ORIGINAL PAPER

Behavioral Evaluation of Sociosexual Dysfunction in Aromatase Knockout Mice

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Abstract. Estrogen has been known to play a critical role in regulation of male sexual behavior. Recent studies demonstrated severe impairment of male mouse sexual and aggressive behavior by targeted disruption of aromatase gene, Cyp19. In the present study, as basis for a loss of sexual behavior, we compared odor difference between wild and aromatase-knockout (ArKO) mice using probe estrous females in sexual preference paradigm. The probe females reacted equally to body smell and soiled bedding of both ArKO and normal mice, suggesting androgen regulation of pheromones and/or attractive odorants emitted by sexually vigorous male mice. Emotionality of those mice was also measured in open field and elevated plus maze. ArKO mice had significantly low activity in open field, but not in elevated plus maze, compared to wild-type mice. We suggest that absence of aggressive behavior is based on their high emotionality (low activity in unfamiliar area).

Key words: Estrogen, Emotion, Pheromone, Knock-out, Sexual differentiation

Introduction

There has been accumulated ample evidence showing critical roles of estrogen in brain differentiation of sexual dimorphism in physiology and behavior. Early exposure to either estrogen or aromatizable androgen in ontogeny produces masculine phenotype such as male copulatory [1, 2] and aggressive behaviors [3, 4], whereas absence of these hormones in the critical period induces female phenotype, lordosis behavior [5] and ovarian functions [6, 7]. Estrogen also plays an important role in expression of copulatory behavior in adult males. Prevention of metabolism from androgen to estrogen by aromatase inhibitors disrupts mount behavior in male rats [8], hamsters [9] and mice [10], whereas treatment of estrogen activates mount behavior in male and female rats [11, 12]. Thus, estrogen exerts both the organizational and priming effects in male animals throughout development.

Recently, transgenic techniques have been well developed for studying neuroendocrine mechanisms of sexual differentiation. Deletion of estrogen receptor gene, both ERs α and β, demonstrates the importance of estrogen in development of reproductive systems in mice. Female mice lacking ER α showed a complete loss of female sexual behavior [13], and males lacking ER α showed partial disruption of male sexual behavior [14–16]. Although ER β had no effect on both female and male sexual behavior [17], double knockout of ERs α and β eliminated male mouse sexual behavior [18]. Presumably, ERs α and β may interact each other to regulate male and female sexual behavior [19].

Another line of studies uses aromatase knockout (ArKO) mice that cannot produce estrogen [20–22]. These ligand-deficiency mice showed severe impairment of copulatory behavior in the male and infertility in the female [21–23]. They also showed a loss of intermale aggression [24, 25] and sex partner preference [26, 27].

In the present study, we investigated how the male mice lacking aromatase (disruption of Cyp19) fail to display sexual behavior and aggression. It is possible that a lack of estrogen in peripheral organs changes the produc-

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tion of pheromone and/or other odorants, causing changes in their attractiveness and female approaches. Then, we examined differences of their body smell between ArKO and wild-type mice using partner/odor preference paradigm. In addition, central estrogen might change their emotional responsiveness on which aggressive behavior is based. Then, we also examined their emotionality measured in open field and elevated plus maze.

Materials and Methods

1) Animals

Animal care and experiments adhere to the Guidelines for Care and Use of Laboratory Animals of the Institution of Laboratory Animal Resources, National Research Council (1996). All animal were maintained in controlled temperature and illumination (lights on 23:00-11:00). Food and water were given ad libitum. ArKO male mice were generated by targeted disruption of Cyp19 gene as previously described [28, 29]. Heterozygous males and females of C57BL/6 strain were bred to generate wild-type (+/+), heterozygous (+/-) and homozygous-null (ArKO) offspring. Nine ArKO, 6 +/- and 5 +/+ males (8 weeks old at beginning) were used for the following behavioral experiments.

As probe females, another 9 C57BL/6 females (8 weeks at beginning) were ovariectomized under ether anesthesia, and injected subcutaneously 48 hours before each behavioral test with 5 μg estradiol benzoate (EB) followed by 0.5 mg progesterone (P) 4 hours before the test.

2) Tests for Body Smell of ArKO Mice

Partner preference paradigm was used for testing their body smells. Probe females were located between ArKO males and +/- males or between ArKO and +/+ males. The acrylic cage (30 cm x 50 cm x 30 cm) divided into 3 areas; right, center and left areas, by the acrylic walls with 5 cm wide pathway (see Fig. 1). The apparatus was bedded with white paper tips (Alpha-dri, Shepherd Specialty Papers, Inc., MI). The back ends of right and left areas had small compartments separated by duplicate opaque panels for experimental males. Each panel, with a 2 cm hole in diameter, bored at different position, were assembled into a partition, which allowed the passage of air but prevented visual and physical interactions. The male compartments

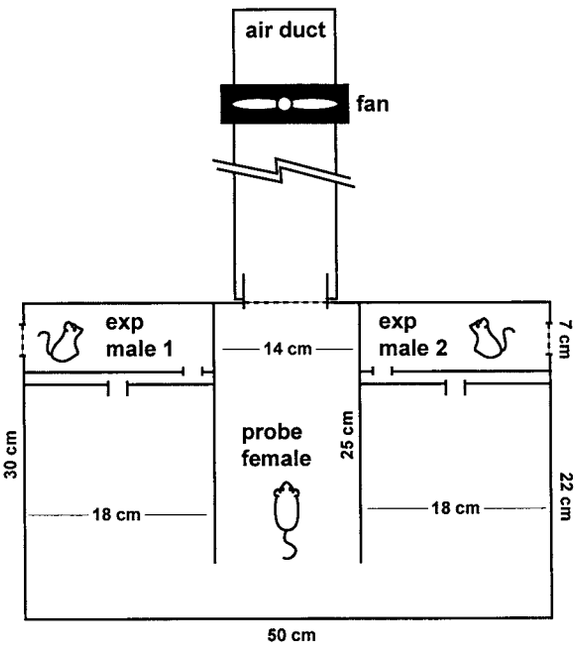

Fig. 1. Schematic representation of the apparatus used in test for body smell in ArKO mice. A probe female was located between ArKO and control male mice, and time spent by the female to stay in the vicinity of each stimulus male was measured.

had air-inlets (mesh-covered holes, 3 cm in diameter), and the center compartment had an air-outlet (10 cm in diameter) at the back. A motor-driven blower, attached to the air-outlet via a flexible duct, introduced air from the left and right compartments into female areas (approximately $0.2m^3$/min).

During acclimation, each of paired experimental males was located in the side compartments, respectively, and a probe female was placed in center of the apparatus. The blower attached in opposite direction to make air flow out from female areas into the male compartments (no presentation of odors). The acclimation period lasted for 10 min.

After the acclimation, the probe female was relocated to the center area and the direction of blower was returned to proper position. The time spent by the female in each area was recorded for 10 min. After every test, the apparatus was cleaned and sprayed with 70% ethnol. Fresh paper beddings were put into the apparatus ready for the next test.

A week after the tests, the same tests with different combinations of paired males were carried out as the second tests.

3) Tests for Excretion-Odors of ArKO Mice

Using their soiled bedding in home cage as stimuli, odor preference test was conducted in an acrylic observation box (30 cm × 50 cm × 40cm). Probe females were located between soiled beddings of ArKO males and clean beddings, between soiled beddings of +/- males and clean beddings, and between soiled beddings of ArKO males and of +/- males. The beddings were collected from each male's home cage immediately before each test. The beddings on a grass Petri dish (12 cm in diameter) were placed in the observation box apart from each other. The time spent for contacting each dish was measured during 5 min.

4) Open Field Test

Open field tests were daily carried out for 3 consecutive days in a white polyvinyl chloride circular chamber (90 cm in diameter with 50 cm walls). The floor was marked by 8 radial lines and a 50 cm circle, which divided the arena into the center and rim. The apparatus was illuminated by overhead fluorescent lighting.

Experimental males were placed in the center of the arena. The number of line crossings, rearings, and feces were measured for 3 min.

5) Elevated Plus Maze Test

One week after open field tests, all males were subjected to elevated plus maze test. The elevated plus maze consisted of a black and white acrylic plus shaped maze elevated 60 cm from floor. Two opposite arms, 5 × 30 cm, were black, and another two arms of the same dimensions were white. The black arms were enclosed by 15 cm high walls (closed arms), and white arms had no wall (open arms). The proportion of time spent onto open arms was measured for 10 min as an index of emotionality. Experimental males received one trial per day on each consecutive 3 days.

6) Statistics

All data were first analyzed by two-way ANOVA with repeated measures. When comparing a pair among the groups in each test, statistical significance was evaluated by t test.

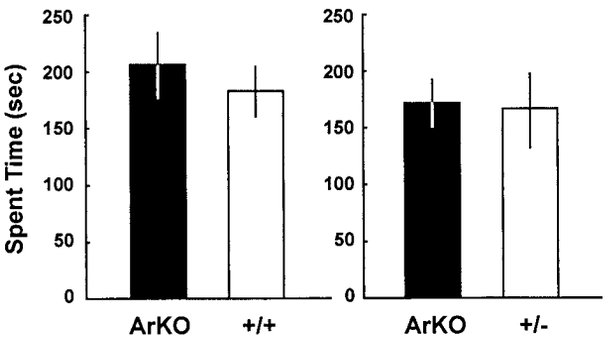

Fig. 2. Mean time spent by probe females in each area in body smell test. The probe females spent almost same time close to ArKO and +/+ male mice (left) and to ArKO and +/- male mice (right). Vertical bars represent SEM.

Results

1) Test for Body Smell

During the acclimation period, the mean time (sec) spent by probe females in each area was not significantly different in both paired stimulus males (ArKO and WT, 123.33 ± 18.62 and 171.28 ± 34.19; ArKO and HET, 147.98 ± 38.58 and 106.12 ± 18.29). In the test period, the means of time spent in each area is shown in Fig. 2. Although the probe females tended to spend longer time in the vicinity of stimulus experimental males, compared to that during the acclimation period, they showed no preference at all. They spent almost same time close to ArKO and +/+ male mice and to ArKO and +/- male mice ($t < 1$, ns).

2) Test for Soiled beddings

Fig. 3 shows the results of tests for soiled beddings of experimental male mice. The probe females explored significantly longer the soiled beddings collected from home cages of ArKO males ($t(8) = 5.78$, $p < .01$) or +/- males ($t(8) = 4.76$, $p < .01$), compared to clean fresh beddings. They spent time for exploring either soiled beddings longer than 3 times of clean beddings.

When presented a pair of soiled beddings collected from ArKO and +/- males, the probe females tended to explore the soiled beddings of ArKO males longer than those of +/- males, but the difference did not yielded statistical significance because of the large variances.

3) Open Field Behavior

In +/+ male mice, highest locomotion activity was observed on Day 1, and the activity gradually decreased

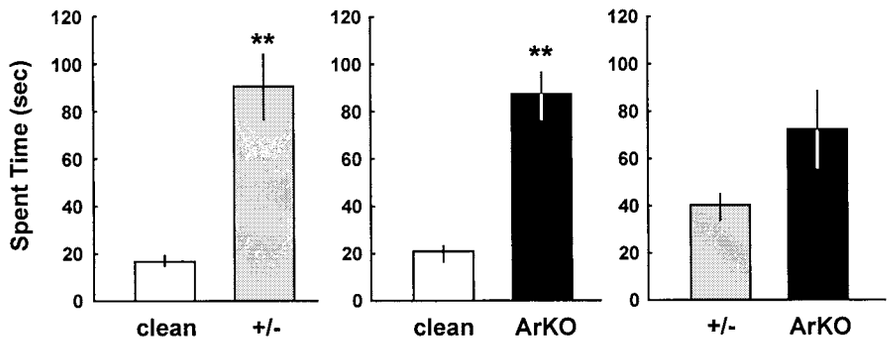

Fig. 3. Mean time spent by probe females for exploring each bedding. The probe females explored significantly longer the soiled bedding collected from ArKO (left) or +/- male's cages (center), compared to clean bedding. No difference was found in test between ArKO and +/- males (right). Vertical bars represent SEM. **p < .01.

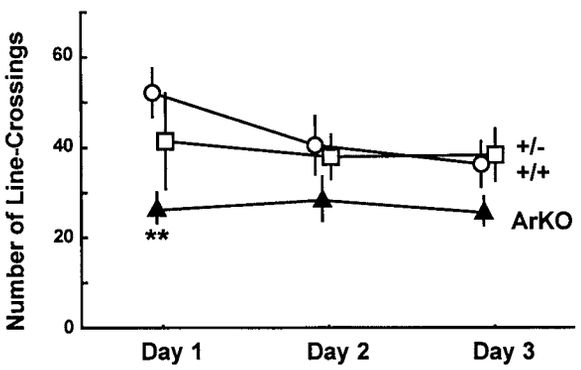

Fig. 4. Mean number of line-crossings in open field test over 3 consecutive days. Locomotion activity of ArKO males was significantly lower than that of +/+ males on Day 1. Vertical bars represent SEM. **p < .01.

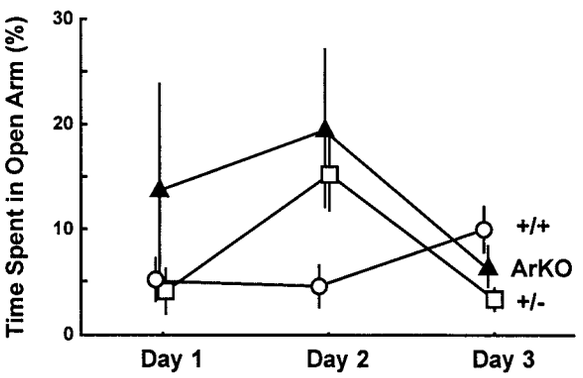

Fig. 5. Mean proportion of time stayed in open arms in elevated plus maze test over 3 consecutive days. No difference was found among ArKO, +/+ and +/- males throughout 3 days. Vertical bars represent SEM.

through the 3 test days (Fig. 4). The tendency was almost same in +/- males, but variance was much larger than those of +/+ males. In contrast to those males, locomotion activity of ArKO males was lower from Day 1 and no change through the 3 test days (Fig. 4). On Day 1, the mean number of line crossings was significantly lower in ArKO males than that of +/+ males ($t(12) = 4.01$, $p < .01$). The mean numbers of rearings and defecations did not differ significantly among all genotypes throughout 3 days (data not shown).

4) Elevated Plus Maze Activity

The results of elevated plus maze test, which are indicated by proportion of time stayed in open arms, are shown in Fig. 5. No difference was found among ArKO, +/+ and +/- males. They all stayed in the closed arms for most of the test period throughout 3 days.

Discussion

The first half of the present study clearly showed that receptive females do not differently react to ArKO and normal male mice. The probe females equally accessed to odors of either ArKO mice or +/+, +/- mice. In addition, the females were also interested in soiled beddings of ArKO males, when compared to clean one.

Chemosensory signals play extremely important roles in sociosexual behavior in mice, as well as other mammalian species [30, 31]. The signals mainly excreted with urine are received via two distinctive systems, olfactory epithelium and vomeronasal organ, and travel into the limbic and hypothalamic regions, regulating sociosexual

behaviors. Recently, a complete loss of sex discrimination and intermale aggression was reported in mice lacking TRP2, a putative ion channel of the transient receptor potential family that is required for sensory activation of vomeronasal neurons and exclusively expressed in those neurons [32]. Thus, chemosensory signals broadly affect social behavior including copulatory, maternal and aggressive behaviors.

There is also evidence revealing that secretion of those pheromonal and attractive chemicals is regulated by gonadal hormones [33]. Attractiveness of male odors to females depends directly on the level of circulating testosterone in voles. Gonadectomy decreases attractiveness of odors to opposite sex conspecifics in both males and females, while replacement of testosterone in males or estrogen in females restores it [34]. ArKO males have no estrogen and elevated level of testosterone in blood [20, 22]. However, our probe females that were highly receptive by treatment with estrogen and progesterone react indifferently to odors of both ArKO and control male mice. This suggests that secretion of male pheromones or attractive odorants is regulated by androgen but not estrogen converted by aromatase.

Our soiled bedding tests indicate that (1) odors from both ArKO males and other controls are attractive, not aversive, because longer time spent for exploration of it compared to clean beddings, and (2) attractive odorants was contained in excretions. Electrophysiological recording in behaving mice indicated activation in some neurons in the accessory olfactory bulb, secondary sensory neurons in vomeronasal system, when their nose contacted to head or face area of stimulus conspecifics [35]. However, substances in urine may be primarily important for sexual attractiveness.

In conclusion, we can reject the hypothesis that copulatory deficit in ArKO males is due to loss of sexual pheromone or attractive odorants to females emitted by male mice. The impairment of copulatory activity in ArKO mice may be purely caused by absence of estrogen action in neural circuits responsible to male sexual behavior.

As to second part of the present study, emotionality of ArKO males, locomotion activity of ArkO males was lower than that of +/+ males in open-field test, suggesting that disruption of aromatase gene resulted in increased emotionality. This result agrees with the previous study showing ArKO males were more anxious than wild or heterozygous males [27].

On the other hand, there was no clear difference between ArKO males and +/+ or +/- males in elevated plus maze. ArKO males seem to spend rather longer time in open arms, but with large variances and no statistical difference, than other controls. Although both open field and elevated plus maze tests has been believed to measure emotionality or anxiety in rodents, these 2 tests may have different sensitivity or measure somewhat different aspect of emotionality. Indeed, there is some contradiction in studies of mice lacking ER α, increased in elevated plus maze [36] and decreased in open field [14]. As ArKO and ER-knockout are not the same, our present results and previous study of ArKO [27] suggest that estrogen rather reduce emotionality in mice.

The present study showed no difference in body smell and high emotionality in ArKO male mice. The high emotionality (nearly equal to timidity) might be related to low aggression in ArKO mice [37]. Presumably ArKO mice may have much more other characteristics in behavior. Further studies are required to understand ArKO mice, and of course, roles of estrogen and aromatase.

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