Journal of Neuroendocrinology
From Molecular to Translational Neurobiology

Journal of Neuroendocrinology **22**, 728–735

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REVIEW ARTICLE

Early Oestrogens in Shaping Reproductive Networks: Evidence for a Potential Organisational Role of Oestradiol in Female Brain Development

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Journal of Neuroendocrinology

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A central tenet of contemporary theories on mammalian brain and behavioural sexual differentiation is that an organisational action of testosterone, secreted by the male's testes, controls male-typical aspects of brain and behavioural development, whereas no active perinatal sex hormone signalling is required for female-typical sexual differentiation. Furthermore, the available evidence suggests that many, although not all, of the perinatal organisational actions of testosterone on the development of the male brain result from the cellular effects of oestradiol formed via neural aromatisation of testosterone. However, a default developmental programme for the female brain has been criticised. Indeed, we review new results obtained in aromatase knockout mice indicating that oestradiol actively contributes to the differentiation of female-typical aspects of brain and behavioural sexual differentiation. Furthermore, we propose that male-typical neural and behavioural differentiation occurs prenatally in genetic males under the influence of oestradiol, which is avoided in foetal genetic females by the neuroprotective actions of α-fetoprotein, whereas female-typical neural and behavioural differentiation normally occurs postnatally in genetic females under the influence of oestradiol that is presumably produced by the ovaries.

Key words: oestrogens, sex differences, progesterone receptor, kisspeptin, sexual behaviour.

doi: 10.1111/j.1365-2826.2010.02016.x

The traditional view of sexual differentiation of the mammalian brain holds that sex differences in the brain and behaviour develop under the influence of gonadal hormones, with the male brain developing under the influence of testosterone secreted by the male's testes, and the female brain in the absence of these hormones. Accordingly, McEwen *et al.* (1) proposed that the female rodent brain needs to be protected from any oestrogens produced by the placenta or by male siblings lying in close approximation, and that α-fetoprotein (AFP), comprising an important plasma protein present during foetal development in high concentrations, is the most likely candidate to achieve this protection because of its high oestrogen-binding capacities. This idea of a default organisational programme for the female brain as well as a protective role for AFP has been challenged. First, there is some behavioural evidence indicating that the normal sexual differentiation of the female brain requires oestradiol (2, 3) and, second, the presence of AFP within neurones in the absence of any local AFP synthesis suggests that AFP can enter the brain. It was therefore proposed by Toran-Allerand (4) that AFP can actually act as a carrier transporting oestradiol into the brain and, by doing so, participates in the sexual differentiation of the female brain. Thus, there have been two opposing hypotheses on the role of AFP and oestradiol in the development of the female brain.

However, we have recently been able to resolve this longstanding dilemma about the role of AFP in brain sexual differentiation using a knockout mouse model for AFP (AFP-KO) (5). Female AFP-KO mice were clearly defeminised with regard to their lordosis behaviour as well as their population of tyrosine hydroxylase neurones in the sexually differentiated anteroventral periventricular area (AVPV) of the hypothalamus, although these behavioural and morphological consequences of the AFP mutation could be reversed by transplacental treatment with an aromatase-inhibiting drug, 1,4,6-androstatriene-3,17-dione (ATD) (6). These results thus confirmed that circulating AFP binds oestradiol in female foetuses so as to protect their brains from the potential defeminising effects of this hormone that would normally occur in the male brain in response to oestradiol locally produced from testosterone after it has been taken up from the circulation.

Although the results obtained in AFP-KO female mice suggest that the principal action of prenatal oestrogen exposure, regardless of whether it occurs in female or male mice, is to defeminise, and to some extent, masculinise the brain and behaviour, there is increasing evidence for a feminising role of oestradiol in the development of the female brain. Indeed, in this review, we present results obtained in aromatase knockout (ArKO) mice showing that oestradiol can have feminising actions on the brain and behaviour. On the basis of the results obtained in both knockout mouse models (i.e. the ArKO and AFP-KO mouse), we propose that the defeminising actions of oestradiol normally occur prenatally in males and are avoided in foetal females because of the protective actions of AFP, whereas the feminising actions of oestradiol normally occur postnatally in genetic females, perhaps when the ovaries start to produce oestrogens (i.e. in the second week after birth) and when circulating concentrations of AFP have diminished so that AFP no longer plays a protective role.

Classical theory of brain and behavioural sexual differentiation

In male mammals, the presence of the *Sry* gene on the Y-chromosome causes the undifferentiated gonads to develop into testes instead of ovaries (7). Testosterone secreted by testicular Leydig cells promotes the development of the Wolffian ducts into the internal male genital structures, whereas anti-Müllerian hormone secreted by testicular Sertoli cells causes regression of the female-typical Müllerian ducts. The penis and scrotum develop under the influence of dihydrotestosterone, which is formed from testosterone by the enzyme, 5α-reductase. In normal female differentiation, the Müllerian ducts develop without any apparent hormonal input into the uterus, fallopian tubes and the distal portion of the vagina. The Wolffian ducts regress and disappear in the absence of androgenic stimulation. A seminal study by Phoenix *et al.* (8) provided the first evidence that the capacity to display sex-specific behaviours in adulthood (and, by inference, the sexual differentiation of the brain) follows the same pattern as that of the genitals. Thus, female guinea pigs treated with testosterone propionate prenatally showed increased levels of male-typical mounting behaviour together with reduced levels of female-typical lordosis behaviour in adulthood after hormonal priming (8). Supportive evidence for a role of perinatal testosterone in the development of the male brain came from subsequent studies by Baum (9) and many others (10, 11) demonstrating that the removal of testosterone by neonatal castration reduced males' later capacity to show male sexual behaviours at the same time as enhancing their ability to show female sexual behaviours. Additional evidence suggested that testosterone secreted by the testes acts perinatally, either directly via androgen receptors or after being aromatised into oestradiol and stimulating oestradiol receptors (12, 13), to masculinise (enhance male-typical sexual responses) and/or defeminise (suppress female-typical sexual responses) the neural substrate that controls sexual behaviour in adulthood. The results of these early studies also implied that the neural mechanisms that control later female-typical sexual behaviour normally develop perinatally in females 'by default' (i.e. without the need for any sex steroid stimulation). Consistent with this view is the observation (14) that the rodent ovary does not secrete significant amounts of oestradiol before postnatal day 7, and that any oestrogens secreted by the mother during gestation will not be available to the foetal (male or female) brain because they are bound with high affinity and capacity to AFP, a plasma glycoprotein produced in high quantities by the foetal liver (15, 16). Thus, it was proposed by McEwen *et al.* (1) that AFP serves to protect the developing female brain from becoming masculinised and/or defeminised by any oestradiol originating from their mother or male siblings lying in close approximation. This hypothesis on a protective role for AFP was recently confirmed by our research group using AFP-KO mice (6).

Role of oestradiol in female-typical brain and behavioural sexual differentiation

The possible importance of ovarian hormones in female-typical brain sexual differentiation was first suggested by some early behavioural studies (3, 17) in which it was shown that female rats ovariectomised on the day of birth had lower lordosis quotients after adult treatment with oestradiol and progesterone than females that either kept their ovaries (17) or were ovariectomised at birth and at the same time implanted with ovaries until postnatal day 60 (3). In addition, Toran-Allerand (18) reported that oestradiol promoted neurite outgrowth from foetal hypothalamic explants of both sexes, suggesting a role for oestradiol in neural differentiation. More recently, Dohler *et al.* (19) showed that neonatal treatment of female rats with tamoxifen, an oestrogen receptor antagonist, decreased their later capacity to show lordosis behaviour, whereas concurrent neonatal administration of a low dose of oestradiol benzoate (EB) prevented this effect. Finally, Baum and Tobet (20) found that female ferrets treated prenatally with the aromatase inhibitor, ATD, and then treated in adulthood with a low or moderate dose of EB, displayed decreased acceptance quotients when paired with a stimulus male.

These various behavioural results suggested that exposure to a low level of oestradiol over a postnatal interval between birth and the age of puberty facilitated the later capacity to display female sexual behaviour; however, they did not provide incontrovertible evidence that oestradiol normally contributes to the development of female sexual behaviour in female mammals. First, the effects of neonatal ovariectomy on the potential to show lordosis behaviour later in life were only transient because any differences in lordosis behaviour disappeared after repeated testing (3). Perhaps more importantly, in addition to its anti-oestrogenic actions, tamoxifen can exert oestrogen-like agonist actions in the brain (21). Therefore, the observed reduction in lordosis behaviour induced by administering tamoxifen neonatally to female rats (19) may actually have resulted from a partial defeminisation of the brain by the oestradiol-like actions of tamoxifen acting on neural oestradiol receptors. Finally, prenatally ATD-treated female ferrets and control females displayed equivalent, high, acceptance quotients when tested after receiving a high dose of EB in adulthood (20), again indicating that ATD-treated females were capable of displaying normal female-typical receptive behaviour. Thus, it has been difficult to provide con-

clusive evidence of a role for oestrogens in the development of the female brain. As a result, the hypothesis languished since the mid-1980s as a result of the absence of a suitable animal model in which to assess rigorously the possible contribution of oestradiol. The creation of the ArKO mouse (22, 23) provided an opportunity to reopen work on this hypothesis. Because ArKO mice cannot produce oestrogens themselves as a result of a targeted mutation in the aromate (*Cyp19)* gene, but do have functional oestrogen receptors, they can respond to exogenous oestradiol at any time during their life span. This means that this model can be used to distinguish between organisational and activational effects of oestradiol on brain and behavioural functions. Here, we present a short overview of the results obtained in the ArKO mouse model that provide evidence for a role of oestradiol in female neural and behavioural differentiation.

Reduced female sexual behaviour in female ArKO mice

In a first experiment, we determined whether lordosis behaviour was affected in female ArKO mice (24). If the female brain indeed develops in the absence of any oestradiol, ArKO female mice should show normal, wild-type levels of lordosis behaviour, provided that they are supplemented with ovarian hormones, such as oestradiol and progesterone, in adulthood because they cannot produce oestradiol. In addition, progesterone levels are affected in ArKO females (22) because they do not show any signs of ovulation (25). Indeed, their ovaries contain follicles but no corpora lutea. In addition, during puberty, their ovaries appear to develop some testicular tissue, as demonstrated by the presence of Sertoli and Leydig cells, suggesting that oestrogens are not only necessary for folliculogenesis, but also to maintain ovarian morphology. Adult treatment with oestradiol restores ovarian morphology in ArKO females (26), but fails to induce ovulation (27). We observed that ArKO females showed less lordotic responses when mounted by the stimulus male (Fig. 1A). There was no genotype differences in the number of mounts received. In addition, this reduction in lordosis behaviour did not disappear with repeated testing as was observed in the early studies using neonatally ovariectomised female rats (3), suggesting thus permanent effects of early oestradiol deprivation on the later ability to show lordosis behaviour.

The absence of lordosis behaviour in ArKO females might have been caused by a partial defeminisation of the brain as a result of the presence of phytoestrogens in the food. By contrast to natural oestrogens, phytoestrogens are generally nonsteroidal and thus have lower affinities for oestradiol-binding plasma proteins such as AFP. They may thus evade the protective actions of AFP and freely enter the brain to interfere with the development of the neural circuits involved in later sexual behaviour. However, these experiments on lordosis behaviour in ArKO females have been repeated when feeding all subjects, including ArKO mice, phytoestrogen-free food (phytoestrogen-free mouse chow D10001 AIN-76A; Brogaarden, Lynge, Denmark) and very similar results were obtained (i.e. a clear significant reduction in lordosis behaviour in ArKO females compared to wild-type females) (O Brock, M.J. Baum, and J. Bakker, unpublished results).

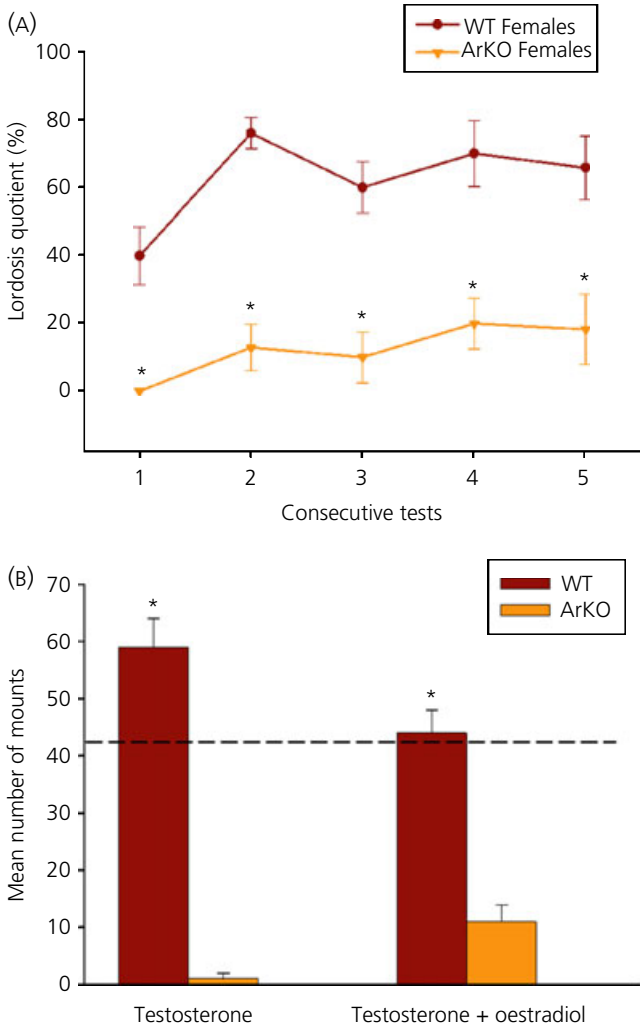

Fig. 1. Sexual behaviour of female wild-type and aromatase knockout (ArKO) mice. (A) Mean ± SEM lordosis quotients calculated by dividing the number of lordotic responses shown by the number of mounts received from the stimulus male (×100%). *P < 0.05 compared to wild-type (WT) females. (B) Mean ± SEM number of mounts (including mounts with intromission-like movements) displayed with an oestrous female. Females were first tested with testosterone and then with testosterone and oestradiol. The dotted line represents number of mounts and intromissions displayed by gonadally intact wild-type male mice under similar testing conditions (48) *P < 0.05 compared to ArKO females. Data adapted from Bakker *et al.* (24).

Another possible explanation for reduced levels of lordosis behaviour in ArKO females could be that they have been partially masculinised by increased concentrations of testosterone during early development. Fisher *et al.* (22) reported that ArKO mice (a different knockout model than the one used in our studies) of both sexes are exposed to increased plasma levels of testosterone during adulthood. These increased levels of testosterone probably results from the lack of negative-feedback action of oestradiol on the hypothalamic-pituitary-gonadal axis, as suggested by the increased levels of circulating luteinising hormone (LH) and follicle stimulating hormone in these mice (22). Alternatively, the increase in plasma

testosterone could be the result of an accumulation of the androgenic substrate because it is no longer aromatised to oestradiol as a result of targeted mutation in the aromatase gene. Because the foetal and neonatal ovaries are not producing high levels of steroid hormones, as well as there being little evidence that the HPG axis is active during early development (28), it is unlikely that this increase in testosterone levels also takes place during early development; however, because no data are available at present to evaluate this question, it could be speculated that increased levels of testosterone contribute to the development of the behavioural phenotype of ArKO female mice. Therefore, to evaluate whether the reduction in lordosis behaviour in ArKO females reflects the masculinising and defeminising actions of testosterone on their brains during early development, female wild-type and ArKO mice were tested for their ability to show male-typical mounting behaviour with an oestrous female. After 3 weeks of testosterone treatment, female ArKO mice showed very little mounting behaviour, whereas wild-type readily displayed mounting behaviour, even at higher frequencies, as observed in gonadally intact wild-type male mice of the same strain (Fig. 1B). Adding oestradiol stimulated some mounting behaviour in ArKO females, but not up to the levels observed in wild-type females. Thus, there is no clear behavioural evidence for brain masculinisation in ArKO females. Indeed, it may be argued that female mounting behaviour is actually a female-typical behavioural characteristic that also needs to be feminised by oestradiol during early development, and thus not, as it is traditionally thought, a typical male behavioural characteristic only. The ethological significance of female–female mounting behaviour is of course less clear when compared to male–female mounting behaviour, although it might signal dominance and reproductive status.

In summary, the results obtained on lordosis behaviour in ArKO females are best explained by assigning an active role for oestradiol in female-typical behavioural differentiation. At present, these results provide the best available evidence for a role of oestradiol in the development of the female brain.

Reduced kisspeptin expression in female ArKO mice

In recent years, the *Kiss1* gene product, kisspeptin, has been proposed as an important upstream regulator of the gonadotrophin-releasing hormone (GnRH) system because human patients did not enter into puberty as a result of a mutation in the *GPR54* gene (29), which encodes the kisspeptin receptor, named GPR54 (now named Kiss1r). Similar results were obtained in mice with a targeted disruption of the *Kiss1* gene (30). Interestingly, kisspeptin expression is sexually dimorphic in rodent species, with females having greater numbers of kisspeptin-expressing neurones than males in the rostral periventricular area of the third ventricle (RP3V) (31, 32) suggesting that kisspeptin may play a sexually dimorphic role in controlling reproductive events. In particular, the RP3V kisspeptin population has been proposed to play a critical role in the positive-feedback actions of oestradiol on GnRH release. Because ArKO females were reported to be unable to ovulate (25, 27), we determined whether the sexual differentiation of the RP3V kisspeptin population was affected in ArKO female mice. In addition, because we observed several changes in olfactory functioning in female ArKO mice (24, 33, 34), we investigated whether the integration of pheromones into the reproductive system was affected in ArKO female mice and thus whether oestradiol may be important in organising sexually dimorphic responses of the reproductive system, such as LH release, to pheromones. Exposure to male pheromones induced Fos protein in RP3V kisspeptin neurones in ArKO female mice, albeit significantly less compared to wild-type females (Fig. 2A). The sexual differentiation of kisspeptin neuronal number was lost in ArKO females (Fig. 2B) (i.e. the number of kisspeptin-immunoreactive neurones in the RP3V of ArKO females is as low as

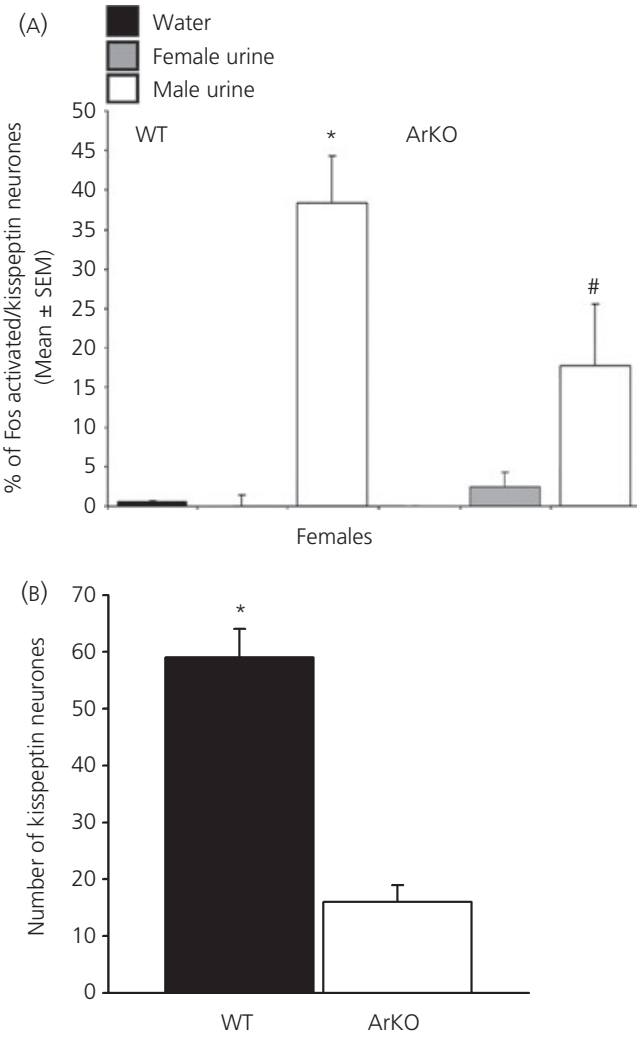

Fig. 2. Effects of aromatase knockout (ArKO) on the sexual differentiation of kisspeptin neuronal numbers and their activation by same versus opposite sex urinary pheromones. (A) Mean ± SEM percentage of fos activated kisspeptin expressing neurones after exposure to either water, intact male or oestrous female urine in the rostral periventricular area of the third ventricle (RP3V) of female wild-type (WT) and ArKO mice. *P < 0.05 compared to water exposure; #P < 0.05 compared to water exposure and wild-type females exposed to male urine. (B) Mean ± SEM number of kisspeptin neurones in the RP3V. *P < 0.05 compared to ArKO females. Data adapted from Bakker *et al.* (35).

that observed in male mice), suggesting that the sex difference in kisspeptin neuronal number in wild-type mice reflects an organisational action of oestradiol in females (35) because ArKO females were supplemented with oestradiol in adulthood for these experiments. This result was confirmed by a recent study performed by Clarkson *et al.* (36), who showed that ovariectomy of female pups at postnatal (P) day 15 resulted in a 70-90% reduction in kisspeptin expression within the RP3V analyzed at either P30 or P60, whereas oestradiol treatment in P15 ovariectomised mice from P15-P30 or P22–P30 resulted in a complete restoration of kisspeptin expression in this brain region. Furthermore, they showed decreased numbers of kisspeptin neurones in the RP3V of adult female ArKO mice, as was also observed in our study (35). These findings can be best explained by organisational rather than activational effects of oestradiol on kisspeptin neurones in the RP3V because earlier work from this group showed that the RP3V kisspeptin population is not present before P25 (31).

Interestingly, kisspeptin neurones in the RP3V were still activated by male pheromones in ArKO females, although this activation was clearly reduced in comparison with wild-type females. Whether there is a link between this reduced activation of kisspeptin neurones and the lack of showing ovulation following adult oestradiol treatment (27) is still unknown. We are currently determining whether concurrent treatment with oestradiol and progesterone, which has been shown to be successful in inducing LH surges in wild-type female mice (37), can induce LH surges in ArKO female mice.

In summary, these results clearly show that oestradiol induces a female-typical population of kisspeptin in the RP3V and thus provides additional evidence for an organisational role of oestradiol in female neural differentiation.

Reduced prepubertal expression of progesterone receptor in the hypothalamus of female ArKO mice

The observation of clear deficits in lordosis behaviour (24), as well as decreased numbers of kisspeptin neurones in the RP3V (35) of female ArKO mice, has resurrected the question of whether oestradiol, acting during the first several weeks of life, actively contributes to female-typical brain and behavioural sexual differentiation, perhaps beginning after postnatal day 7 when the ovary first produces oestradiol (38) and when the AFP concentration has diminished to such an extent that it no longer plays a neuroprotective role against oestrogens. One approach to the question of whether oestradiol actively contributes to female development would be to compare the expression of an oestradiol-dependent gene in ArKO female mice, which produce no oestradiol in any tissue, and in wild-type control females. The expression of the progesterone receptor (PR) in the hypothalamus of adult female rodents is well known to be dramatically up-regulated by oestradiol (39, 40). Likewise, Wagner and colleagues have conducted an extensive series of experiments showing that the number of PR-immunoreactive (-ir) cells in the medial preoptic area (MPOA), the lateral part of the ventromedial hypothalamus (VMHvl) and the AVPV of both rat (41) and mouse (42, 43) is significantly greater in neonatal males than in females. Additional work from this group (40, 43) showed that oestradiol, formed via the neural aromatisation of testosterone in male neonates, is responsible for the higher, male-typical levels of PR expression in the MPOA. Finally, another study from this group (44) showed that administration of the aromatase inhibitor ATD reduced PR-ir in the MPOA of male rats killed at the end of gestation. Therefore, based on these results, we used the expression of PR-ir in three different hypothalamic regions (i.e. AVPV, MPOA and VMHvl) as an index of the action of oestradiol action in the female mouse brain across the first 25 days of postnatal development (45). We found that the amount of PR-ir in the AVPV and MPOA was significantly lower in ArKO female mice than in wild-type females at several prepubertal ages, including postnatal day P15, P20 and P25, but not neonatally at P0, P5, or P10 (Fig. 3) (45). Similarly, PR-ir was significantly lower in the VMHvl at P25 in ArKO versus wild-type females but not at earlier postnatal ages (45). We also observed that PR-ir was consistently higher in male than in female wild-type mice in the AVPV and MPOA over P0–P10 and in the VMHvl over P0–P20. In addition, PR-ir in ArKO males was significantly lower than in wild-type males in these brain areas across these latter ages, and resembled the values observed in wild-type females, confirming previous stusies indicating that oestradiol formed in the male hypothalamus by aromatisation of testosterone is responsible for inducing male-typical levels of neural PR expression.

These results thus support the view that oestradiol contributes prepubertally to at least one female-typical aspect of development in both the MPOA and AVPV, namely the expression of PR (45). However, more research is needed to determine whether this prepubertal increase in neural PR expression observed in wild-type females is simply a passive, inconsequential response to increased

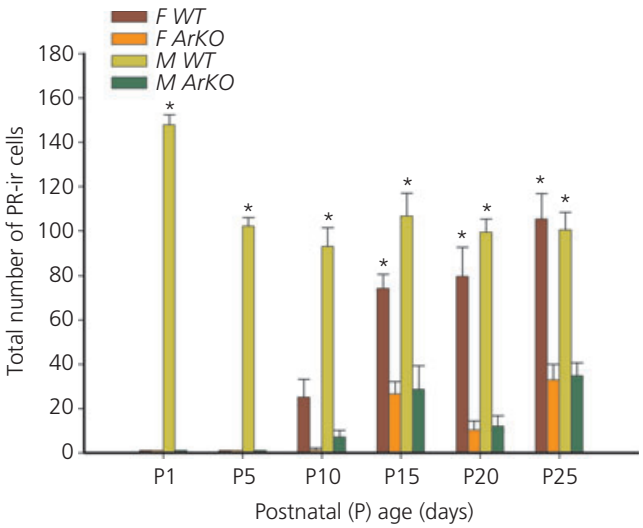

Fig. 3. Reduced prepubertal expression of progesterone receptor (PR) in the hypothalamus of aromatase knockout (ArKO) mice. (A) The total number of PR-immunoreactive (-ir) cells in the medial preoptic area (MPOA) at several postnatal ages in female and male wild-type (WT) and ArKO mice. *P < 0.05 compared to ArKO mice of both sexes. Data adapted from Brock *et al.* (45).

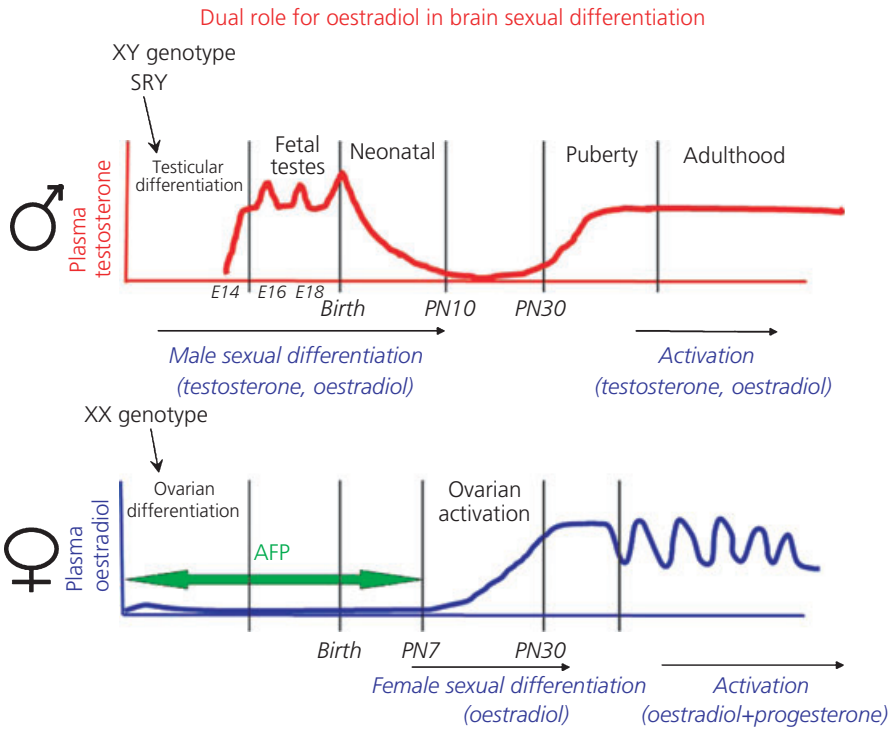

Fig. 4. Working hypothesis on how oestradiol can have both feminising and defeminising effects on brain sexual differentiation. Thus, male-typical neural and behavioural differentiation occurs prenatally in genetic males under the influence of testosterone and oestradiol, which is avoided in foetal genetic females by the neuroprotective actions of α-fetoprotein (AFP), whereas female-typical neural and behavioural differentiation probably occurs postnatally in genetic females under the influence of oestradiol that is presumably produced by the ovaries and at the time that AFP no longer plays a neuroprotective role.

ovarian production of oestradiol or, alternatively, whether long-lasting morphological and/or behavioural neuroendocrine consequences of this prepubertal oestrogenic stimulation of PR expression can be identified in the female rodent MPOA and AVPV, such as the development of the female-typical kisspeptin population.

Interestingly, these results also indicate that oestradiol induces both female- and male-typical expression of PR in the mouse hypothalamus, with the male-typical population developing prenatally and the female-typical population developing postnatally (Fig. 4). These sex differences in expression (and particularly in the induction of PR expression) may simply reflect gonad development, with the brain responding passively, and perhaps not some inherent brain sex difference or sex difference in critical period.

Conclusions and future directions

The results obtained in female ArKO mice suggest an active contribution of oestrogens to the development of the female brain. Initially, these results are at odds with our results obtained in AFP-KO mice demonstrating that the principal action of prenatal oestrogen exposure, regardless of whether it occurs in female or male mice, is to defeminise and to some extent masculinise brain and behaviour. Thus, both knockout mouse models show feminising and defeminising actions of oestradiol. Such a dual role of oestradiol in brain sexual differentiation was earlier suggested by Dohler *et al.* (19) who introduced the 'progressive hypothesis of brain sexual differentiation', which asserts that, under the influence of moderate levels of oestradiol, female-typical neural and behavioural traits develop, whereas, under the influence of high levels of oestradiol, male-typical neural and behavioural traits develop. However, our results obtained in AFP-KO mice clearly show that any prenatal oestrogen action is blocked by AFP during development and, thus, in order for oestradiol to influence brain sexual differentiation, it has to be produced locally from testosterone by aromatisation. So the next question would be that if oestradiol is acting prenatally in the female brain, what might be the sources of this oestradiol? It may be *de novo* synthesis (44) but, at present, the evidence to support is still lacking. Another possibility is that female-typical neural and behavioural characteristics develop postnatally at the time that the ovaries have begun to produce oestradiol (after P7) and when the amount of AFP has decreased substantially and AFP no longer plays a protective role (Fig. 4). Our results obtained on PR expression clearly show that the female-typical expression of PR develops after P10, which suggests that feminising actions of oestradiol take place prepubertally and not perinatally. Supportive evidence comes from the study by Clarkson *et al.* (36) on the sexual differentiation of the RP3V kisspeptin population, showing that prepubertal treatment with 17β-oestradiol between P15 and P30 was able to restore the decrease observed in kisspeptin expression in P15-ovariectomised female mice. However, the possibility cannot be ruled out that some prenatal or early

neonatal event is still necessary for mice to be able to later show a female-typical response to oestradiol with regard to hypothalamic kisspeptin and PR expression. The ArKO mouse will be a great model to test whether there is a specific critical period for female brain development because they can be supplemented with oestradiol at various times during development. Studies are currently underway aiming to determine whether deficits in lordosis behaviour can be reversed by prepubertal treatment with oestradiol. If normal wild-type levels of lordosis behaviour can be induced in female ArKO mice by treating them with exogenous oestradiol over a specific postnatal period, then these results would provide the best evidence for an active contribution of oestradiol to female neural and behavioural sexual differentiation.

Finally, the view that testosterone and/or oestradiol are solely responsible for the development of sex differences in the mammalian brain has been challenged as well. Several sex dimorphisms have been identified that are difficult to attribute solely to the perinatal actions of sex steroids in the nervous system. Thus, it has been proposed that different doses of genes expressed off the X chromosome in XY (male) versus XX (female) mice influence aspects of brain and behavioural sexual differentiation (46), perhaps reflecting a nonhormonal, genetic signalling mechanism that actively promotes female-typical brain sexual differentiation. For example, De Vries *et al.* (47) showed that the male-typical profile of vasopressin innervation of the lateral septum depends on the presence of a Y-chromosome. XY males and XY female mice (i.e. females with a deletion of the Sry gene) were more masculine than XX mice with respect to the density of vasopressin-ir fibres in the lateral septum. Our finding (6) of a female-like vasopressin innervation in the lateral septum of female AFP-KO mice is intriguing because mice had been exposed to high defeminising levels of oestradiol during prenatal development. Thus, more research is needed to determine the contribution of chromosome genes to the sexual differentiation of the brain.

At present, there is increasing evidence for an organisational role of oestradiol in female-typical neural and behavioural sexual differentiation, thereby challenging the old dogma of a default organisational programme for the female brain. However, more research is needed on when oestradiol is precisely acting in the female brain to induce feminisation (i.e. is there a specific critical time window for female brain differentiation as has been shown for the development of the male brain?). In addition, questions remain on the mechanisms by which oestradiol induces feminisation (i.e. is there a specific role for progesterone receptors as well as progesterone itself in the development of the female brain?).

Acknowledgements

This work was supported by National Institutes of Health grant HD044897 and Fonds National de la Recherche Scientifique (FNRS) MIS F.4502.07. J.B. is a research scientist of the Belgian FNRS.

Received: 2 April 2010,
revised 28 April 2010,
accepted 28 April 2010

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