A loss of aggressive behaviour and its reinstatement by oestrogen in mice lacking the aromatase gene (*Cyp19*)

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Abstract

Aromatase P450 (CYP19) is an enzyme responsible for conversion of androgens to oestrogens. We generated CYP19 knockout (ArKO) mice by targeting disruption of the CYP19 gene and observed that the ArKO males exhibited a complete loss of aggressive behaviour against intruder mice when examined using a resident–intruder paradigm. The defect in the behaviour of ArKO males was reinstated when the mice received supplements of 17β-oestradiol soon after birth. Nevertheless, the cumulative duration of the behaviour displayed by the treated mice during the test period of 15 min was 19 ± 10 s, which was much shorter than that displayed by wild-type males, 90 ± 17 s. When the supplementation was started at 7 days after birth, the defect was not restored. These findings illustrate an absolute requirement for oestrogen during the neonatal stage of a male's life for the development of the potential for aggression observed in adulthood. Furthermore, the present study demonstrates that ArKO males are a useful model in which to investigate the neural mechanisms by which aggressive behaviour is controlled.
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Introduction

Aggression of male mice against other males is a form of social behaviour in which adult males fight to establish dominance relationships (Moyer 1976). Removal of testicular testosterone by castration results in a decrease in aggression as well as a loss of dominance (Barfield *et al.* 1972, Albert *et al.* 1986*b*). Furthermore, replacement by daily testosterone injections or by subcutaneous implants of testosterone-filled silastic capsules to castrated animals reinstates the intermale aggression (Albert *et al.* 1987*a*, Brain & Haug 1992, Monteica-Heino *et al.* 1993). These findings strongly indicated that testosterone plays an essential role in facilitating the display of intermale aggressive behaviour. A number of studies documented that the medial preoptic area (Albert *et al.* 1986*a*) and/or the medial hypothalamus (Albert *et al.* 1987*b*) is the site containing the testosterone-sensitive neural circuitry which modulates intermale aggression, because lesions in those brain areas resulted in suppression of intermale aggression.

While testosterone undoubtedly plays an important role in aggressive behaviour, castration–replacement studies in mice have shown that oestrogen-sensitive regulatory pathways, which are distinct from androgen-sensitive pathways, also participate in the promotion of intermale aggression (Cologer-Clifford *et al.* 1999). The importance of the oestrogens/oestrogen receptor (ER) signalling pathway in intermale aggression was also reported in a study on mice lacking one of the ERs, ERα (ERα knockout mice, αERKO), in which males rarely displayed aggression against olfactory bulbectomised wild-type males (Ogawa *et al.* 1997, 1998). Furthermore, higher activity of aromatase, an enzyme responsible for the conversion of androgen to oestrogen (Simpson *et al.* 1994), was detected in the amygdala of more aggressive mice during early ontogeny (Compaan *et al.* 1994). Participation of oestrogen in aggressive behaviour was also implicated in other vertebrates such as song birds, showing that inhibition of aromatase activity abolishes male aggressive behaviour during the non-breeding season (Soma *et al.* 2000).

Recently, we generated mice lacking aromatase (aromatase knockout (ArKO) mice) by targeted disruption of the aromatase P450 gene (*Cyp19*). Female ArKO mice are totally infertile and show features similar to those seen in ovariectomised mice, such as diminution in size of the uteri and decreased density of bones. In ArKO males, we observed a reduced reproductive ability and the development of hepatic steatosis, which is attributable, at least in part, to down-regulation of enzymatic activities involved in fatty-acid β-oxidation reactions in hepatocytes (Nemoto *et al.* 2000).

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In the present study, we investigated ethological aspects of oestrogen actions by analysing aggressive behaviour of ArKO mice using a resident–intruder test. In addition, we examined the effects of supplementation with 17β-oestradiol (E_2) on this behaviour. We found that the disruption of *Cyp19* resulted in a complete loss of aggressive behaviour against an intruding male, and that the loss was effectively reversed by E_2 supplementation when it was initiated within three days after birth at a relatively high dose.

Materials and Methods

Animals

All animals were maintained on a 12 h light/darkness cycle at 22 °C–25 °C. A standard rodent chow (NMF; Oriental Yeast, Tokyo, Japan) and water were available *ad libitum*. For comparison of phenotypes, wild-type and knockout mice from the same litters were used. Animal care and experiments were carried out in accordance with institutional animal regulations. We took special care to avoid any animals being injured during the intermale aggression experiments.

E_2 supplementation

E_2 was dissolved in sesame oil. The schedule for E_2 supplementation was determined empirically as follows. A group of mice received subcutaneous injections 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=9), and 15 μg (n=7). The injections were carried out every fourth day until day 21 after birth. Twenty-five microlitres of sesame oil were injected into mice as controls (n=7). Then, mice received weekly injections of 0·75 μg E_2 (experimental) or vehicle alone (control) until the end of the experiments. Mice that received 7·5 ng E_2 were also given weekly injections of 7·5 ng E_2 after day 21 following birth. In another group of mice, the injections of 7·5 μg E_2 were initiated on day 3 (n=13), day 5 (n=14), day 7 (n=10) or day 15 (n=12) after birth. The injections were repeated every fourth day until day 21 after birth. Thereafter, they received weekly injections of 0·75 μg E_2 until the end of the experiments. The third group of males (n=10) was supplemented with 7·5 μg E_2 on the day of birth and on day 4 after birth without any other E_2 injections. Analyses were performed on animals at 12–16 weeks of age.

Analysis of intermale aggression

The aggressive behavioural test was performed in a standard polycarbonate mouse cage (23 × 16 × 13 cm) in a dimly-lit room between 1800 and 2000 h. A resident–intruder test was employed to evaluate the intermale aggression (Ogawa *et al.* 1998). Wild-type and ArKO males kept individually for two weeks prior to the test were used as residents. ArKO or wild-type mice, housed in a group, were used as intruders. Since ArKO males were not aggressive towards wild-type males and did not themselves initiate any fights, they were used as intruders when examining the effects of E_2 supplementation on ArKO males. An intruder was transferred to the home cage of a resident and the behaviour was tape-recorded for 15 min. The cumulative duration of aggressive behaviour such as wrestling, biting attacks, lateral threats, and tail rattling was determined. The effects of E_2 on intermale aggression of ArKO mice were evaluated by counting the number of mice showing aggressive behaviour towards intruders during the test.

Statistical analysis

Data were analysed by the Kruskal–Wallis test for multiple comparisons. The P value obtained was <0·0001 (see Figs 1 and 2).

Results and Discussion

Wild-type male mice showed aggressive behaviour against an intruder mouse under the experimental conditions as shown in Fig. 1. The cumulative durations of the attacks were about 2·5 min and 1·5 min against wild-type and ArKO intruder mice respectively. In contrast, ArKO males did not show such behaviour.

When neonatal ArKO males were given E_2 at a concentration of 7·5 μg/mouse or more, the mice showed aggression against an intruder as they grew to 12–16 weeks of age. Although the treatment apparently restored male aggressive behaviour, the cumulative duration of the behaviour displayed by the treated mice was 19 ± 10 s (n=9). This duration was significantly shorter than that of the wild-type mice (90 ± 17 s), indicating that the conditons for E_2 supplementation employed in the present study were suboptimal. Indeed, the highest levels of aromatase activity in the brain are detected during the prenatal stage in rodents, approximately two to three days before birth (Lephart 1996). Thus, we assumed that a more profound effect of E_2 on aggressive behaviour would be expected if we administered E_2 during the prenatal stage.

Supplementation with reduced amounts of E_2 exerted marginal effects on aggressive behaviour (Fig. 2A). The efficiency of restoration of aggressive behaviour by E_2 depended on the time when the supplementation was initiated after birth (Fig. 2B). When it was initiated at 7 days after birth, only three of ten mice showed aggression and when initiated at 15 days after birth, no mice exhibited aggression. It has previously been reported that

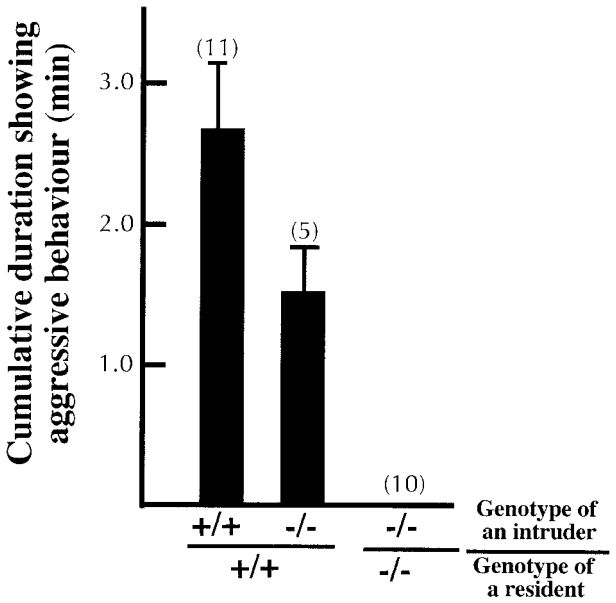

Figure 1 Loss of aggression in ArKO mice. Cumulative duration of aggressive behaviour was scored by means of a resident–intruder test. Wild-type mice (+/+) were examined with a group-housed wild-type (+/+) or ArKO (−/−) mouse. ArKO mice (−/−) were examined only with an ArKO (−/−) mouse. Numerals in parentheses are the number of mice examined. $P<0{\cdot}0001$ (by the Kruskal–Wallis test).

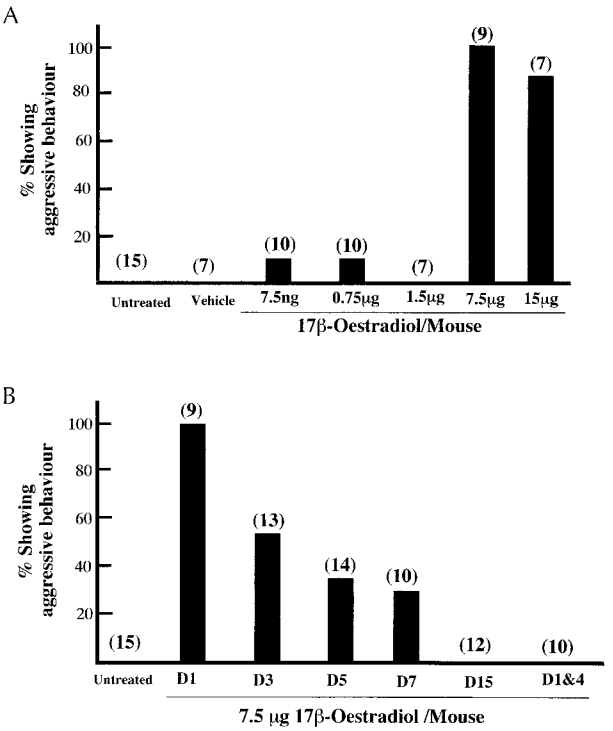

Figure 2 Reinstatement of the aggressive behaviour of ArKO mice with E_2. (A) Dose-dependency of the reinstatement. Mice were supplemented on the day of birth with various amounts of E_2 as indicated. The supplementation was repeated every fourth day until day 21, and was followed by weekly injections of 0·75 μg E_2/mouse until the experiment ended. 'Vehicle' indicates a group of ArKO males injected with 25 μl sesame oil in a similar fashion. 'Untreated' indicates a group of ArKO males that did not receive any injection. (B) ArKO mice were supplemented with 7·5 μg E_2 per mouse on day 1, 3, 5, 7 or 15 after birth (as indicated by D1, D3, D5, D7 or D15). Then the supplementation was repeated as described above. The mice in the group shown by D1&4 were given E_2 only on days 1 and 4 after birth. The untreated mice and mice treated from day 1 are the same as those used in the experiments for examining the dose-dependency. The numerals in parentheses over each bar indicate the numbers of mice examined. The reinstatement rate was calculated by dividing the number of mice showing aggressive behaviour against an intruder by the number of mice examined and was expressed as a percentage. $P<0{\cdot}0001$ (by the Kruskal–Wallis test).

male mice castrated on the day of birth are less aggressive than males castrated 10 days later when both are given androgens as adults and tested for aggression (Edwards 1969, Peters *et al.* 1972, Monteica-Heino *et al.* 1993). These findings indicate that endogenous testicular androgens have their greatest effect on the organisation of mechanisms for aggressive behaviour during the first few days after birth (Monteica-Heino *et al.* 1993). The findings of the present study support the importance of the first few days after birth for hormonal stimuli that trigger the process for development of the potential for adult aggressive behaviour. Nevertheless, the present studies demonstrate that the functional molecule needed to regulate the process appears to be oestrogen rather than testicular androgen.

Mice supplemented with E_2 only twice during the neonatal stage, namely on the day of birth and on day 4 after birth, did not show aggression. These results indicate that E_2 might be required continuously from birth. Alternatively, there might be other critical periods, in addition to the neonatal stage, at which E_2 plays an essential role in the development of the potential for adult intermale aggression. The requirement for steroid hormones during a stage of life other than the perinatal period was suggested by the studies of castration–replacement experiments, where the perinatal surge in plasma testosterone was interpreted as providing an initial stimulus for triggering the postnatal process essential for adult aggressive behaviour, rather than making a unique contribution (Monteica-Heino *et al.* 1993.). A loss of aggression was also reported in αERKO (Ogawa *et al.* 1997, 1998), but not in βERKO mice (Krege *et al.* 1998). This suggests that oestrogens regulate male aggressive behaviour through actions of ERα in the brain.

Mice generated by disruption of genes encoding monoamine oxidase A (Cases *et al.* 1995), adenosine receptor type A_{2a} (Ledent *et al.* 1997), 5-hydroxytryptamine receptor 1B (Ramboz *et al.* 1996), neuronal nitric-oxide synthase (Nelson *et al.* 1995) or

α-calcium-calmodulin-kinase II (Chen *et al.* 1994) exhibited more aggressive behaviour than their wild-type littermates. Thus, expression of one or several of these genes might be regulated either directly or indirectly in an oestrogen-dependent manner in the brain. Chemosensory perception unquestionably plays an important role in intermale aggression in mice (Bean 1982, Clancy *et al.* 1984) and bilateral removal of the olfactory bulbs eliminates intermale fighting (Edwards *et al.* 1993). Thus, it is also possible that not only the central nervous system, but also the olfactory system, including the sensors for pheromones, might be restored by E_2 supplementation. Whatever the sites of actions of E_2, the experimental conditions established in the present study highlight a method for the search and characterisation of genes playing important roles in aggressive behaviour.

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