Published in final edited form as:

J Neuroendocrinol. 2007 October ; 19(10): 767–772. doi:10.1111/j.1365-2826.2007.01585.x.

Effects of Organisational Oestradiol on Adult Immunoreactive Oestrogen Receptors (α and β) in the Male Mouse Brain

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Abstract

Steroid hormones act on developing neural circuits that regulate the hypothalamic-pituitary-gonadal axis and are involved in hormone-sensitive behaviours. To test the hypothesis that developmental exposure to oestradiol (E_2) organises the quantity of adult oestrogen receptors ($ER\alpha$ and $ER\beta$), we used male mice with a targeted mutation of the aromatase enzyme gene (ArKO) and their wild-type (WT) littermates. These mice are unable to aromatise testosterone to E_2 , but still express both $ER\alpha$ and β . To evaluate adult responsiveness to E_2 , gonadectomised males were implanted with Silastic capsules containing E_2 , or an empty implant, 5 days prior to sacrifice. Immunoreactivity for $ER\alpha$ and $ER\beta$ was quantified in the caudal ventromedial nucleus (VMN) and the medial preoptic area (POA). Regardless of genotype, adult treatment with E_2 reduced $ER\alpha$ -immunoreactive (ir) and $ER\beta$ -ir cell numbers in the POA, as well as $ER\beta$ -ir, but not $ER\alpha$ -ir, cell numbers in the VMN. Genotype, and thus endogenous exposure to E_2 , produced opposite effects on ER expression in the two brain areas. In the VMN, ArKO males had more $ER\alpha$ -ir and $ER\beta$ -ir cells than did WT males. In the POA, ArKO males had fewer $ER\alpha$ -ir and $ER\beta$ -ir cells than did WT males. Thus, numbers of immunoreactive neurones containing both ERs in the adult ArKO male were enhanced in the POA, but decreased in the VMN, and most likely these patterns were established during the developmental critical period. Furthermore, although both $ER\alpha$ and β -ir cell numbers are altered by the disruption of the aromatase gene, $ER\beta$ is altered in a more robust and region-specific manner.

Oestradiol (E_2) is a steroid hormone produced by the gonads and also in the brain where it affects neural structure and function both in perinatal and adult individuals (1–3). Like other steroid hormones, E_2 serves as ligand for specialised receptors that affect gene transcription. To date, two oestrogen receptors ($ER\alpha$ and $ER\beta$) have been cloned (4,5) and characterised in the murine brain (6). The regulation of ERs by ligand has been examined using a number of techniques including binding assays, autoradiography, *in situ* hybridisation and immunocytochemistry (7–12). In accordance with the principles of negative feedback, when the primary source of E_2 (i.e. the gonads) is removed, ER expression levels increase, and E_2 replacement causes a subsequent decline in ER (11). However, during the rat oestrous cycle, different neural regions exhibit differences in the concentrations of ER mRNA (11). Another dimension of the relationship between E_2 , $ER\alpha$ and $ER\beta$ is that exposure to E_2 during development (during gestation, shortly after birth and/or puberty) may influence the feedback dynamics of each ER to its ligand in the adult brain. For example, when male rat pups are deprived of E_2 just after birth and analysed as adults, levels of ER are elevated compared to

untreated littermates (7,8). Thus, the quantity of neural ER is closely related to and influenced by endogenous levels of oestradiol during development.

In the present study, we utilised a new approach to re-examine a question that has been asked previously: how do perinatal and adult E₂ exposure differentially affect the concentrations of adult neural ERs? First, to remove both pre- and postnatal endogenous production of E₂, without using pharmacology or surgery, we conducted these studies in the aromatase knockout (ArKO) mouse. These mutants carry targeted disruptions in both the transcriptional and translational sites of the *CYP19* gene, do not produce aromatase enzyme, and are unable to convert testosterone to E₂ (13). Both ER α and ER β are present in ArKO brains and they are capable of responding to exogenous oestradiol (13,14). Second, although many of the studies examining developmental actions of E₂ on adult receptor function compared males and females, we chose to examine males only. By using males, we not only can examine effects resulting from the lack of oestradiol during the hormone critical period, but also circumvent potential confounds attributable to sex and sex chromosome genes that manifest at different time-points (15,16). Finally, we used immunocytochemistry to examine the impact of E₂ on both ER α and ER β in the same individuals in consecutive sections. Few studies have asked about the relationship between E₂ and ER β (17,18) or assessed both receptors in the same individuals (6,19).

We predicted that if E₂ acts during neural development to establish adult ER levels, then castrated adult male ArKO mice should have different numbers of ER immunoreactive cells compared to wild-type (WT) littermates. After the administration of E₂ to adults we predicted that ArKO males would be less responsive to E₂ and have a blunted decrease in ERs compared to WT males. We focused our analysis on two areas that have been examined in previous studies: the caudal aspect of the ventromedial nucleus of the hypothalamus (VMN) and the medial preoptic area (POA). These two regions are highly involved in regulating adult displays of reproductive behaviour, are oestrogen-sensitive, and exhibit sexually dimorphic characteristics known to be organised by developmental hormone exposure. In addition, several studies suggest suboptimal sexual behaviour in ArKO mice (13,14,20,21) and ER functioning is certainly essential for normal expression of these behaviours.

Materials and methods

Animals

Mice used in this study were produced by heterozygous breeding pairs; each mouse had one normal and one disrupted copy of the aromatase enzyme gene (13). At the time of this experiment, animals had been back-crossed at least three times into the C57BL/6J strain. Animals were genotyped by polymerase chain reaction amplification of tail DNA (13). All mice were born and housed in the University of Virginia Animal Care Facility, located in Gilmer Hall, under a 12: 12 h light/dark cycle (lights on at 00.00 h).

Mice were given food and water *ad libitum*. All animals were fed a standard laboratory mouse chow that contains phytoestrogens (Harlan Teklad Global Diet 8604) (Harlan, Bicester, UK)). We selected this diet because it is a standard rodent chow and we did not want to complicate the interpretation of the data by introducing dietary phytoestrogens as another variable. Moreover, standard phytoestrogen-containing diets have been used for most studies of sexual differentiation. The groups formed included WT (n = 13) and ArKO (n = 19) mice. Adult mice (age 10–16 weeks) were gonadectomised and, 1 week later, received a Silastic implant (1.98 mm inner diameter $\times$ 3.17 mm outer diameter) containing 50 μ g 17- β -oestradiol dissolved in 25 μ l sesame oil or an empty implant. Implants were placed subcutaneously in the midscapular region. All animals were sacrificed 5 days later. In total, the four groups of castrates had the

following compositions; WT + blank (n = 8), WT + E₂ (n = 5), ArKO + blank (n = 9) and ArKO + E₂ (n = 10).

At the time of brain collection, animals were deeply anaesthetised with sodium pentobarbital and brains were removed and fixed via a 5% acrolein immersion (22). Following overnight immersion in 0.1 M of phosphate buffer containing 30% sucrose, serial coronal sections (30 μ m) were made through the forebrain and stored in cryoprotectant (containing sucrose, PVP-40, and ethylene glycol) at -20 °C. One in four sections were processed for immunocytochemical analysis of ER α and another quarter for ER β .

Immunocytochemistry

Brain sections were removed from cryoprotectant and rinsed in 0.2 M Tris buffered saline (5 $\times$ 10 min), pH 7.8. These rinses were followed by a 30-min incubation in a 1% NaBH₄ solution and a 10-min incubation in 0.3% H₂O₂, with three 10-min rinses in between. Sections were incubated at 4 °C for 48 h in a primary antibody directed against either ER α or β . For ER α , we used a polyclonal antibody directed against the last 14 amino acids of the rat ER α C-terminus (C1355, 1: 5000; generously provided by Dr Margaret Shupnik, University of Virginia) (23). One reason for selecting this antibody is that immunoreactivity is not influenced by ligand availability (24). For ER β detection, we used a polyclonal antibody directed against the C-terminus of the mouse ER β protein (1: 1000; Z8P Zymed Laboratories, San Francisco, CA, USA). Control studies on the effects of ligand on numbers of immunopositive ER β cells have not been conducted with this antiserum; thus, it is possible that some of the effects of oestrogen might be caused by altered efficiency of antibody binding rather than a decrease in receptor expression or its degradation. Both antibodies have been used in mouse brain previously (25, 26). Next, tissue was incubated for 1 h in a biotinylated goat antirabbit secondary antibody (1: 500; Vector Laboratories, Burlingame, CA, USA). Following rinses, sections were incubated for 90 min in avidin-biotin complex (1: 1000; Vectastain Elite; Vector Laboratories). After 30 min of rinsing, tissues were incubated for 5 min with a nickel intensified diaminobenzidine (DAB) solution (0.25% nickel ammonium sulphate and 0.05% DAB) activated by 0.001% hydrogen peroxide. All sections were processed for ER α and β in the same staining run.

Image analysis and statistics

Oestrogen receptor immunoreactive cell bodies were quantified in one section from each brain using Metamorph Image Analysis (Molecular Devices, Chester, PA, USA). Sections were best matched based on landmarks defined in the Franklin and Paxinos mouse brain atlas (27). As described previously (26), areas quantified were the POA (0.14 mm anterior to bregma) and the caudal aspect of the VMN (-1.82 mm posterior to bregma) (27). In the POA, the two major fibre tracts served as landmarks when choosing sections where the optic tract is barely present at the base of the brain, and the anterior commissure is present at its greatest lateral extent. Within the VMN, matching sections were chosen based on the shape of the third ventricle and arcuate nucleus in addition to the lateral and caudal appearance of the ER α staining within the VMN. We intentionally excluded the region of the POA representing the anteroventral periventricular nucleus by avoiding counting immunoreactive cells located within a distance of ten cell bodies from the ventricle. Relationships among groups were assessed using a two-way ANOVA for each ER with genotype and hormone condition as the two factors. Differences among groups were analysed using Bonferroni's planned comparison tests. $P < 0.05$ was considered statistically significant.

Results

Developmental exposure to, and adult treatment with, E₂ decreases numbers of both ER α -immunoreactive (ir) and ER β -ir neurones in the VMN

In the VMN, numbers of ER α -ir cells were affected by genotype [$F(1,31) = 5.92$, $P < 0.02$], with ArKO males possessing more ER α -ir cells than WT males in this area. Hormone treatment had no independent influence [$F(1,31) = 0.10$], however, an interaction between genotype and hormone treatment was observed [$F(1,31) = 6.61$, $P < 0.02$]. Planned comparisons revealed that the effect was caused by ArKO castrates that did not receive E₂ treatment in adulthood ($P < 0.05$; Fig. 1). For ER β , genotype [$F(1,31) = 5.82$, $P < 0.022$] and hormone treatment [$F(1,31) = 23.7$, $P < 0.0001$] both had independent effects on cell numbers, and an interaction was also found [$F(1,31) = 7.01.92$, $P < 0.01$]. Again, ArKO males had more ER β -ir cells than did WT males and E₂ treatment decreased ER β -ir cell numbers. Planned comparisons revealed that the castrated ArKO mice that did not receive E₂ in adulthood had more ER β -ir cells in the VMN than males in any of the other groups ($P < 0.05$; Figs 1 and 2).

Developmental exposure to E₂ increases, while adult E₂ decreases, numbers of both ER-ir neurones in the POA

Oestrogen receptor α -ir cell numbers were affected by genotype [$F(1,31) = 7.13$, $P < 0.01$] and adult hormone condition [$F(1,31) = 16.01$, $P < 0.001$], but no interaction between these factors was revealed [$F(1,31) = 0.51$]. The same pattern was found for ER β -ir cells; independent effects of genotype [$F(1,29) = 10.90$, $P < 0.005$] and hormone [$F(1,29) = 17.53$, $P < 0.001$], but no interaction between the two [$F(1,29) = 1.13$], was observed. Post-hoc analysis showed that ArKO mice had fewer ER α -ir and ER β -ir cells in the POA compared to WT males (Fig. 3). Furthermore, WT and ArKO males that received E₂ treatment had lower numbers of ER-ir cells than untreated castrates. Both effects were more striking for ER β -ir than ER α -ir in part because there are ten-fold more ER α than ER β containing neurones in this region.

Discussion

Our results show that endogenous E₂ production in males during perinatal development and/or puberty affects adult neural ER-ir cell number in a receptor and region-specific manner. In the VMN, males that lacked both developmental E₂ and received no adult hormone treatment after castration (ArKO males) had the largest quantities of both ER α -ir and ER β -ir cell numbers. Adult treatment with E₂ down-regulated both oestrogen receptors in ArKO brains. Interestingly, in the WT male VMN, ER α -ir cells were unaffected by administration of E₂ to castrated adults. The implication here is that developmental exposure to E₂ decreases adult responses to the same hormone in the adult VMN. Also, although adult E₂ treatment did reduce ER β -ir cell numbers in both genotypes, there were fewer ER β -ir neurones in the brains of WT males; the castrated untreated ArKO males had three- to four-fold more ER β -ir than did the WT untreated castrates. Thus, the lack of E₂ during development leads to an augmented expression of this receptor in adult VMN. By contrast, in the POA, the lack or presence of endogenous E₂ prior to adult castration did not change the response to E₂; after castration, we noted a decrease in both ER α -ir and ER β -ir cell numbers. Furthermore, in castrates that did not receive E₂, ArKO males had fewer ER-ir cells than did WT males, thus showing region-specific differences in constitutive ER content. This relationship was more striking for ER β than ER α .

The only immunocytochemical data similar to these were collected from gonad-intact male ArKO and WT mice, and ER α was the only ER examined (9). In this previous study, ArKO mice had more ER α -ir cells in the POA and arcuate/VMN region of the hypothalamus compared to WT littermates (9). None of our data are directly comparable with these in terms

of the ArKO mouse strain used, hormone milieu, ER α antibody or brain regions examined. For example, the arcuate/VMH region previously examined contained both the rostral and caudal VMN as well as the part of the arcuate nucleus, whereas our analysis was localised to the caudal VMN. The only meaningful comparison that we can make is between the POA data. We can consider data from gonad-intact ArKO males in the previous study as being comparable with our ArKO castrate males given an empty implant (both lack E₂), and the gonad-intact WT in the previous study as being comparable with our E₂-treated WT males. When we made this comparison, we noted a strong trend for more ER α in the ArKO POA compared to the E₂-treated WT male. Thus, similar patterns were observed in both studies.

In male rats, postnatal administration of an aromatase inhibitor (on the day of birth through 21 days of age) produces a significant increase in ER α -ir concentration in the POA and VMN of adults compared to control castrates (7). Our data in the VMN are in agreement with this report but, in the POA, our findings are in the opposite direction. It is possible that two critical periods exist for the POA (i.e. before and after birth), and only one of these is affected by the postnatal drug treatment. It is also possible that species differences between rats and mice can account for these discrepancies.

In addition to the ArKO mouse, studies using ERKO mice have been conducted in which the effects of E₂ on the WT ER were examined (17,18). The assumption behind these experiments is that the lack of one ER provides information about its role in the actions of E₂ on the remaining ER. When ER β is knocked out in male mouse brain, down-regulation of ER α -ir by long-term E₂ treatment is blocked in both the POA and the VMN (18). These results do not match the present data in the ArKO male showing that ER α -ir cell numbers can be reduced by E₂ in both these regions. Thus, selective blockade of the actions of ER β is not equivalent to complete E₂ deprivation. Male reproductive behaviour in ER β KO males is in agreement with this conclusion (28,29).

Although the removal of one ER may not be equivalent to complete E₂ deprivation, it does enable us to parse out combined actions of the receptors. For example, ER β -ir has been quantified in adult male WT and ER α KO mice (17). Adult males were either gonad-intact or castrated and given short- or long-term E₂ treatment. In the WT males, castrates had higher ER β -ir numbers in the POA and VMN than did the WT given long-term E₂, although the effect was only significant in the POA. This is in agreement with the present findings. The lack of functional ER α reduced ER β -ir in the POA regardless of hormone treatment. In the VMN, ER α KO males in all the groups without gonads had very high ER β -ir counts. Considered along with the present data, these studies suggest a role for ER α in regulating ER β in the POA and VMN. Although ArKO males display a decrease in ER β -ir in response to adult E₂, ER α KO males fail to do so (17). The ER β KO male has a reduced response to E₂ in terms of ER α -ir in the POA, and the ArKO male as a similar response but not as blunt; thus, E₂ can decrease ER α -ir significantly when both ERs are activated.

Brain region-specific discrepancies in ER regulation following ligand administration are well documented and are dependent upon several factors. Previous studies using rodents provide evidence for both up and down regulation of neural ERs during the cycle or following adult E₂ treatment (11,30,31). Within the rat brain, ER α distribution in the postnatal POA and VMN is comparable to that seen in adults. Moreover, female rats display more robust ER α -immunoreactivity than do males, suggesting that neonatal testosterone aromatised to E₂ decreases ER α -ir cell numbers (32). Pharmacological disruption of the aromatisation of testosterone to E₂, however, alters neural ER-ir cell numbers. For example, in male rat brains, there is a down regulation of ER α following testosterone treatment that can be reversed upon coadministration of the non-steroidal aromatase inhibitor, fadrozole (33). These data indicate

that perinatal disruption of ER activation causes permanent alterations in adult hormone receptor content.

The ArKO model has proven useful in parsing out organisational and activational effects of oestradiol in the rodent brain. Although these animals are incapable of synthesising E₂ from testosterone, the classic ER α and β are still present. Several behaviours in the male ArKO mouse are disrupted, but these can be rescued by adult treatment with E₂. This suggests a degree of plasticity which makes it important to evaluate how and whether ER dynamics in the adult brain are altered by the absence of endogenous ligand and/or restoration of ligand in adults. The VMN is essential for the expression of adult female sexual receptivity, a set of behaviours that are highly sexually dimorphic; male mice appropriately primed to do not show lordosis (34). Our data suggest that VMN-related behaviours may differ between adult WT and ArKO males. Recently, we have reported that male ARKO mice, raised on a low phytoestrogen containing chow, tend to show more lordosis behaviour than do WT males on the same diet (21). In the case of behaviours involving the POA, our data predict that WT and ArKO males treated with E₂ may be equivalent or have only small differences. In the case of male aggression and copulatory behaviours, this appears to hold true (14,35,36). In summary, some of the specificity of the actions of oestradiol are produced by regional differences in the ER-containing cell populations and by the two different ER subtypes within these populations.

Acknowledgements

The authors would like to thank Ms Aileen Wills and Ms Savera Shetty for technical assistance. This work was supported by NIH grants R01 MH57759 and K02 MH01349 (E.F.R.). A.E.K. was supported by an NIH training grant T32 HD072323 and an individual NRSA F31 MH70092.

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Fig. 1.

Mean numbers of oestrogen receptor-immunoreactive (ER-ir) neurones in the ventromedial nucleus (VMN) in adult male aromatase knockout (ArKO) mice and wild-type (WT) littermates. Animals were castrated and given an oestradiol implant or a blank implant for 5 days prior to sacrifice. (A) Numbers of ERα-ir neurones. (B) ERβ-ir cell counts. *Significantly more ER-ir cells than males in any of the other groups ($P < 0.05$). Numbers of animals per group: WT + blank ($n = 8$), WT + E₂ ($n = 5$), ArKO + blank ($n = 9$), ArKO + E₂ ($n = 10$).

Fig. 2. Photomicrograph of the ventromedial nucleus. Both oestrogen receptor (ER) α -immunoreactive (ir) cells (A,B) and ER β -ir neurones (C,D) are shown. Brains were collected from castrated adult males of two genotypes; aromatase knockouts (B,D) and their wild-type littermates (A,C). Scale bar = 100 μ m.

Mean numbers of oestrogen receptor-immunoreactive (ER-ir) neurones in the medial preoptic area (POA) in adult male aromatase knockout (ArKO) mice and wild-type (WT) littermates. Animals were castrated and given an oestradiol implant or a blank implant for 5 days prior to sacrifice. (A) Numbers of ERα-ir neurones. (B) ERβ-ir cell counts. *Significantly reduced ER-ir cell numbers in animals with oestradiol treatment ($P < 0.05$). **Significantly more ER-ir cells in WT than ArKO males ($P < 0.05$). Numbers of animals per group: WT + blank ($n = 8$), WT + E₂ ($n = 5$), ArKO + blank ($n = 9$), ArKO + E₂ ($n = 10$).