Oestrogen at the neonatal stage is critical for the reproductive ability of male mice as revealed by supplementation with 17β-oestradiol to aromatase gene (*Cyp19*) knockout mice

K Toda, T Okada[1], K Takeda[2], S Akira[2], T Saibara[3], M Shiraishi[4], S Onishi[3] and **Y Shizuta**

Departments of Medical Chemistry, [1]Anatomy and [3]Internal Medicine, and [4]Institute for Laboratory Animals, Kochi Medical School, Nankoku, Kochi 783–8505, Japan

[2]Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565–0871, Japan

(Requests for offprints should be addressed to K Toda, Department of Medical Chemistry, Kochi Medical School, Nankoku, Kochi 783–8505, Japan; Email: todak@kochi-ms.ac.jp)

Abstract

Aromatase P450 (CYP19) is an enzyme responsible for the conversion of androgens to oestrogens. We generated CYP19 knockout (ArKO) mice by targeted disruption of *Cyp19* and studied the role of oestrogens in male reproductive ability. Approximately 85% of ArKO males were unable to sire offspring. However, no obvious difference was found in testicular and epididymal weights, numbers of sperm in the epididymis or the ability of sperm to fertilize eggs *in vitro* between wild-type and ArKO males. An examination of mating behaviour demonstrated that ArKO males showed an impairment in mounting behaviour against sexually mature females. The inability of more than 90% of ArKO males to sire offspring was reversed by repeated subcutaneous injections of 17β-oestradiol when initiated on the day of birth. The effects of 17β-oestradiol on reproduction were concentration dependent and evident when supplementation was initiated on day 7, but not on day 15 after birth. These findings suggest that oestrogens acting during neonatal life are required for normal mating behaviour in adulthood.

Journal of Endocrinology (2001) **168,** 455–463

Introduction

Aromatase P450 (CYP19) catalyzes the conversion of androgens to oestrogens (Simpson *et al.* 1994). In mice, expression of *Cyp19* is found predominantly in the gonads and the brain (Yamada *et al.* 1993). We have previously reported the isolation of a full-length cDNA encoding mouse aromatase P450 and the determination of its nucleotide sequence (Terashima *et al.* 1991). Recent studies have demonstrated that transcription of *Cyp19* is regulated by tissue-specific promoters coupled with alternative splicing events (Lephart 1996), as is the case for the human homologue (Simpson *et al.* 1994).

The physiological roles of oestrogens in the male have been reviewed recently (Sharpe 1998). Defects in reproductive function were frequently observed when male mice were exposed to high doses of oestrogens during the prenatal or neonatal period (McLachlan *et al.* 1975, Newbold *et al.* 1986, Toppari *et al.* 1996). Furthermore, studies on mice lacking one of oestrogen receptors, ERα (αERKO), have suggested that the oestrogen/ERα signalling pathway is not only important for reproductive function (Eddy *et al.* 1996, Hess *et al.* 1997, Couse & Korach 1999), but also for male reproductive behaviour such as intromission and ejaculation (Wersinger *et al.* 1997, Ogawa *et al.* 1997, 1998).

In addition to the regulatory function of oestrogens in the reproductive system, a number of studies have clarified their roles in the central nervous system, which controls development of sexual behaviour in males. Testicular testosterone was first proposed as a regulator of male reproductive behaviour (Stone 1939, Davidson 1966). However, oestradiol has been demonstrated to mimic the effects of testosterone (Davis & Barfield 1979, Vagell & McGinnis 1997). Furthermore, administration of dihydrotestosterone, a non-aromatizable androgen, was ineffective in restoring male sexual behaviour in castrated rats (McDonald *et al.* 1970) and treatment of neonatal male rats with androst-1,4,6-triene-3,17-dione, an aromatase inhibitor, resulted in modulation of androgen-induced sexual behaviour (Christensen & Clemens 1975, Beyer *et al.* 1976, Booth 1978). These studies proposed a hypothesis that, in rodents, testosterone of testicular origin is aromatized in the brain to oestradiol, which acts as the principle biologically active molecule responsible for brain sexual differentiation at the perinatal stage in the male

0022–0795/01/0168–455
Online version via http://www.endocrinology.org

(Arnold & Gorski 1984, Lephart 1996). This stage is called the critical period. While the exact mechanisms by which oestrogens control the organization of neural networks during the critical period in the brain are not well understood, it is known that oestrogens modulate prenatal/postnatal neuronal maturation and formation of neural circuits. These actions of oestrogens are thought to be important for the formation of sexual dimorphism observed in some parts of the brain such as the preoptic area, ventromedial hypothalamic nuclei and medial amygdala, all of which are generally recognized as sites involved in the control of sexual behaviour (McEwen *et al.* 1982, Arnold & Gorski 1984, Lephart 1996, Beyer 1999).

We recently generated mice lacking aromatase (ArKO mice) by targeted disruption of *Cyp19*. The female ArKO mice were totally infertile and the size of their uteri and bone density was decreased. These phenotypes are very similar to those seen in ovariectomized mice. The ArKO males showed massive accumulation of fat within the gonadal fat pads and develop hepatic steatosis with aging. Biochemical and histological studies have revealed that the hepatic steatosis is attributable, at least in part, to suppression of hepatic enzyme activities involved in fatty acid β-oxidation (Nemoto *et al.* 2000). Furthermore ArKO males exhibit an impairment in intermale aggressive behaviour (Toda *et al.* 2000, 2001). In the present study, we use ArKO male mice to clarify the physiological roles of oestrogens in male reproduction. We observed that the reproductive ability of the ArKO males was severely impaired, however, this impairment was reversed by supplementation with 17β-oestradiol (E_2) when the treatment was initiated soon after birth.

Materials and Methods

Animals

Animal care and experiments were carried out in accordance with institutional animal care regulations. All animals were maintained on a 12 h light:12 h darkness cycle at 22–25 °C and fed with a standard rodent chow (NMF; Oriental Yeast, Tokyo, Japan) and water *ad libitum*. Mice lacking functional CYP19 due to targeting disruption of *Cyp19* were generated as described (Nemoto *et al.* 2000, Toda *et al.* 2000). Briefly, a targeting vector was designed based on the structure of *Cyp19*, in which an 87 bp fragment, located within exon 9, corresponding to nucleotide position 1124–1210 relative to the translational start site in the mature mRNA (corresponding amino acid residues numbers 375–403) (Terashima *et al.* 1991), was replaced with a neomycin-resistance gene derived from pMC1-neo (Thomas & Capecchi 1987). The deletion of the 87 bp sequence from the gene was verified as inactivating the gene function completely by *in vitro* expression studies. The linearized target vector was electroporated into embryonic stem cells (E14–1) (Takeda *et al.* 1996). Selected embryonic stem cells were then microinjected into C57BL/6J blastocysts to generate chimeric mice. Chimeric male mice were mated with C57BL/6J female mice to generate mice heterozygous for the mutation, which were then inter-crossed to generate mice homozygous for the *Cyp19* mutation (ArKO) (Toda *et al.* 2000). For comparison of phenotypes, wild-type and knockout mice from the same litters were used.

Expression of Cyp19

Total RNA was prepared from testes and brains of wild-type and ArKO mice at 14 weeks of age according to the method of Mirkes (1985). One microgram of each RNA was reverse transcribed with 10 pmol of an oligo dT primer in a final volume of 20 μl using Moloney murine leukaemia virus-reverse transcriptase (Gibco-BRL, Rockville, MD, USA). Subsequent analyses with polymerase chain reaction (PCR) were carried out with 1 μl of the cDNA (equivalent to 0·05 μg total RNA) with a set of primers to amplify fragments containing nucleotide sequences of exons 8 and 9 of *Cyp19* (expected fragment size: 284 bp) (sense 5′-GCAGCTCCTGACACCATGTC-3′; antisense 5′-CTTCCGATGTTCAGAATGATGTT-3′), exons 6 and 7 of *Cyp17* (319 bp) (sense 5′-TGGTGCACAATCCTGAGGTG-3′; antisense 5′-TTGGATCTAAGAAGCGCTCA-3′), and a fragment of transcripts of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (210 bp) (sense 5′-CGGATTTGGTCGTATTGG-3′; antisense 5′-TCCTGGAAGATGGTGATG-3′). Thirty-eight cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 74 °C were used for amplification of the fragments. Portions of the products were analyzed by electrophoresis on 5% polyacrylamide gels. Amplified fragments were verified by determination of the nucleotide sequence following subcloning into the plasmid, pBluescript II SK(−).

Histological examination

Histological studies were carried out using five mice of each genotype. Testes from wild-type and ArKO mice at 14 weeks of age were fixed in 10% buffered formalin solution for 2 days before dehydration and then embedded in paraffin. Samples were cut in sections of 5 μm thickness and stained with haematoxylin and eosin. The thickness of seminiferous epithelium was measured in 30 randomly selected seminiferous tubules cross-sections at stage VII of the spermatogenic cycle for each individual. The data were analyzed statistically with the Mann–Whitney U-test. The results are expressed as the mean thickness ± S.D.

Evaluation of sperm function

Five and nine mice were used for counting the numbers of sperm of wild-type and ArKO males at 9–16 weeks of age

respectively. One of the two epididymides was dissected and used for counting. Sperm in each epididymis were allowed to disperse into 1 ml phosphate-buffered saline. A 1:10 dilution was used for haemocytometric count. Three wild-type C57Bl/6J female mice at 4 weeks of age were injected with pregnant mare serum gonadotrophin (i.p. 5 IU/mouse) and then given human chorionic gonadotrophin (i.p. 5 IU/mouse) 48 h later. Oocyte/cumulus masses were surgically extracted from oviducts 18 h after the second injection. They were then incubated for 5 h at 37 °C with sperm at a concentration of 1–2 × 10^5 sperm/ml, derived from the wild-type or ArKO males (Hogan *et al.* 1994) to evaluate the ability of sperm to fertilize the eggs. In the *in vitro* fertilization assays, sperm were collected from both epididymides from a mouse of each genotype. We repeated this experiment three times and obtained the same results. In one experiment, we used ArKO males at 10 months of age, instead of young males.

E_2 supplementation

Oestradiol (Sigma–Aldrich, Kyoto, Japan; minimum purity 98%) was dissolved in sesame oil. In the first group of mice, subcutaneous injections were initiated on the day of birth with the following amounts of E_2 in a volume of 25 μl: 7·5 ng (n=10 ArKO males), 0·75 μg (n=10), 1·5 μg (n=7), 7·5 μg (n=16) and 15 μg (n=7). The treatment was repeated every fourth day for 3 weeks. The ArKO mice in the control group (n=7) were administered 25 μl sesame oil vehicle. After 3 weeks, the mice that had been injected with 7·5 ng E_2 from birth continued to receive once weekly injections of 7·5 ng E_2, whilst the other experimental groups of mice were injected with 0·75 μg E_2 or vehicle (controls) once a week until the end of the experiments. In the second group of mice, injections of 7·5 μg E_2 were initiated from day 5 (n=12), day 7 (n=10) and day 15 (n=12) after birth and administered every fourth day until the mice were 3 weeks of age after which the mice were treated with 0·75 μg E_2 once a week until the end of the experiments. The third group of ArKO males (n=11) was only supplemented with 7·5 μg E_2 on the day of birth and on postnatal day 4. Analysis was performed using animals at 12–24 weeks of age.

Evaluation of male reproduction

Mice at 16–20 weeks of age were used for the evaluation of reproductive ability. Each wild-type (n=25) or ArKO (n=20) female was mated with a wild-type or heterozygous male. Untreated ArKO (n=28) and ArKO males supplemented with E_2 as described (vehicle (n=7), 7·5 ng E_2 (n=10), 0·75 μg E_2 (n=10), 1·5 μg E_2 (n=7), 7·5 μg E_2 (n=16), and 15 μg E_2 (n=7), 7·5 μg E_2 initiated on day 7 (n=10), day 15 (n=12), and days 1 and 4 (n=11)) were also used for evaluation of reproductive ability. Each male was housed with two females known to be fertile. When males did not sire litters during a continuous mating period of 2 months, they were considered to be infertile. Males that sired litters more than twice were considered to be fertile.

Analysis of mounting behaviour

Behaviour tests were performed in a standard polycarbonate mouse cage (23 × 16 × 13 cm) in a dimly lit room between 1800 and 2000 h. Twelve wild-type and 18 ArKO males at 16–24 weeks of age were used for the analysis. Mounting behaviour was analyzed during a 30-min test with a wild-type C57BL/6J female mouse at 8 weeks of age in the home cage of the male. The females were ovariectomized and injected subcutaneously with 10 μg E_2 (48 h prior to the test) and 500 μg progesterone (4–6 h prior to the test). The number of mounts and latencies to first mount of each male were scored at least twice with a weekly interval.

Statistical analysis

Data were analyzed by Wilcoxon rank-sum test, Wilcox signed rank test, chi-square test or Mann–Whitney U-test. The differences were regarded to be significant when P<0·05.

Results

We first examined the expression of CYP19 mRNA by means of RT-PCR using testicular and brain RNAs prepared from wild-type and ArKO mice. As shown in Fig. 1, the transcripts encompassing nucleotide sequence of exon 8 to exon 9 of *Cyp19* were detected in the cDNAs derived from the testicular as well as the brain RNA of the wild-type mice, but not in those of the ArKO mice. In contrast, comparable amounts of transcripts of *Cyp17* and the GAPDH gene were detected in the testes and brains of both genotypes respectively. Thus, these results established that the full-length transcript of *Cyp19* was absent from the testes and brains of ArKO mice.

The reproductive ability of ArKO mice was analyzed together with wild-type mice and mice heterozygous for the *Cyp19* mutation (Table 1). No apparent fertility problems were observed in the wild-type and heterozygous mice. In contrast, ArKO females were totally infertile and ArKO males showed reduced reproductive ability. We observed that mice homozygous for the mutation were born with an expected ratio of the Mendelian frequency when crossed between heterozygous mice (data not shown). Histological analysis of the testes of wild-type and ArKO mice aged 14 weeks revealed that seminiferous epithelial layers in the testes of ArKO males (thickness at stage VII of the spermatogenesis cycle:

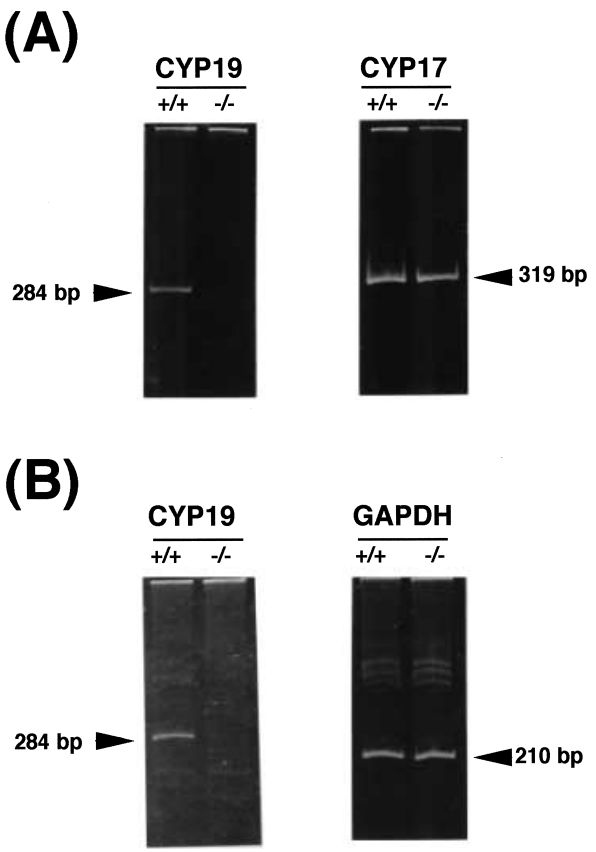

Figure 1 Expression of *Cyp19* in the testes and brains. Total RNA prepared from the testes (A) or brains (B) of wild-type (+/+) or ArKO (−/−) mice was reverse transcribed. A portion of cDNAs was used for PCR amplification of a fragment encompassing exon 8 to exon 9 (284 bp) of *Cyp19*, exon 6 to exon 7 (319 bp) of *Cyp17* or GAPDH (210 bp).

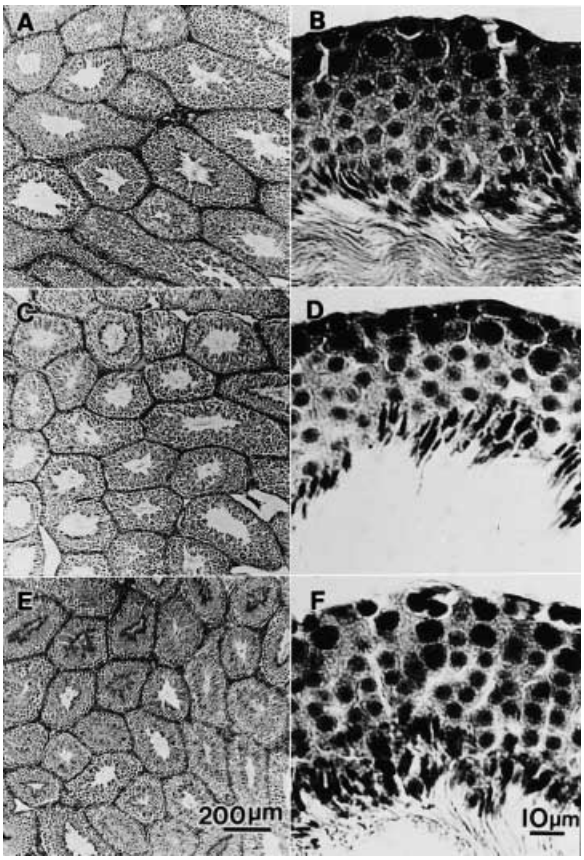

Figure 2 Testicular histology of wild-type and ArKO mice aged 14 weeks. Haematoxylin and eosin staining of the testes from a wild-type mouse (A and B), an ArKO mouse (C and D), and an ArKO mouse treated with 7·5 μg E_2/mouse on the day of birth and on every fourth day thereafter until 3 weeks of age, then the mouse was given 0·75 μg E_2 once a week (E and F). Seminiferous tubule cross-sections at stage VII of the spermatogenic cycle are shown (B, D and F). Thicknesses are (B) 64·4 ± 4·0 μm, (D) 55·1 ± 5·5 μm (P<0·001 vs wild-type male) and (F) 65·2 ± 9·6 μm (P>0·05 vs wild-type male). Bars: 200 μm (A, C and E) and 10 μm (B, D and F). Values are mean thickness ± S.D.

55·1 ± 5·5 μm) were apparently thinner than those of wild-type mice (64·5 ± 4·0 μm, P<0·0001) (Fig. 2). However, we could find no gross difference in the thickness of the seminiferous epithelium at other stages of the spermatogenic cycle in the mice of each genotype examined (n=5). Furthermore, we observed no abnormalities such as atrophic or degenerating seminiferous tubules, or dilation of the seminiferous tubule lumens which were reported in αERKO mice (Eddy *et al.* 1996). We found no statistically significant difference in testicular and epididymal weights, or the numbers of sperm between wild-type and ArKO mice at 9–16 weeks of age as shown in Table 2. We also observed that sperm of both ArKO and wild-type males were functional and able to fertilize eggs when examined using an *in vitro* fertilization assay (Fig. 3). We obtained essentially the same results with ArKO males at 10 months of age in the *in vitro* fertilization assay as well as in the testicular histology to those of the ArKO mice aged 14 weeks (data not shown). It was concluded, therefore, that sperm are concentrated

Table 1 Reproductive ability which was calculated by dividing the number of litters by the number of mice mated and expressed as % fertility

Female	Male	Number of mice mated	Number of litters	% fertility
+/+	+/+	10	10	100
+/+	+/−	15	15	100
−/−	+/+	10	0	0
−/−	+/−	10	0	0
+/−	+/−	16	16	100
+/+	−/−	28	4	14
−/−	−/−	12	0	0

Table 2 Testicular and epididymal weights, and sperm number. One of the testes and caudae epididymides of each mouse were used to evaluate the weights and numbers of sperm respectively. Values are means ± S.E.M.

	Testicular weight	**Epididymal weight**	**Sperm number** (per epididymis)
Wild-type	128 ± 20·2 mg (n=13)	46·2 ± 1·3 mg (n=5)	0·9 ± 0·06 × 10^7 (n=5)
ArKO	119 ± 17·1 mg (n=15)	44·6 ± 5·2 mg (n=9)	1·2 ± 0·6 × 10^7 (n=9)

normally in the epididymis of ArKO males and that their function remains unaffected.

As shown in Fig. 4, the sexual behavioural study of ArKO males revealed an impairment in mounting behaviour. Over 90% of wild-type males (11 of 12) showed mounting against ovariectomized females during the test period (30 min), whereas only 10% (2 of 18) of the ArKO males aged 16–24 weeks showed mounting behaviour (P<0·0001 vs wild-type males). Latency to first mount of wild-type males was 480 ± 231 s, whereas in ArKO males it extended to 670 ± 348 s (P<0·0001 vs wild-type males). To confirm the reduction of mounting frequency in ArKO males, we changed the duration of the test by extending its time in some groups to 60 min. However, neither an increase in the number of mounts nor signs of intromission were observed in ArKO males under such modified conditions. In addition, we did not observe any plug formation during housing of ArKO males with females for 1 week (n=7). These results indicated that the apparent reduction in reproduction of ArKO males was due, at least in part, to the change in mounting behaviour.

We examined the effect of E_2 supplementation on the reproductive ability of ArKO males. As shown in Fig. 5A, when E_2 was administered at a concentration of 7·5 μg/mouse, the reproductive function of ArKO males returned to the levels of wild-type mice (P<0·0001 vs untreated ArKO males). The effect of E_2 was evident at a concentration of 1·5 μg E_2/mouse (6 of 7 males were fertile, P<0·01 vs untreated ArKO males), but only a marginal effect was observed at concentrations less than 0·75 μg E_2/mouse (5 of 10 males were fertile, P>0·05 vs untreated ArKO males). When supplementation was initiated on day 7 after birth, E_2 was also able to support reproduction in ArKO males (P<0·0002 vs untreated ArKO males) (Fig. 5B). However, when it was initiated on day 15 after

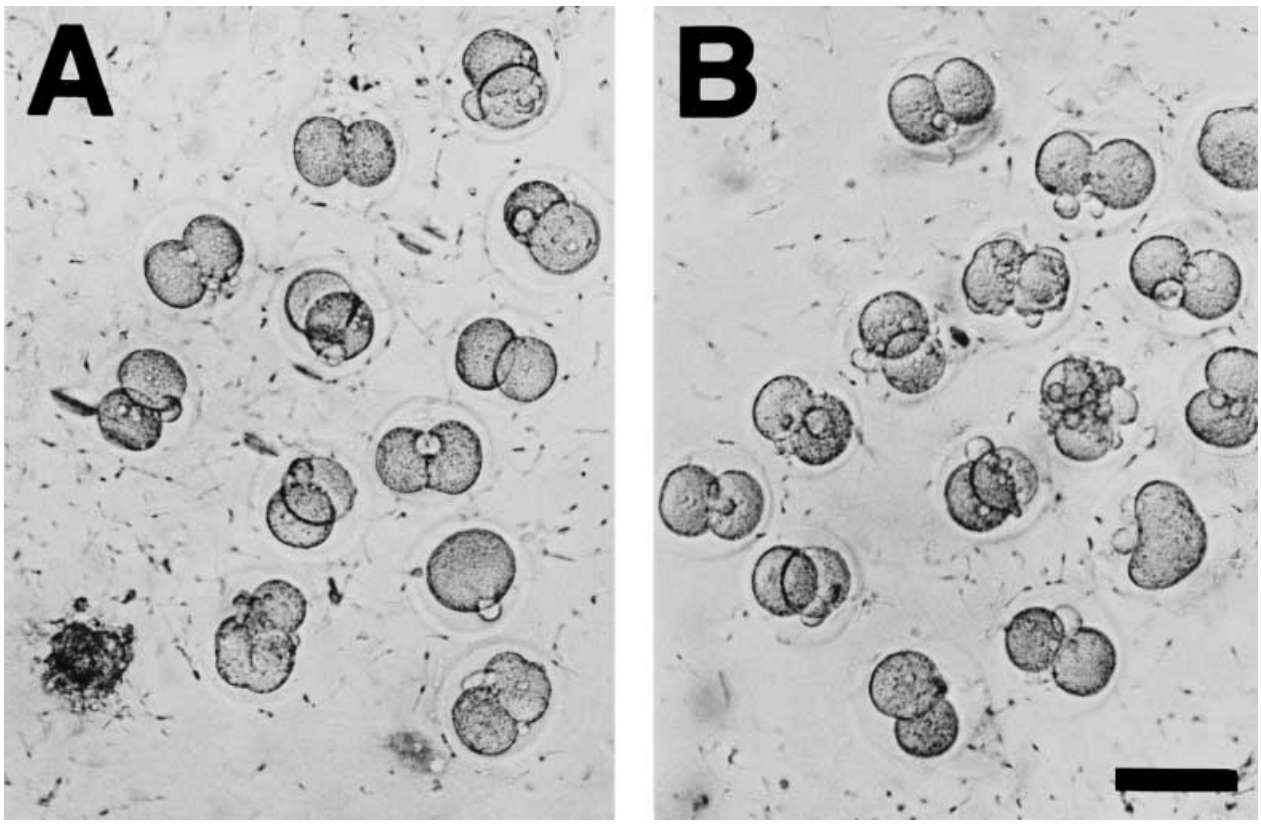

Figure 3 Fertilization with sperm from wild-type and ArKO mice *in vitro*. Eggs of wild-type females were mixed with sperm of wild-type (A) or ArKO (B) mice and incubated at 37 °C for 1 h. Note that after 1 h, polar bodies were observed equally in both cases. Bar: 100 μm.

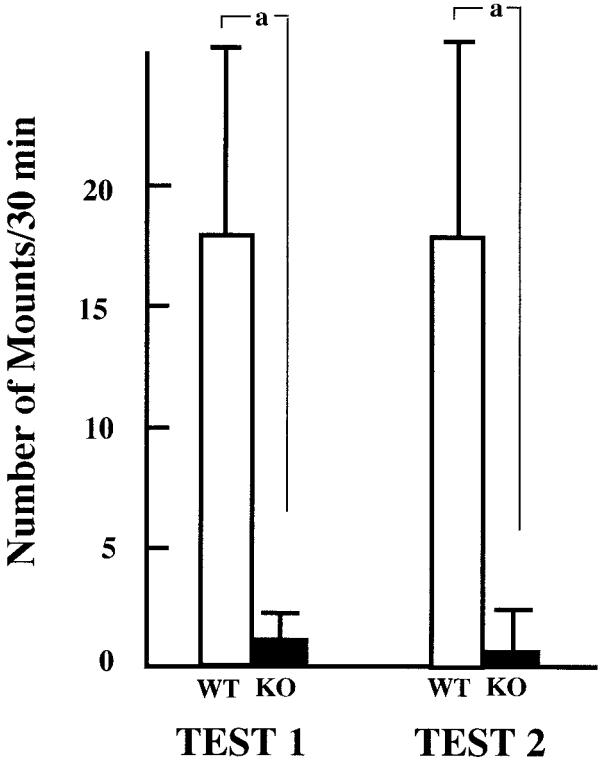

Figure 4 Impairment of mounting behaviour in ArKO males. Mounting behaviour was examined by counting the number of mounts performed by each mouse against a sexually mature female mouse during the test period of 30 min. The mean numbers of mount per wild-type (n=12) and ArKO (n=18) mouse are shown as open and solid bars respectively. Vertical bars represent S.E.M. a, P<0·0001.

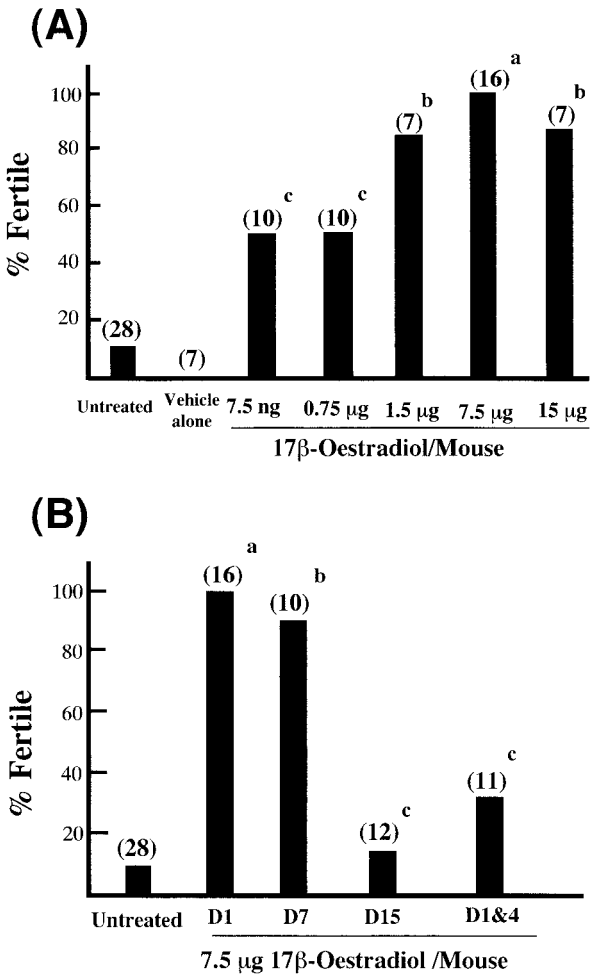

Figure 5 Reversal of the impairment in reproductive ability of ArKO males with E_2 supplementation. (A) Dose-dependent reversal of the reduced reproductive ability of ArKO males. Mice (numbers shown in parentheses) were supplemented with various amounts of E_2 as indicated on the day of birth. The supplementation was repeated every fourth day until day 21, and then followed by weekly injections of 0·75 μg E_2/mouse until the end of the experiments, except for the mice given 7·5 ng E_2, which received weekly injection of 7·5 ng E_2 after day 21 until the end of the experiments. Vehicle alone indicates a group of ArKO males injected with 25 μl sesame oil in the same fashion. Untreated indicates a group of ArKO males that did not receive any injection. a, P<0·0001; b, P<0·01; c, P>0·05 vs untreated ArKO males. (B) Existence of sensitive periods for reversal of the reduced reproductive ability by E_2. ArKO mice were supplemented with 7·5 μg E_2 on the day after birth as indicated (D1, D7, D15 and D1&4). The supplementation was then repeated as above. D1&4 indicates ArKO males treated with 7·5 μg E_2 only on the day of birth and on postnatal day 4. When these mice were 16–20 weeks of age, they were housed with wild-type females. The ArKO males supplemented with E_2 from day 1 are the same mice as those described in (A). The numbers in parentheses indicate the number of mice examined. Improvement of reduced reproductive ability of ArKO males was calculated by dividing the number of mice siring litters more than twice with the total number of mice examined and expressed as % fertility. a, P<0·0001; b, P<0·0002; c, P>0·05 vs untreated ArKO males.

birth, the effects of E_2 were negligible (P>0·05 vs untreated ArKO males). In contrast, 80% of ArKO males (9 of 11) given E_2 on the day of birth and on postnatal day 4 were fertile, although only four mice were able to sire offspring more than twice. Histological analysis demonstrated that supplementation with 7·5 μg E_2/mouse resulted in an increased thickness of seminiferous epithelial layers compared with that in the untreated ArKO males (65·2 ± 9·6 μm) (Fig. 2E and F, P<0·0001 vs untreated ArKO males).

Discussion

We generated ArKO mice in which an 87 bp fragment in exon 9 of *Cyp19* was replaced with the neomycin-resistance gene to disrupt the gene function. Until now, three kinds of ArKO mice have been generated independently. These characteristic phenotypes are presented in Table 3. On the whole, the phenotypes of these mice appear to be similar; however, differences have been

Table 3 Comparison of phenotypes of ArKO mice generated

	Fisher *et al.*	**Honda *et al.***	**Toda *et al.***
Disrupted sites in the gene	Exon IX	Exon I and II	Exon IX
Name of ES cells used (mouse strain from which the ES cells are derived)	KG (129/sv)	CCE (129/sv)	E14-1 (129/sv)
Host blastocyst	C57BL/6J	C57BL/6	C57BL/6J
External phenotype	Coats are dull at 3 weeks of age; No development of labial folds in the females	Indistinguishable from wild-type	Indistinguishable from wild-type
Accumulation of abdominal fat	Yes	Yes	Yes
Fertility			
Female	Infertile	Infertile	Infertile
Male	Fertile until 14 weeks of age, but infertile at 7 months of age ($n=3$)	1 out of 10 mice is fertile (14–17 weeks of age)	4 out of 28 mice are fertile (10–18 weeks of age)
	Spermatogenesis: At 12–14 weeks of age; normal At 4·5 months of age; 3 of 4 are normal and one has no sperm At 1 year of age; a general reduction in the number of sperm in the cauda epididymis	Spermatogenesis: At 12–16 weeks of age; active sperm in the testis and epididymis	Spermatogenesis: At 9–16 weeks of age; normal in the number and function in the cauda epididymis ($n=9$)
Behaviour	N.D.	Reduction of mounting behaviour against females	Reduction of mounting behaviour against females
References	Fisher *et al.* (1998) Robertson *et al.* (1999)	Honda *et al.* (1998)	This study

N.D., not described; ES cells, embryonic stem cells.

reported with respect to male reproductive ability. The reason for these differences is not clear. As suggested previously (Robertson *et al.* 1999), the difference in composition of the diet might be one of the reasons. Not all ArKO males generated by us and by others (Honda *et al.* 1998) are infertile. This heterogeneous phenotype is also unaccountable at the moment. Oestrogens supplied maternally during gestational as well as lactational periods are possible sources that might affect the development of the male reproductive system, especially in the case of pups unable to synthesize oestrogens by themselves. In addition, oestrogens produced by wild-type or heterozygous littermates might also influence ArKO males *in utero*. Another possibility which may explain the differences in male reproductive function between the various ArKO models is the potential for compensation for the lack of E_2 by other steroid metabolites. It was reported that some of the ArKO mice had elevated testosterone and androstenedione levels (Fisher *et al.* 1998, Robertson *et al.* 1999). Elevation in serum testosterone levels in ArKO male aged 12–20 weeks was also observed in the present study ($2{\cdot}63 \pm 0{\cdot}7$ ng/ml for wild-type males ($n=5$) and $5{\cdot}0 \pm 1{\cdot}2$ ng/ml for ArKO males ($n=5$), means ± S.D.).

In the present study, we have demonstrated that the suppression of normal mating behaviour in male ArKO mice can be ameliorated by E_2 supplementation when it is initiated within 7 days after birth. The histological study on the testes of ArKO males revealed that E_2 supplementation resulted in an increase in the thickness of seminiferous epithelial cell layers in ArKO mice. The effects of oestrogens on the seminiferous epithelium were recently reported using a hypogonadal mouse, in which oestrogen was shown to increase the total volume of the seminiferous epithelium as well as the total seminiferous tubule volume in addition to the induction of spermatogenesis (Ebling *et al.* 2000). Furthermore, E_2 at physiological concentrations has been demonstrated to effectively inhibit apoptosis of human testicular germ cells *in vitro* (Pentikäinen *et al.* 2000). Additional evidence supporting the relative importance of oestrogenic actions in the testis was obtained from the studies on αERKO mice, which showed a defect in spermatogenesis caused, in part, by

impaired fluid reabsorption in the efferent ductules (Eddy *et al.* 1996, Hess *et al.* 1997). Nevertheless, in the present study, the disruption of *Cyp19* did not result in any obvious alteration of testicular function. In addition, atrophy of the seminiferous epithelium in the testes of ArKO mice aged up to 14 weeks was not observed in this study or in a previous study (Robertson *et al.* 1999) as was reported in αERKO mice. These findings suggest that ERα regulates the expression of some genes which are essential for testicular function in an oestrogen-independent manner or that oestrogens or oestrogenic ligands are produced by some unidentified gene products other than *Cyp19* in ArKO mice as suggested by the study on αβERKO mice (Couse *et al.* 1999).

Whilst testicular function appeared normal in the ArKO males, a severe impairment in sexual behaviour was observed when mounting behaviour against sexually mature females was examined. This is consistent with a study reported recently (Honda *et al.* 1998). Thus, the normal development of the nervous system, which is known to be sensitive to oestrogen during neonatal life (Arnold & Gorski 1984, Lephart 1996), is critical for male reproduction. Whilst the E_2 supplementation reversed the inability of ArKO males to achieve impregnation, only marginal effects of the treatment were observed on the mounting behaviour of ArKO males when examined under experimental conditions employed (data not shown). These observations might imply that we need to supplement ArKO mice with E_2 during the prenatal stage to reverse sexual behaviour, because the highest aromatase activity in the brain is observed 2 to 3 days before birth in rodents (Lephart 1996).

Studies on the sexual behaviour of αERKO males have demonstrated that the males showed normal mounting behaviour but were impaired in the induction of ejaculation (Couse & Korach 1999). This suggests that E_2 is required for mounting behaviour in an ERα-independent manner. Nevertheless, the present findings support the view that oestrogens are required during the perinatal period (critical period) to irreversibly organize the neural circuits essential for control of reproductive activity in males.

In conclusion, the ArKO mouse model is a useful tool with which to study the molecular nature of the critical period during which oestrogens regulate the formation of neural circuitry related to the control of sexual behaviour.

Acknowledgements

This work was supported in part by research grants provided by the Ono Medical Research Foundation, and Toyota High-Tech Research Grant Program (to K T).

References

Arnold AP & Gorski RA 1984 Gonadal steroid induction of structural sex differences in the central nervous system. *Annual Review of Neuroscience* **7** 413–442.

Beyer C 1999 Estrogen and the developing mammalian brain. *Anatomy and Embryology* **199** 379–390.

Beyer C, Morali G, Naftolin F, Larsson K & Perez-Palacios 1976 Effects of some antiestrogens and aromatase inhibitors on androgen induced sexual behavior in castrated male rats. *Hormones and Behavior* **7** 353–363.

Booth JE 1978 Effects of the aromatization inhibitor androst-4-ene-3,6,17-trione on sexual differentiation induced by testosterone in the neonatally castrated rat. *Journal of Endocrinology* **79** 69–76.

Christensen LW & Clemens LG 1975 Blockade of testosterone-induced mounting behavior in the male rat with intracranial application of the aromatase inhibitor androst-1,4,6-triene-3,17-dione. *Endocrinology* **97** 1545–1551.

Couse JF & Korach KS 1999 Estrogen receptor null mice: What have we learned and where will they lead us? *Endocrine Reviews* **20** 358–417.

Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, Davis BJ & Korach KS 1999 Postnatal sex reversal of the ovaries in mice lacking estrogen receptors α and β. *Science* **286** 2328–2331.

Davidson JM 1966 Activation of male rat's sexual behavior by intracerebral implantation of androgen. *Endocrinology* **79** 783–794.

Davis PG & Barfield RJ 1979 Activation of masculine sexual behaviour by intracranial estradiol benzoate implants in male rats. *Neuroendocrinology* **28** 217–227.

Ebling FJP, Brooks AN, Cronin AS, Ford H & Kerr JB 2000 Estrogenic induction of spermatogenesis in the hypogonadal mouse. *Endocrinology* **141** 2861–2869.

Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB & Korach KS 1996 Targeting disruption of the estrogen receptor gene in male mice causes alternation of spermatogenesis and infertility. *Endocrinology* **137** 4796–4805.

Fisher CR, Graves KH, Parlow AF & Simpson ER 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp 19* gene. *PNAS* **95** 6965–6970.

Hess RA, Bunick D, Lee K-H, Bahr J, Taylor JA, Korach KS & Lubahn DB 1997 A role for oestrogens in the male reproductive system. *Nature* **390** 509–512.

Hogan B, Beddington R, Costantini F & Lacy E 1994 Manipulating the mouse embryo. *A Laboratory Manual*, edn 2. Cold Harbor: Cold Harbor Laboratory Press.

Honda S, Harada N, Ito S, Takagi Y & Maeda S 1998 Disruption of sexual behaviour in male aromatase-deficient mice lacking exons 1 and 2 of the *cyp 19* gene. *Biochemical and Biophysical Research Communications* **252** 445–449.

Lephart ED 1996 A review of brain aromatase cytochrome P450. *Brain Research Reviews* **22** 1–26.

McDonald P, Beyer C, Newton F, Brien B, Baker R, Tan HS, Sampson C, Kitching P, Greenhill R & Pritchard D 1970 Failure of 5α-dihydrotestosterone to initiate sexual behaviour in the castrated male rat. *Nature* **227** 964–965.

McEwen BS, Biegon A, Davis PG, Krey LC, Luine VN, McGinnis MY, Paden CM, Parsons B & Rainbow TC 1982 Steroid hormones: humoral signals which alter brain cell properties and functions. *Recent Progress in Hormone Research* **38** 41–92.

McLachlan JA, Newbold RR & Bullock B 1975 Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. *Science* **190** 991–992.

Mirkes PE 1985 Simultaneous banding of rat embryo DNA, RNA, and protein in cesium trifluoroacetate gradients. *Analytical Biochemistry* **148** 376–383.

Nemoto Y, Toda K, Ono M, Fujikawa-Adachi K, Saibara T, Onishi S, Enzan H, Okada T & Shizuta Y 2000 Altered expression of fatty acid-metabolizing enzymes in aromatase-deficient mice. *Journal of Clinical Investigation* **105** 1819–1825.

Newbold RR, Bullock BC & McLachlan JA 1986 Adenocarcinoma of the rete testis: diethylstilbestrol-induced lesions of the mouse rete testis. *American Journal of Pathology* **125** 625–628.

Ogawa S, Lubahn DB, Korach KS & Phaff DW 1997 Behavioral effects of estrogen receptor gene disruption in male mice. *PNAS* **94** 1476–1481.

Ogawa S, Washburn TF, Taylor J, Lubahn DB, Korach KS & Phaff DW 1998 Modifications of testosterone-dependent behaviors by estrogen receptor – a gene disruption in male mice. *Endocrinology* **139** 5058–5069.

Pentikäinen V, Erkkilä K, Soumalainen L, Parvinen M & Dunkel L 2000 Estradiol acts as a germ cell survival factor in the human testis *in vitro*. *Journal of Clinical Endocrinology and Metabolism* **85** 2057–2067.

Robertson KM, O'Donnell L, Jones MEE, Meachem JS, Boon WC, Fisher CR, Graves KI, McLachlan RI & Simpson ER 1999 Impairment of spermatogenesis in mice lacking a functional aromatase (*cyp 19*) gene. *PNAS* **96** 7986–7991.

Sharpe RM 1998 The roles of oestrogen in the male. *Trends in Endocrinology and Metabolism* **9** 371–377.

Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, Mendelson CR & Bulun SE 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocrine Reviews* **15** 342–355.

Stone CP 1939 Copulatory activity in adult male rat following castration and injection of testosterone propionate. *Endocrinology* **24** 165–174.

Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, Nakanishi K, Yoshida N, Kishimoto T & Akira S 1996 Essential role of Stat 6 in IL-4 signalling. *Nature* **380** 627–630.

Terashima M, Toda K, Kawamoto T, Kuribayashi I, Ogawa Y, Maeda T & Shizuta Y 1991 Isolation of a full-length cDNA encoding mouse aromatase P450. *Archives of Biochemistry and Biophysics* **285** 231–237.

Thomas KR & Capecchi MR 1987 Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51** 503–512.

Toda K, Okada S, Nakamura K, Nishihara M, Yokotani K & Shizuta Y 2000 Concentrations of monoamines and acetylcholine in the brains of mice lacking the aromatase cytochrome P450 gene. In *Molecular Steroidgenesis*, Frontiers Science Series No. 29, pp 141–143. Eds M Okamoto, Y Ishimura & H Newada. Tokyo: Universal Academic Press Inc.

Toda K, Saibara T, Okada T, Onishi S & Shizuta Y 2001 A loss of aggressive behaviour and its reinstatement by oestrogen in mice lacking the aromatase (*CYP19*) gene. *Journal of Endocrinology* **168** 217–220.

Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ Jr, Juegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Mueller J, DeMeyts ER, Scheike T, Sharpe R, Sumpter J & Skakkebaek NE 1996 Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives* **104** 741–803.

Vagell ME & McGinnis MY 1997 The roles of aromatization in the restoration of male rat reproductive behavior. *Journal of Neuroendocrinology* **9** 415–421.

Wersinger SR, Sannen K, Villalba C, Lubahn DB, Rissman EF & De Vries GJ 1997 Masculine sexual behaviour is disrupted in male and female mice lacking a functional estrogen receptor α gene. *Hormones and Behavior* **32** 176–183.

Yamada K, Harada N, Tamaru M & Takagi Y 1993 Effects of changes in gonadal hormones on the amount of aromatase messenger RNA in mouse brain diencephalon. *Biochemical and Biophysical Research Communications* **195** 462–468.

Received 3 October 2000
Accepted 10 November 2000