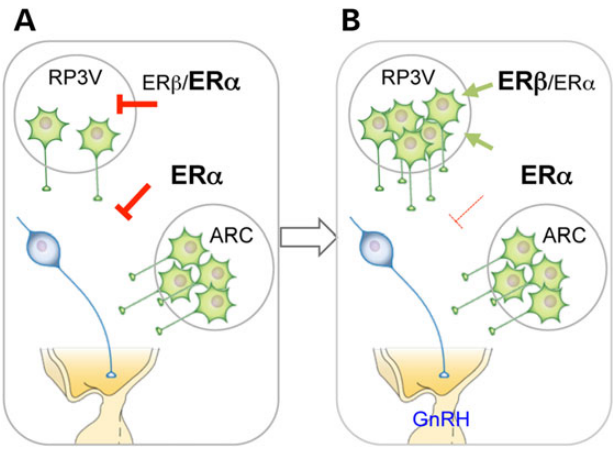

Figure 8. Schematic representation of the potential role of the ERβ signalling pathway in oestradiol-induced pubertal maturation. (**A**) During the postnatal period, oestradiol may maintain through ERα an inhibitory effect on kisspeptin expression in the RP3V and kisspeptin neuron activity in the ARC nucleus (full red lines). (**B**) The emergence of ERβ signalling pathway during the prepubertal period may counteract ERα effects by promoting kisspeptin expression in the RP3V (green arrow). A stimulatory phase would follow, involving reinforcement or potentiation of kisspeptin expression in the RP3V and decreased sensitivity to the ERα-mediated inhibition (dashed red lines), which then participate to trigger GnRH secretion. Kisspeptin neurons are illustrated in green and GnRH cells in blue.

expression and/or neuronal activity. The emergence of prepubertal ERβ signalling due to increased production of ovarian oestradiol and/or increased expression of ERβ (58) or downstream components, would promote *Kiss1* expression in the RP3V (Fig. 8). This positive input might thereafter be followed by decreased inhibition exerted by ERα on kisspeptin cell activity (13,36) and by other processes including activation of neuro-glial networks underpinning pubertal maturation (59). The possible cooperation between ERα and ERβ signalling pathways in oestradiol-induced pubertal maturation is in good agreement with a human study showing that combined polymorphism of both ERα and ERβ might influence the age of menarche in girls (9). Analyses of these interactions in transgenic models may help to better understand the physiological mechanisms underlying the regulation of puberty onset and predict the potential effects of exposure to molecules with oestrogenic or anti-oestrogenic activities.

Materials and Methods

Animals

All experiments were performed in agreement with the European guidelines for the use of experimental animals (Decree 87-848, 86/609/ECC). Littermates were group-housed under a controlled photoperiod (12:12-h light–dark cycle—lights on at 7 am), maintained at 22°C, with free access to food and water.

Generation and genotyping of ERβ^{NesCre} mouse line

Control and mutant females were obtained in the same litters in a C57BL/6J genetic background by crossing females in which exon 3 of *ERβ* was flanked by loxP sites (15), with floxed *ERβ* males expressing Cre recombinase under the control of the nestin promotor (NesCre) and a neural enhancer (28,60). As previously reported, Cre-mediated excision of floxed exon 3 of the *ERβ* gene allows the deletion of all *ERβ* transcripts (15). The used NesCre transgene permits such deletion in neural precursor cells by embryonic day 10.5, before gonadal differentiation (60). The selective *ERβ* deletion was determined by PCR as previously described (15,28). RT-PCR was carried on total RNAs (2 μg) extracted from the ovary and brain using the Superscript III first strand Synthesis System (Invitrogen). PCR reactions were performed using the resulting cDNA, Taq DNA pol (In vitrogen), dNTPs (10 nM each) and primers for ERβ (15) or GAPDH in a MyCycler Thermal Cycler (Bio Rad, Marne la Coquette, France). The amplified cDNA fragments were separated by electrophoresis through a 1.5% agarose gel and stained by ethidium bromide.

Reproductive physiology

Female mice were examined daily for evidence of vaginal opening and first oestrus from postnatal day 15. Vaginal smears flushed with physiological saline were taken from juvenile mice to determine the day of first oestrus and from adult females (2–3 months of age) for 3–4 weeks to assess oestrous cyclicity. The oestrus cycle phase was identified by microscopy after hematoxylin-eosin coloration of the vaginal smears. Body and uterine weights of juvenile and adult mice were measured. Levels of circulating oestradiol and LH were analysed using RIA (ultra-sensitive oestradiol kit; Beckman Coulter, Villepinte, France) and immunoassay (ultra-sensitive Elisa kit; Endocrine Technologies, Newark, CA, USA), respectively, as previously described (28,61). Bone mineral density and percentage of fat mass were determined by using a Piximus densitometer (Lunar Corporation).

Quantitative RT-PCR

Fresh mice brains were rapidly embedded in Tissue-Tek, frozen in a −30°C isopentane solution and stored at −80°C until use. Frozen tissue punches were recovered through the preoptic area and MBH with a 1 mm diameter canula. These tissue punches encompassed respectively the RP3V and the ARC nucleus where kisspeptin neurons are located. Total RNA was extracted using the PicoPure RNA isolation kit from Arcturus (Exilone SARL, Vicq, France). RNA was reverse transcribed using the Promega kit (Charbonière les Bains, France). Real-time PCR analysis was performed using iQ SYBR Green Supermix (Biorad, Hercules, CA, USA). The PCR reaction was performed using primers previously described for *Kiss1* (62), *Pol2r* (63), *GnRH* and *GAPDH* (64), *ERα* (65) and *TAC2* (66). Two different endogenous reference genes were chosen for their insignificant variation across experimental groups: *GADPH* and Polr2a. PCR specificity was verified by melting curve analysis and agarose gel electrophoresis. For each assay, a standard curve was created using 2-fold serial dilutions of cDNA, from which PCR efficiencies for each gene were calculated. All experiments had efficiencies between 90 and 106%. Each sample was run in triplicate to obtain an average cycle threshold (Ct) value and relative expression of each target gene was determined using the comparative Ct method (67). The data were normalized to either *GADPH* or *Polr2a* expression levels. Results were expressed as fold differences in relative gene expression with respect to ERβ$^{fl/fl}$ mice at P20 or P25.

Immunohistochemistry

Coronal sections (30 μm) from female brains at P25 or adulthood (2–3 months of age) were prepared for immunohistochemistry as previously described (61). For dual-label immunofluorescence kisspeptin/ERα or kisspeptin/GnRH, sections were blocked for 30 min with 2% normal donkey serum (Aurion, Wageningen, Netherlands)

in phosphate buffered saline (PBS) containing 0.3% Triton X-100 and 0.1% human albumin, then incubated with sheep anti-kisspeptin antibody AC053 [1:10 000; (68)] and either rabbit anti-ERα antibody (1:250; Santa Cruz Biotechnology, Dallas, USA) or rabbit anti-GnRH #19900 [1:10 000; (69)] for 72 h at 4°C. Dual-label immunofluorescence was carried out for 2 h at room temperature using an Alexa Fluor 488-conjugated donkey anti-sheep secondary antibody (1:500; Life Technologies) and an Alexa Fluor 555-conjugated donkey anti-rabbit secondary antibody (1:500; Life Technologies) for kisspeptin and GnRH or ERα immunostaining, respectively. After rinses in PBS, sections were immersed in a Hoechst stain bath (2 μg/ml in water; Invitrogen) for 5 min, rinsed again in PBS, dried and mounted with fluoromount G (Southern Biotech, Birmingham, AL, USA) under a coverslip.

The number of kisspeptin-immunoreactive cells was counted in anatomically matched section of the AVPV nucleus (plates 28–29 of the Mouse Brain Atlas of Paxinos et Franklin (70); two sections per animal) and the rostral (plate 30; two sections per animal) and caudal (plates 31–32; two sections per animal) regions of the RP3V as previously described (61). Total kisspeptin-immunoreactivity (voxel counts) was carried out on anatomically matched sections of the anterior (plate 44; one section per animal) and posterior (plate 51; one section per animal) areas of the ARC nucleus. Quantification of GnRH-immunoreactive cell bodies was carried out on two anatomically matched sections selected at the level of the MS (plate 22), the DBB (plate 24) and the rPOA (plate 26). Total GnRH immunoreactivity was carried out on three anatomically matched sections of the rostral, medial and caudal levels of the median eminence (plates 45, 47 and 49, one section per animal) within an area of 100 × 30 μm. ERα immunoreactive neurons were counted at the three subdivisions of the RP3V. All cells within 100 μm of the ventricle were counted. Two brain sections at each level of the RP3V were analysed in each mouse. The number of ERα immunoreactive neurons was also counted at the anterior and posterior levels of the ARC nucleus within an area of 200 × 150 μm, and at the level of the ventromedial nucleus within an area of 400 × 300 μm (plate 44; one section per animal). Dual-label immuno-cyto-chemical analysis of ERα and kisspeptin co-expression was carried out at the three levels of the RP3V. Double labelled neurons were identified as clearly exhibiting a green cytoplasm and a red nucleus.

Behaviours

Locomotor activity and O-maze tests

Females were primarily screened for locomotor activity in a computed circular corridor as previously described (28). Briefly, mice were introduced in a circular corridor made of two concentric cylinders crossed by four diametrically opposite infrared beams (Imetronic, Pessac, France). The locomotor activity was counted when animals interrupted two successive beams and thus had travelled a quarter of the circular corridor.

The anxiety state was measured in the O-maze paradigm (56 cm diameter, 5.5 cm width, 65 cm height). Two parts of the device, each representing a quarter of the ring, were enclosed by walls (17 cm height). At the beginning of the test, females (2–3 months of age) were place in the closed arm. The number of entries and time spent in the open arms were recorded for 9 min. Vaginal smears were taken for 2 weeks before the test in order to determine the stage of the oestrus cycle on the day of experiment. According to the result, females were separated into two groups: females in proestrus/oestrous of the follicular phase (high oestradiol levels) and females in dioestrous of the luteal phase (low oestradiol levels).

Sexual behaviour

Females (2–3 months of age) were OVX under general anaesthesia and supplemented with subcutaneous Silastic implants containing 50 μg of oestradiol benzoate (Sigma-Aldrich) in 30 μl sesame oil as previously reported (28). Two weeks later, females were given a subcutaneous injection of 1 mg progesterone (Sigma-Aldrich) in 100 μl sesame oil 4–5 h before the mating test. Animals were tested twice, with an interval of 1 week between tests. Sexually experienced males were used as partners. Tests ended when females had received 20 mounts from males. The lordosis quotient was calculated for each subject in response to male mounting (61).

Olfactory preference was tested in an enclosed Plexiglas Y-maze. Females were allowed to adapt to the apparatus without any stimuli for 5 min on two consecutive days. On the third day, they were tested by placing an anaesthetized receptive female and gonadally intact male in the boxes, one at the distal end of each arm. The time spent sniffing each goal box was scored over the 5-minute test. Results were expressed as a percentage of the total time spent sniffing male and female cues. The maze was cleaned with 10% ethanol between trials.

Statistics

Data were expressed as mean ± S.E.M. Student's *t*-tests were used to determine the effect of genotype on *ERβ* expression, the time of vaginal opening and first oestrous, oestradiol levels and on body composition. For gene expression analysis, univariate t-tests were used, taking the value of 1 for controls as the theoretical average. As variances were not homogeneous between groups, kisspeptin, GnRH and ERα immunoreactivity as well as fertility were analysed with the Mann–Whitney nonparametric test. Two-way ANOVA was used to analyse the main effects of genotype and age on body and uterine weights, genotype and ovariectomy on LH levels, genotype and oestrous stage on oestrous cyclicity and anxiety-like behaviour, genotype and experience on lordosis behaviour or genotype and stimulus on olfactory preference. Tukey post-hoc tests were used to determine group differences. P-values of less than 0.05 were considered to be significant.

Supplementary Material

Supplementary Material is available at *HMG* online.

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