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Neurological effects of aromatase deficiency in the mouse☆

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Abstract

In the brain, the conversion from androgen into estrogen is an important process for the differentiation of the brain function in male rodents. The aromatase is expressed in some nucleus of the brain. To assess the functional significance of the aromatase gene in development and activation of sex-specific behavior, we analyzed behavioral phenotypes of the aromatase knockout (ArKO) male mice. ArKO males obviously decreased their fertility and showed deficits in male sexual behavior including mount, intromission and ejaculation. Noncontact penile erection was not significantly affected by defect of the aromatase gene. A reduction of aggressive behavior against male intruders was also observed in ArKO males, while they tend to exhibit aggression toward estrous females during male copulatory tests. Moreover, the infanticide toward the pups was observed in the ArKO males, whereas characteristic parental behavior, but not infanticide was observed in wild-type males. These results indicate that aromatase gene expression is a critical step not only for motivational and consummatory aspects of male sexual behavior, but also for aggressive and parental behaviors in male mice.

Keywords: Estrogen; Brain; Sexual differentiation; Cytochrome P450; Knockout mouse

1. Introduction

Aromatase (the *cyp19* gene product) playing an important role in reproductive processes is mainly expressed in gonadal tissues such as ovary [1], testis [2]. The enzyme is also localized in extra-gonadal tissues such as brain [3], placenta [4], skin fibroblasts [5], bone [6], adipose [7] and vascular tissues [8], where it is involved in various physiological functions through paracrine or autocrine rather than endocrine actions.

Testosterone secreted from the testes reaches the brain as a prohormone [9,10], and exerts its effects mainly via conversion to estrogen in the neural tissues in many male mammals [11,12]. This local conversion to estrogen is catalyzed by aromatase, which is highly expressed in specific brain regions including the hypothalamus and limbic areas during the period of development [13,14] and adulthood [15,16]. Therefore, aromatization is considered an important process of physiological action of testicular androgens on the brain [17–20]. This aromatizaton hypothesis was first proposed by Naftolin et al. [3,21] and is now thought to be responsible for the organization of neural circuitry underlying male sexual behavior during the limited period of time when sexual differentiation occurs in rodents. Later in adult life, aromatase and its inductive estrogen are also required for full activation of male copulatory behavior.

Current generation of estrogen deficiency models in mice with targeted disruption of the aromatase [22–24] or the estrogen receptor (ER) gene provided insight into the role of estrogen in male reproductive physiology and behavior. A detailed analysis of ERα [25–28], ERβ [29] and ERαβ [30] knockout (αERKO, βERKO or αβERKO) mice has revealed that ERs play essential roles in male sexual behavior as well as aggressive and parental behaviors in male mice. In humans, several cases of mutations of the gene encoding aromatase have been reported [31–36]. Recently, Carani et al. [37] showed that male sexual behavior in man with aromatase deficiency was improved after treatment with estrogen, suggesting that aromatase and estrogen also have roles in male sexual activity in humans.

Aromatase knockout (ArKO) mouse, a new model of the study for estrogen function, provides critical findings of contribution of estrogen to sexual behavior [22,38–41]. In our initial experiments, a great reduction of mounting activity was observed in ArKO male mice lacking exons 1 and 2 of the *cyp19* gene [22]. However, the precise role of aromatase not only on the consummatory aspect of copulatory behavior, but also on aggressive and parental behaviors has not been evaluated. In these experiments, to understand the functional role of aromatase on male-specific behaviors, a series of tests for male sexual behavior, noncontact erection,

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aggressive behavior and parental behavior were performed in gonadally intact ArKO male mice.

2. Materials and methods

2.1. Animals and serum steroids concentration

Gonadally intact male ArKO and wild-type mice from a mixed percentage of C57BL/6 and 129/SvEv strains were used. ArKO mice were prepared by pairing heterozygous mutant animals which were generated by targeted disruption of the aromatase gene. The offspring was genotyped by PCR analysis of tail DNA with three kinds of primers as follows: an antisense primer (AromR-primer; 5′-TTACCATGTCCTAATCTTCAC-3′) and a sense primer (AromF-primer; 5′-CTTGTCTAAGTGTCCAATCAC-3′) for the mouse aromatase gene (504 bp product), and an antisense primer (Neo-primer; 5′-TAAAGCGCATGCTCCAGACT-3′) for the neomycin-resistant gene (180 bp product). The wild-type allele of the aromatase gene was amplified by PCR using the AromF-primer and the AromR-primer, whereas the mutated allele was amplified by PCR using the Neo-primer and the AromR-primer. PCR was carried out for 30 cycles (94 °C, 40 s; 58 °C, 40 s; 72 °C, 30 s) with 40–50 ng of genomic DNA. PCR products were separated on 1.5% NuSieve 3:1 agarose (TAKARA, Kyoto). Mice were singly housed with controlled photoperiod (light/dark 12 h:12 h, lights off at 18:00 h) and temperature (22–24 °C) throughout the tests. Food and water were given ad libitum. Behavioral tests were conducted during the dark phase of the light/dark cycle starting 2 h after lights off except for parental behavior test. The time schedule of the behavioral tests examined are indicated in Fig. 1.

The present study was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

The concentrations of the serum steroid hormones were determined by RIA at Mitsubishi Kagaku Bio-Clinical Laboratories, Inc. Wild and ArKO mice of 9–15 weeks of age were used for the serum steroid assays.

2.2. Male fertility test

To evaluate male fertility, 8-week-old ArKO ($n = 11$) and wild-type ($n =$) males were cohabited with normal female mice (C57BL/6) for 4 weeks in the male's home cage. Only female mice that had showed more than two regular estrous cycles were used as partners. Cages were monitored daily for an additional 25 days and the number of litters was recorded.

2.3. Male sexual behavior test

Male mice were tested twice for sexual behavior for during a 30 min test with estrous female mice (C57BL/6) in the male's home cage. As stimuli, the female mice were ovariectomized and injected with estradiol benzoate (30 and 15 μg at 48 and 24 h before the tests, respectively) and progesterone (500 μg at 4–7 h before the tests) and only females that showed lordosis in response to the stud male's mounting were subjected to the experiment. The measures of male sexual behavior were recorded as follows: frequency of mount (MF), intromissive behavior (IF) and ejaculatory behavior (EF) in 30 min; the latency (s) to first mount (ML), intromissive behavior (IL) and ejaculatory behavior (EL).

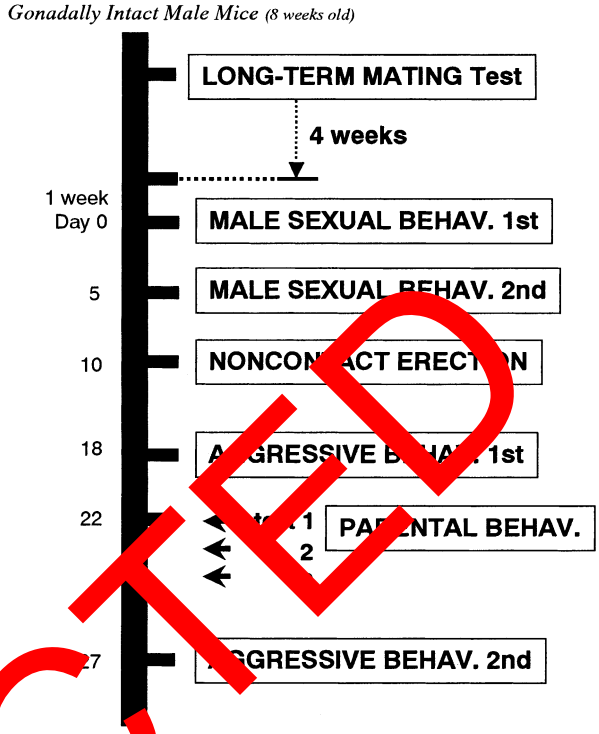

Fig. 1. Schematic presentation of the series of behavioral tests in the present study. The mice used in this study were gonadally intact. The analyses were started at 8 weeks of age. The time schedule and the behavioral test examined are also indicated in the figure.

2.4. Noncontact erection test

Tests were conducted in an observation chamber that was divided in half by two clear plastic partitions with 18 small holes (2 cm in diameter) alternately punched on either side. The barrier prevented direct contact between animals, but allowed reciprocal auditory, visual and olfactory communication. A mirror placed at an angle below the cage permitted simultaneous lateral and ventral viewing. After a 5 min adaptation period, the test was started by placing an estrous female into other side. The tests continued for 15 min after the first erection. Chamber and barrier were washed with ethanol, rinsed with water and dried after each test. Erections were scored when the penis emerged from the penile sheath, which was accompanied by penile grooming and hip flexion.

2.5. Male aggressive behavior test

Each male was tested in his home cage (as the resident) against a group-housed olfactory-bulbectomized (OBX) male (C57BL/6) intruder or estrous female mouse for 15 min. For each experimental male, the latency to the first aggressive behavioral act, the cumulative duration of aggressive bouts and the total number of aggressive bouts were scored. An aggressive bout was defined as a consecutive series of behavioral acts separated by less than 3 s. Aggressive acts consisted of tail rattling, chasing, boxing, biting, offensive attack and wrestling.

2.6. Parental behavior test

Mice were tested for 15 min on 3 consecutive days in their home cages. On the day before the tests, they were given 300 mg of cotton on their cages and allowed to make nests. The test was started by gently placing three foster (C57BL/6) pups (1–4 days old) at the farthest end from the nest. The retrieving, nursing and crouching behaviors were recorded.

2.7. Statistical analysis

The Mann–Whitney's *U*-test was used for the comparison of the behavioral data between wild and ArKO mice. The differences in the incidence of the various behaviors were analyzed by the χ^2-test or Fisher's exact probability test. We considered differences significant, if *P*-values <0.05.

3. Results

3.1. Level of serum steroid hormones in the wild and aromatase knockout mice

Several steroid hormones such as testosterone are generally taken to be factors for the sexual behavior. To assess the change of steroid level, we determined the concentration of some of them in the serum of the wild and the ArKO mice. As expected, the concentrations of estradiol in the serum of ArKO mice are below the limit of detection (data not shown). The specific difference of progesterone levels between wild and ArKO mice can not be observed in the males or females. The serum testosterone levels tend to increase in male and female ArKO mice as compared with those of the wild types. Especially, the testosterone of wild type female mice are almost undetectable but those of the ArKO females elevated to 0.9–4.0 ng/ml (Table 1).

Table 1
The concentrations of the steroid hormones in the serum

Gene genotype	Testosterone (ng/ml)		Progesterone (ng/ml)	
	+/+	−/−	+/+	−/−
Male	0.9	4.4	0.8	0.9
	15.8	13.2	6.3	3.7
	N.D.	0.4	2.0	0.5
	1.1	9.9	3.3	1.5
	0.4	7.4	3.8	0.8
Female	N.D.	4.0		10.2
	0.1	0.9	2.3	4
	0.7	3.2	19.6	14
	N.D.	0.9	1.1	4.6
	N.D.	1.6	1.3	7.

N.D.:not detected.

3.2. Male-type sexual behavior

In a long-tem fertility test, there is an obvious lower fertile ability of ArKO male mice (23%) as compared to that of the wild type (90%). Because an ArKO male of 8–12 weeks of age is capable of spermatogenesis, this low fertility in ArKO males is caused by the other factors. Aromatase gene disruption has an influence on the typical activities of male sexual behavior, i.e. mount, intromission, and ejaculation (Fig. 2). All of the wild-type mice observed displayed mount and intromission activities, which resulted in ejaculation in 80% of these mice. On the contrary, only 24% of ArKO mice displayed mount, and only 10% intromission, and no ArKO mouse showed ejaculation. Moreover, ArKO mice also showed significant decreases in the mount and intromission frequencies. To assess the alteration of sexual reflex in ArKO male, we examined the activity of noncontact erection. ArKO male mice tended to show marginally fewer noncontact erections (Fig. 3).

3.3. Male aggressive behavior

To assess the activity of voluntary attacks on intruder males, we adopted a resident-intruder paradigm. Because the OBX male intruder mice do not attack other males, we can examine the volitional aggressive activity of the test animals. ArKO mice have significantly reduced male aggressive behavior in comparison to wild-type mice (Fig. 4A). In the second aggressive behavior test, 90% of wild-type males tested offensively attacked the OBX intruder male within 15 min, and showed typical aggressive behavior with biting and wrestling (see Section 2). In contrast, the incidence of aggressive behavior in the ArKO male is substantially low. Both the bout and the attack duration of the ArKO mice showed appreciable attenuation as compared with those of wild-type mice.

Under normal conditions, wild-type male mice do not generally attack the estrous female mice. In this study, we demonstrated that ArKO mice showed high aggression to the estrous female in comparison to the wild-type male (Fig. 4B).

3.4. Parental behavior

The aromatase gene deficiency, even in the male, greatly altered the parental response to infant mice. Normal male

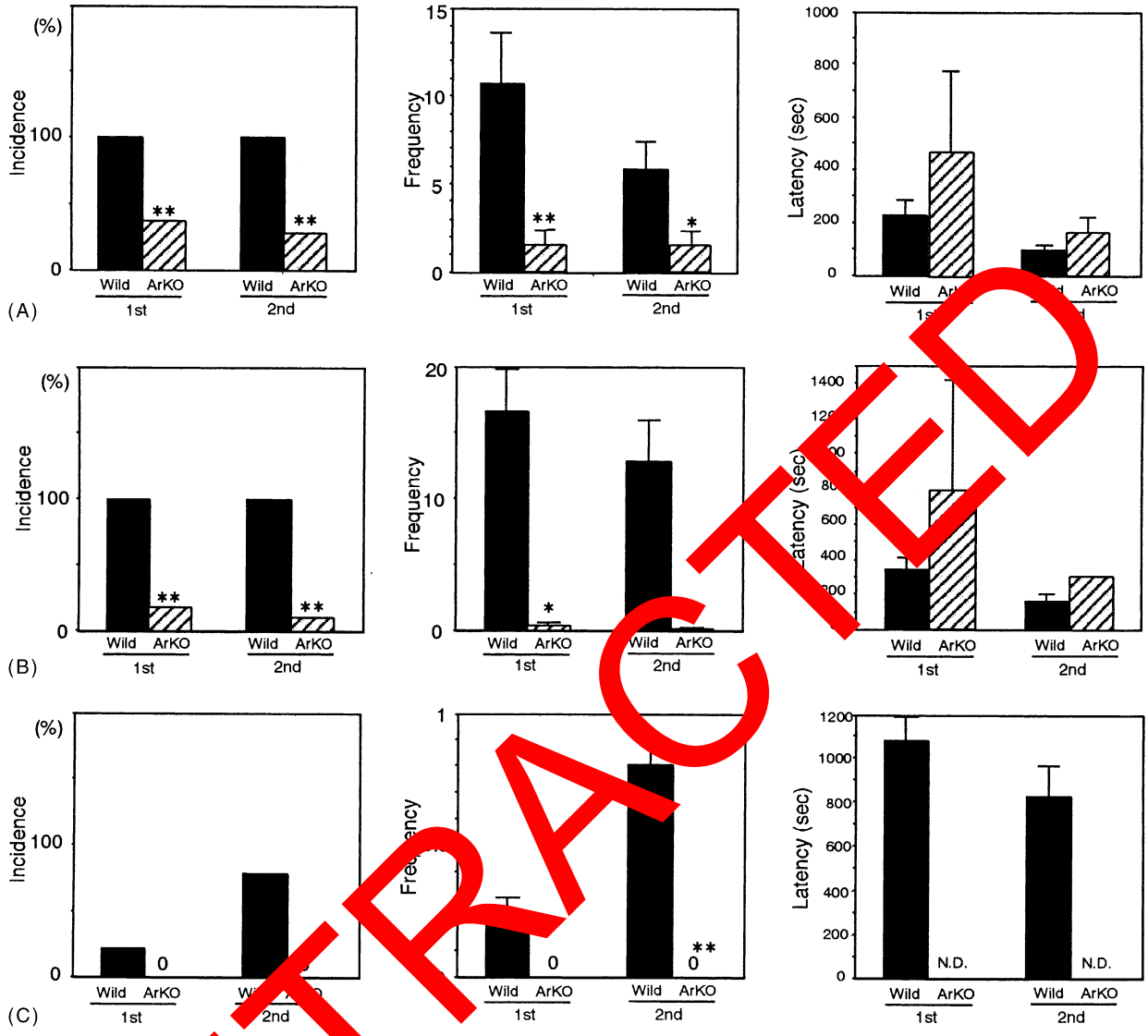

Fig. 2. Effects of targeted disruption of the aromatase gene on activities of male sexual behavior. This figure shows the mount (A), intromission (B), and ejaculation (C) relative to incidence (left column), frequency (middle column), and latency (right column) during a 30 min test. The incidence is given as a percentage, and was statistically analyzed by the χ^2-method. The frequency and the latency to the first act of each behavior are indicated by the mean ± S.E.M. Significant differences when compared with the data for wild-type animals are indicated in the figure (** $P < 0.01$, * $P < 0.05$). N.D.: not determined.

mice showed maternal behavior toward pups. In contrast, most ArKO male mice showed infanticide (Fig. 5A). In addition, ArKO male mice failed to exhibit certain parental behavioral activities such as retrieving (Fig. 5B) and nursing (Fig. 5C). The decreased incidence of parental behaviors was accompanied by increased infanticide. This analysis revealed that ArKO male was not significantly different from wild type male in the incidence of the crouching behavior.

4. Discussion

In the present study, we demonstrate the notable disorder of male sex-specific and its related behaviors in ArKO male mice. ArKO males showed impairment of all components of sexual behavior including mount, intromission and ejaculation. These results indicate that aromatase gene expression is a critical step for induction of male copulatory behavior. Ogawa et al. [25] reached some similar conclusions by conducting behavioral studies with estrogen receptors knockout mice. Modification of different components of male sexual behavior was reported in ERKO mice. αERKO males showed normal levels of mount and reduced levels of intromission, and virtually no ejaculation [25]. All three components of sexual behavior are normal in βERKO animals [29], but αβERKO males did not exhibit any sexual behavior [30]. Taken together, these findings provide genetic evidence for the brain aromatization hypothesis and indicate

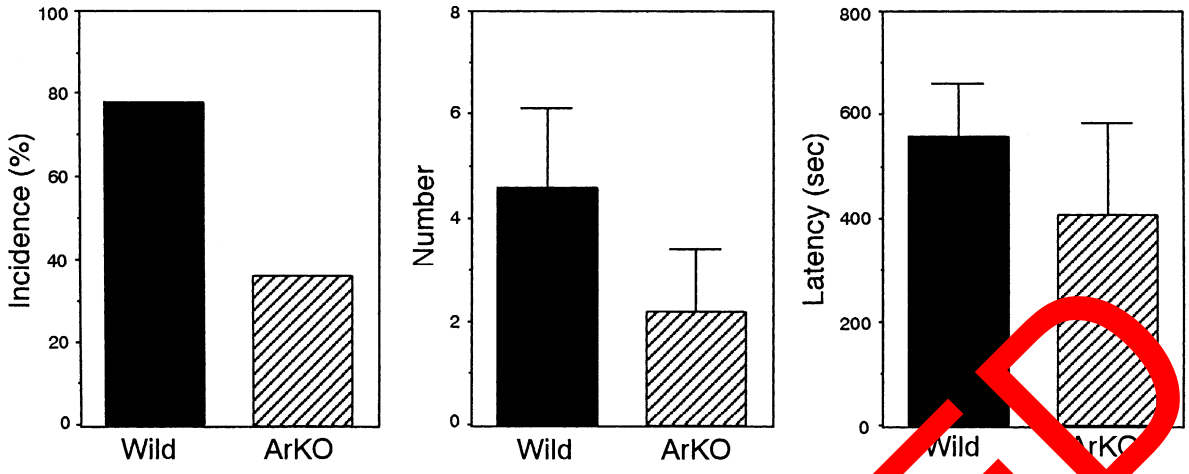

Fig. 3. Effects of targeted disruption of the aromatase gene on non-contact erection. The incidence is shown [illegible] percentage, and was statistically analyzed by the χ^2-method. The number and the latency are indicated by the mean ± S.E.M.

that aromatase and inductive estrogen are essential for motivational and consummatory aspects of male copulatory behavior.

On the contrary, disruption of the aromatase gene did not significantly reduce noncontact penile erection, indicating that some aspects of brain mechanisms concerned with the male erectile response to estrous females still operate in ArKO males. It has been suggested that hormonal activation of noncontact erection largely depends on non-aromatizable androgens such as 5α-dihydrotestosterone, but not estrogen in adult male rat [42–45]. Moreover, perinatal treatment of female rats with 5α-dihydrotestosterone is sufficient to fully masculinize their neural potency for non-contact erection. Accordingly, it is possible that estrogen is not required for sexual arousal in noncontact erection in rodents. Further research on other contexts of erection in ArKO mice would clarify the functional significance of estrogen in the physiology of erectile function.

In a fertility test, 7% of ArKO male mice were infertile at 8–12 weeks of age. Additionally, only 1 of 10 ArKO males at the 14–17 weeks of age were fertile in our initial experiments. To date, ArKO mice lacking different exons have been generated in different laboratories. In contrast to our results, Fisher et al. reported that male mice at the age of 12–14 weeks were fully fertile and repeatedly sired litters even after targeted disruption of exon 9 of the *cyp19* gene [23]. The reason for the discrepancy is not clear, but taking into account the infertility of ERKO males, it might be assumed that inactivation of both exons 1 and 2 including the transcriptional and translational initiation sites could lead to more severe functional loss of aromatase than does inactivation of exon 9.

Although, deletion of the gene encoding aromatase caused severe impairment in male copulation in the majority of animals, a small number of ArKO males are still fertile and could show a complete pattern of male sexual behavior including ejaculation. This was unexpected, because αβERKO male mice were completely infertile and did not show any component of sexual behavior [30]. One possible explanation is that the maternal environment in utero permitted exposure of ArKO mice to steroid hormones. The developing ArKO male fetus could be affected by testicular androgens diffused from nearby male littermates and/or maternal estrogen secreted from the heterozygous pregnant female. If so, some wild-type females might concomitantly acquire the latent capacity for an ejaculatory pattern. Nonetheless, it is well known that there is a clear sexual dimorphism in male sexual behavior and normal female rodents cannot display an ejaculatory pattern in response to androgen treatment in adulthood except under special circumstances. Further study is needed to evaluate relationships among intrauterine positions with respect to the sexual attributes of littermates, the circulating estrogen level of the fetus and male sexual behavior in ArKO mice.

Since aggressive behavior in mice is mainly regulated by olfactory cues, the OBX intruder with intact gonads rarely shows aggression spontaneously, but it can fight back against the resident male's attack. For this reason, we used OBX mice as male intruders in order to assess the intrinsic ability of resident ArKO mice for male aggression. Recently, Toda et al. reported that male mice lacking exon 6 of the *cyp19* gene displayed a deficit in aggressive behavior against male intruders with intact olfaction [46]. However, results obtained in such cases might be due to the absence of aggressiveness in intruder mice toward ArKO mice. It is, however, an open question whether the result obtained in such cases is due to a loss of aggressiveness in ArKO mice. In fact, disruption of the aromatase gene causes the male mice to diminish their male-typical behavior and to exhibit a female-like behavioral phenotype such as lordosis. Hence ArKO males are treated more like females, thereby reducing the probability of attack by intruder mice with intact olfaction.

Interestingly, instead of copulatory behavior, ArKO males in this study exhibited aggression toward estrous female

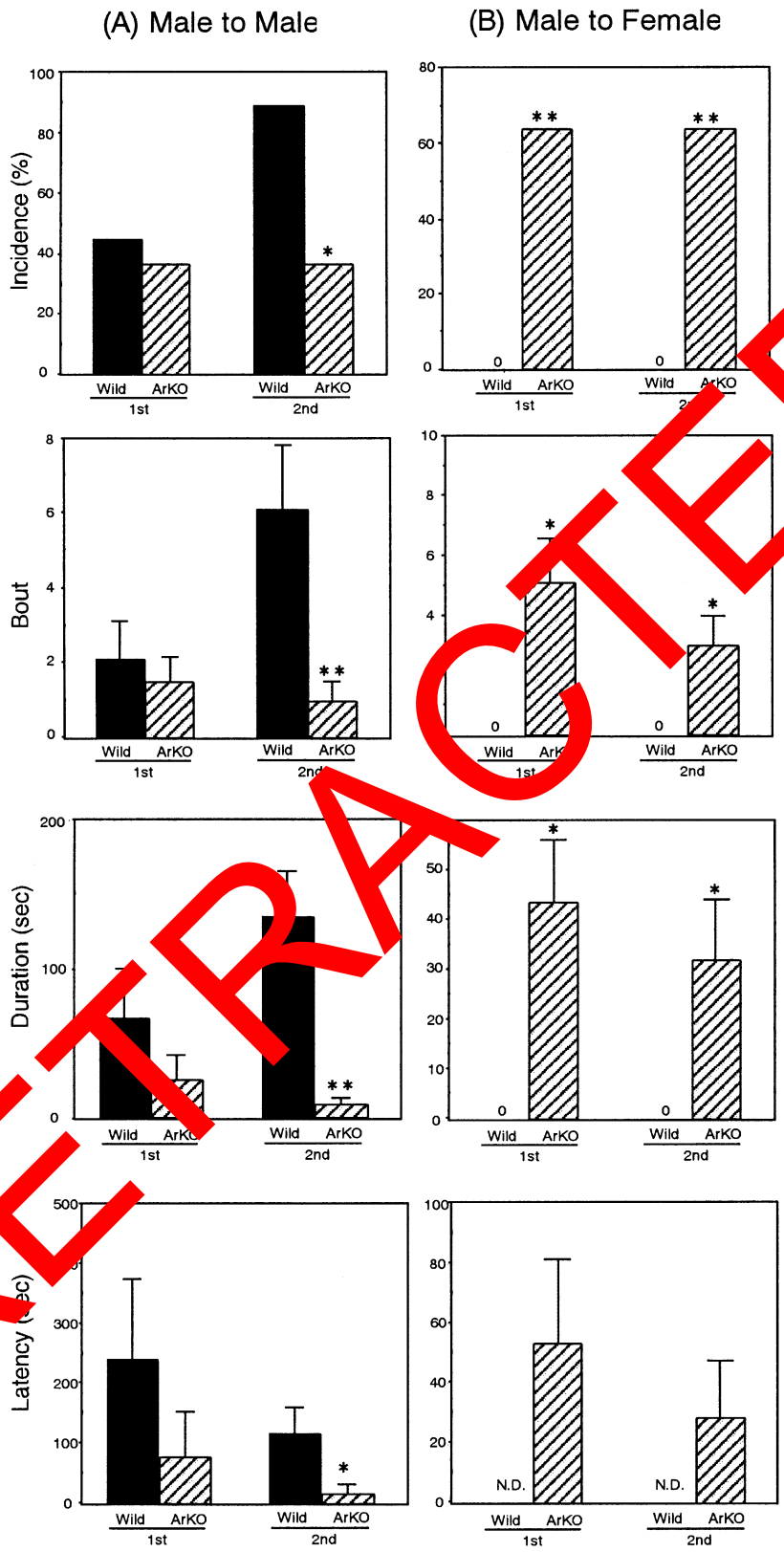

Fig. 4. Effects of targeted disruption of the aromatase gene on male aggressive behavior in the resident intruder paradigm test. The test male was examined in his home cage (as the resident) against a group-housed olfactory-bulbectomized (OBX) male intruder (left side) or estrous female (right side). The incidence is shown as a percentage, and was statistically analyzed by the χ^2-method. The number of bouts, the duration, and the latency to the first act of aggressive behavior are indicated by the mean ± S.E.M. Significant differences when compared with the data for wild-type animals are shown in the figure ($^{**}P < 0.01$, $^{*}P < 0.05$). N.D.: not determined.

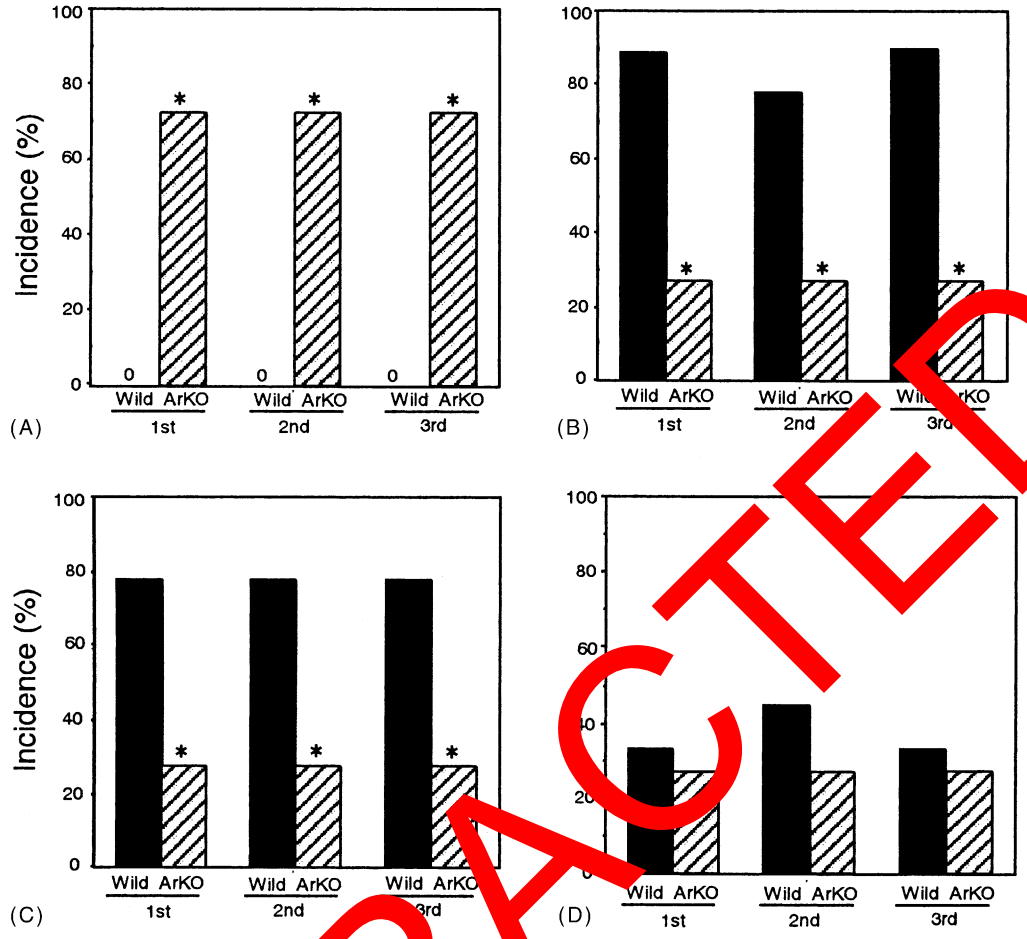

Fig. 5. Effects of targeted disruption of the aromatase gene on activities of parental behavior in male mice. The incidences of infanticide (A), retrieving (B), nursing (C), and crouching (D) activities are shown in Fig. 1. The incidence is shown as a percentage, and was statistically analyzed by the χ^2-method. Significant differences when compared with the data for wild-type animals are shown in the figure ($^*P < 0.05$).

mice during male copulatory tests, although no such aggressive behavior was observed in wild-type males. Nevertheless, intermale aggression against OBX male intruders was drastically reduced in ArKO males in a resident-intruder paradigm test. These phenotypic features are similar to ERKO mice. Ogawa et al. reported that αERKO or αβERKO males displayed a great reduction of aggressive behavior toward OBX male intruders [25,30]. In addition, half of the αβERKO males showed aggression against female mice during male mating tests [30]. The most probable explanation is that loss of function in estrogen biosynthesis in neural tissues during the perinatal period leads to demasculinization of the neural circuit regulating aggressive behavior, so that the aggressive orientation is reversed in male mice. It will be of interest to examine this possibility in the future detailed experiments.

Characteristic patterns of parental behavior, but not infanticide, were observed in wild-type mice, although variability of parental behavior exists in mice across different genetic backgrounds. In contrast, a high proportion of ArKO males, like αERKO males, exhibited infanticidal behavior. This phenotypic feature in ArKO mice might be a reflection of several changes induced by deletion of the aromatase gene. First, the high frequency of pup-killing behavior may be caused by lack of the activational action of estrogen. This possibility is supported by our preliminary observation that postpubertal estrogen replacement prevented ArKO males from showing infanticide. Another explanation is that activation of the androgen receptor signaling pathway in perinatal and adult life causes a behavioral change toward pups, since the serum level of testosterone is slightly elevated in ArKO males [23]. However, effects of castration or administration of physiological doses of testosterone on parental behavior in ArKO mice have not been examined yet. Finally, several studies showed that hormonal manipulation during the perinatal period also affect testosterone-inducible infanticide later in adulthood. Neonatal castration tends to promote infanticidal behavior in male mice. Conversely, perinatal treatment of female mice with androgen results in a reduction of such behavior and this suppressive effect of androgenization is mediated at least via aromatization to estrogen. Therefore, we cannot rule out the possibility that a depletion of

estrogen biosynthesis during the perinatal period may contribute to the subsequent high level of infanticide in adulthood.

ArKO male mice represent a characteristic behavioral profile including impairment of male sexual behavior, male aggression and parental behavior. Although, it remains unclear whether deficits in sex-specific behaviors in ArKO mice are attributed to a lack of organizational or activational action of estrogen, these behavioral changes are particularly interesting in light of aromatase deficiency in humans. A recent study showed that male sexual behavior in man with aromatase deficiency was improved after treatment with estrogen, although loss of aromatase itself did not affect gender-identity and male sexual orientation [34,37]. These findings suggest the possibility that aromatase and estrogen also have a role in male sexual activity in humans. Therefore, further studies with estrogen replacement at specific lifetime stage in ArKO mice will provide an opportunity for a better understanding of estrogen deficiency in male reproductive physiology and behavior.

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