Tsukuba Psychological Research
2013. 45. 1-9

Loss of neuronal ERα abolishes sexual and aggressive behaviors in male mice

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The estrogen receptor α knockout mice (αERKO) underscores the importance of ERα expression in the regulation of social behaviors by estrogen. We investigate the neuronal contribution to both social and nonsocial behaviors by using male mice in which the ERα gene is deleted solely in neurons. Overall, these mice showed similar behavioral changes as those previously reported in αERKO mice. Both sex and aggressive behaviors were completely abolished in these mice. ERα deficient mice also tended to show higher levels of locomotor activity measured in open field test and tended to be heavier than wild-type control mice. These findings suggest that neuronal ERα may play a major role in the regulation of male social behaviors.

Key words: nuclear receptor, glia, estradiol, testosterone, exploratory behavior

Introduction

17β-estradiol, as an aromatized metabolite of testosterone, play a pivotal role in the development and regulation of sex-specific behaviors such as aggressive and sexual behaviors in male rodents, by acting on two estrogen receptor (ER) isoforms, ERα and ERβ (Nilsson & Gustafsson, 2011; Ogawa, Korach, & Pfaff, 2002; Ogawa, Nomura, Choleris, & Pfaff, 2006). The critical requirement for signaling via the ERα is demonstrated by severe disruption of aggressive and sexual behaviors in the ERα male knockout mouse (αERKO) (Ogawa, Lubahn, Korach, & Pfaff, 1997; Ogawa, Washburn, Taylor, Lubahn, Korach, & Pfaff, 1998; Ogawa, Chester, Hewitt, Walker, Gustafsson, Smithies, Korach, & Pfaff, 2000; Scordalakes & Rissman, 2003). In addition to the loss of social behaviors, non-social behaviors were also affected. αERKO male mice exhibited higher locomotion and less anxiety compared to the wild-type (α ERWT) mouse in the open field test (Ogawa et al., 1997).

Though ERα is expressed in both neurons and glia in several regions in the central nervous system (CNS; Garcia-Ovejero, Veiga, Garcia-Segura, & DonCarlos, 2002; Santagati, Melcangi, Celotti, Martini, & Maggi, 1994; Shughrue, Lane, & Merchenthaler, 1997), the steroidal regulation of these behaviors via ERα is assumed to occur in neurons (Lin, Boyle, Dollar, Lee, Lein, Perona, & Anderson, 2011; Wen, Gotze, Mai, Schauer, Leinders-Zufall, & Boehm, 2011). Recently, however, a number of studies have reported that glial cells may be involved in reproductive physiology and development of sexually dimorphic brain areas (Garcia-Segura, Lorenz, & DonCarlos, 2008; Johnson, Schneider, DonCarlos, Breedlove,

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1) We thank Drs. T. M. Wintermantel, J. Elzer, G. Schutz (Molecular Biology of the Cell I, German Cancer Research Center, Heidelberg, Germany) for a kind gift of knockout mice. This study was supported by Grant-in-Aid for Scientific Research (17052001, 17330151, 23240057) and University of Tsukuba Research Project Grant to SO.

& Jordan, 2012; Krebs-Kraft, Hill, Hillard, & McCarthy, 2010). Studies using αERKO male cannot distinguish if the behavioral alterations were due to the deletion of ERα expression in the neurons, glial cells, or both. To examine if behavioral alterations in αERKO male mice was due to the loss of ERα in neurons, we obtained mice that lack ERα selectively in neurons of the CNS but possess ERα in glia and peripheral tissues (ERα fl/fl mutant mice). In these mice, we tested social behaviors including aggressive and sexual behaviors, and behaviors that denote anxiety and exploratory activity in unfamiliar situation.

Materials and Methods

Mice: Three male $ER\alpha^{fl/fl}$; CamKIIα-Cre (KO) mice that lack ERα expression selectively in neurons of the CNS (Wintermantel, Campbell, Porteous, Bock, Grone, Todman, Korach, Greiner, Perez, Schutz, & Herbison, 2006; Elzer, Muhammad, Wintermantel, Regnier-Vigouroux, Ludwig, Schutz, & Schwaninger, 2010), 8 wild-type $ER\alpha^{fl/fl}$ (WT), and 12 heterozygous (HZ) males were used. Adult male mice between 25-37 weeks of age were maintained at constant temperature (24°C) under 12:12 light/dark cycle (lights off at noon) with food and water provided *ad libitum*. All procedures were approved by the Animal Experiment Committee as well as the Recombinant DNA Use Committee at University of Tsukuba, and conducted strictly in accordance with their regulations. All efforts were made to minimize the number of animals and their suffering.

Experimental Design: Seven days before the first behavioral test, mice were singly housed and tested in four different behavioral paradigms in the following order: 1) open-field test (in two consecutive days), 2) light-dark transition test, 3) sexual behavior test (for three times), and 4) resident-intruder paradigm aggression test (in three consecutive days). There was an interval of 2-7 days between different kinds of tests. After the last aggression test, all mice were gonadectomized. Ten days later, a 20 mm silastic capsule containing 1 μg estradiol benzoate (EB) dissolved in sesame oil was subcutaneously implanted into each mouse. Starting on 14 days after EB implants, all mice were tested again for aggression in two consecutive days. All behavioral testing, except the open field test, was done during the dark phase of the light/dark cycle starting 2 hr after lights off. Mice were transferred to the testing room from their housing room 2-4 hr before the testing. Mice were sacrificed by intracardiac perfusion 5-17 days after the last behavioral test for immunohistochemical examination of ERα expression.

Open field behavior test: Mice were tested for two consecutive days for 10 min in an open field apparatus (60×60 cm, 30 cm high opaque walls; O'Hara & Co., Ltd, Japan) illuminated by white lights (50 lux) during the light phase (2-4 hr before lights off) of the light/dark cycle. The floor of the apparatus was hypothetically divided into 25 square sections (12×12 cm each) with 9 inner squares designated as the center area (48×48 cm). At the beginning of each 10 min trial, the mouse was placed in the same corner with its head facing the corner. Activity were monitored and analyzed on a Macintosh computer using Image OFC 2.03 (O'Hara & Co., Ltd), modified software based on the public domain NIH Image program (developed at the U.S. National Institute of Health and available on the internet at *http://rsb.info.nih.gov/nih-image/*). The total horizontal moving distance (total distance), number of vertical activity (leaning and rearing), and time spent in center area were recorded and averaged for two tests for each mouse.

Light-dark transition test: Each mouse was tested once for its anxiety-related behaviors in the light-dark transition test apparatus (O'Hara & Co., Ltd) for 10 min. The test apparatus consisted of enclosed dark (0 lux) and open-top light (350 lux) compartment boxes (40×20 cm, 25 cm opaque high wall each) connected by an inner doorway (2×5 cm) in the center. The test mouse was gently introduced in the dark compartment, and 5 sec later, the doorway between the two boxes automatically opened. Latency to enter the light compartment, cumulative time spent in the light compartment, and number of transitions between two compartments were recorded using Image J LD2 software (O'Hara & Co., Ltd), modified software based on the public domain Image J program.

Sexual behavior test: Each male mouse was tested

in its home cage for sexual behavior toward ICR/Crl female mice for 30 min. All female stimuli mice were ovariectomized and subcutaneously injected with 10 μg of EB at 48 hr and 24 hr before, and 500 μg of progesterone 4 to 6 hr before the testing to ensure high receptivity. Each male was tested three times with intervals of 3 to 7 days, and encountered a different receptive female for each test. All behavioral tests were video recorded with a digital camera. For each male, the number of mounts, intromission, and ejaculation, and latency to each behavior were scored by an experimenter unaware of the genotype of the test mouse using a digital event recorder (Recordia 1.0b, O'Hara & Co., Ltd). Latency to any mount and total number of mounts and intromission were averaged for three tests in each mouse and used for statistical analysis.

Resident-intruder paradigm aggression test: Each mouse was tested for aggressive behavior in its home cage against a weight-matched olfactory-bulbectomized ICR/Crl male intruder mouse (resident-intruder paradigm) for 15 min. All mice were tested while gonadally intact once a day in three consecutive days. After gonadectomy, they were tested again on two consecutive days starting 14 days after EB implants. Each test mouse encountered a different intruder mouse in each test. All behavioral tests were digitally video recorded. Cumulative duration of aggressive bouts including at least one of the aggressive behavioral acts (chasing, biting, tail rattling, boxing, wrestling and lateral attack) as well as number of sexual behavior toward male intruder mice (mount and intromission pattern) were recorded for each mouse by an experimenter unaware of the genotype of the test mouse using a digital event recorder. Data from three tests as intact and two tests after gonadectomy were averaged respectively in each mouse and used for statistical analysis.

Measurement of body weight: Body weight were measured weekly during the end of the light phase for 8 weeks as gonadally intact and once at 2 weeks after gonadectomy.

Immunohistochemistry: After the completion of all behavioral tests, mice were deeply anesthetized with a solution of 1:1 mixture of sodium pentobarbital (60 mg/kg) and heparin (1000 units/kg) and transcardinally perfused with 0.1 M phosphate-buffered saline (pH 7.2) followed by 4% paraformaldehyde in 0.1 M phosphate-buffer (PB; pH 7.2). Brains were removed and post-fixed overnight at 4°C in the same fixative solution. They were then rinsed with 0.1M PB and cryoprotected in 0.1M PB containing 30% sucrose. Free-floating coronal sections (30 μm thickness) were prepared on a freezing microtome. They were then incubated in TBS-X (0.05 M tris-buffered saline, pH 7.2 and 0.2% Triton X-100) containing 1% hydrogen peroxide for 20 min to inhibit endogenous peroxidase activity. After thoroughly rinsed with TBS-X, they were blocked in an incubation buffer (3% normal goat serum and 3% bovine serum albumin in TBS-X) for 1 hr at room temperature. They were incubated in a rabbit polyclonal anti-ERα antiserum (1:25,000; C1355, Upstate, USA) dissolved in the incubation buffer for 48 hr at 4°C. Sections were then treated with a 1:250 dilution of biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, USA) in the incubation buffer for 2 hr at room temperature, followed by 1 hr incubation with avidin-biotin complex (Vectastain ABC Elite kit, Vector Laboratories) in TBS. Sections were rinsed with TBS-X or TBS between each incubation step. After the completion of antiserum reaction procedures, sections were visualized with 0.02% diaminobenzidine, 0.15% nickel ammonium sulfate, and 0.003% hydrogen peroxide in TBS. Sections were then rinsed in TBS and mounted on gelatin-coated slides, air-dried, dehydrated through ascending alcohol series, which were cleared with xylene, and coverslipped with Permount (Fisher Scientific, USA).

ERα immune-stained cells and fibers were observed under an Olympus light microscope (BX61, Olympus, Japan) and photographed with a digital camera (DP21, Olympus).

Statistics: Data from the open field and the light dark transition tests were analyzed by a one-way analysis of variance (ANOVA), followed by a Tukey-Kramer post-hoc test. Data for sexual and aggressive behavior were analyzed by a Kruskal-Wallis test followed by a Mann-Whitney nonparametric test. Body weight data were analyzed by a two-way ANOVA for repeated measurements for the main effects of genotype and day. Significant differences were considered

when $p<0.05$. SPSS 14.0J statistical package (SPSS Inc., USA) was used for all statistical analyses.

Results

Non social behaviors: There was a marginally significant genotype group difference in total distance [$F(2,20)=3.46$, $p=0.05$] and KO mice traveled longer distance than WT mice during 10 min open field tests (Figure 1-A). In the number of leaning and rearing ([$F(2,20)=1.60$, n.s.]; Figure 1-B) and the time spent in the center area ([$F(2,20)=0.53$, n.s.]; Figure 1-C), there were no overall statistically significant differences among three genotypes of mice.

In the light-dark transition test, KO mice did not show any alteration in the latency to the first entry to the light compartment ([$F(2,20)=2.09$, n.s.]; Figure 2-A), the time spent in light the compartment

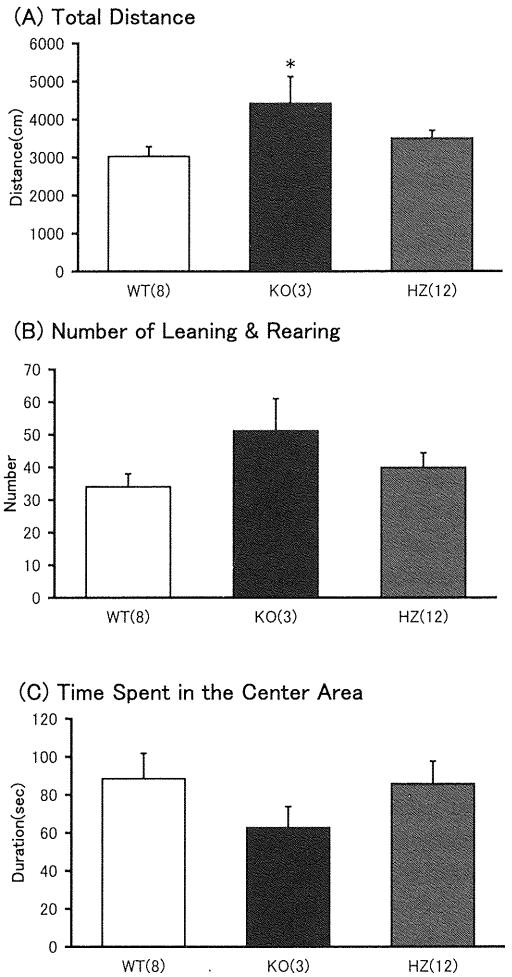

Figure 1. Total distance (A), number of leaning and rearing (B), and cumulative time spent in the center area during 10 min open field test. WT: wild type, KO: neuron specific ERα knockout, HZ: heterozygous mice. Numbers in parentheses indicate the number of mice used in each genotype group. Data presented as Mean + SEM. *: $p<0.05$ vs WT.

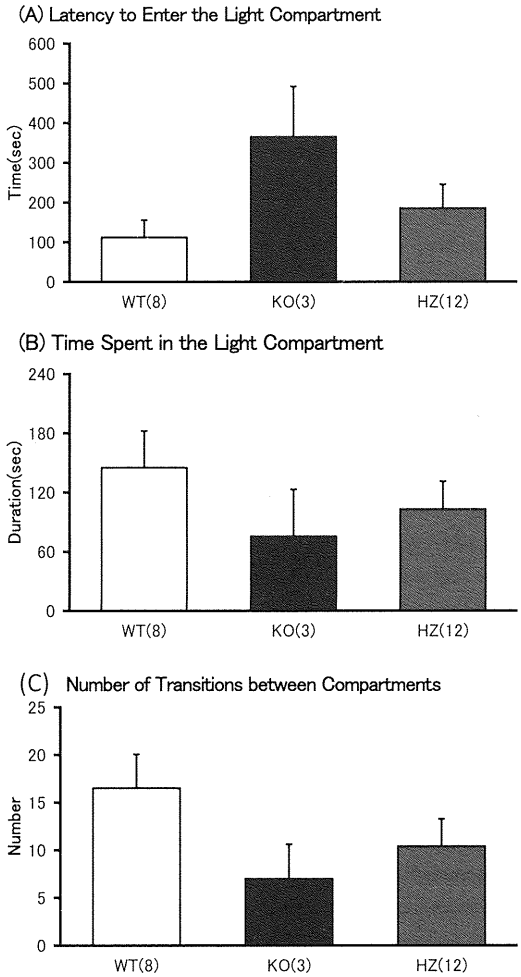

Figure 2. Latency to enter the light compartment (A), cumulative time spent in the light compartment (B), and number of transitions between two compartments during 10min light-dark transition test. WT: wild type, KO: neuron specific ERα knockout, HZ: heterozygous mice. Numbers in parentheses indicate the number of mice used in each genotype group. Data presented as Mean + SEM.

Table 1
Summary of results in sexual and aggresssive behavior tests

		Gonadally Intact			Gonadectomized and EB Treated		
		WT	KO	HZ	WT	KO	HZ
Sexual Behavior Tests	**Latency to the First Mount**						
	756 ± 232.82[a]	1800 ± 0.00+	1353 ± 126.49*				
	171 - 1800[b]	-	533 - 1800				
	Number of Mounts and Intromission						
	13 ± 3.82	0 ± 0.00+	5 ± 1.81				
	0 - 31	-	0 - 18				
	6/8[c]	0/3	8/12				
Aggressive Behavior Tests	**Cumulative Duration of Aggressive Bouts**						
	33 ± 12.75	0 ± 0.00*	9 ± 4.06*	36 ± 17.37	0 ± 0.00	20 ± 9.02	
	0 - 106	-	0 - 34	0 - 114	-	0 - 85	
	5/8	0/3	3/12	5/8	0/3	6/12	
	Sexual Behavior toward Intruder Mice during Aggression Tests						
	2 ± 1	0 ± 0.00	1 ± 0.3	2 ± 0.82	0 ± 0.00	1 ± 0.37	
	0 - 8	-	0 - 3	0 - 7	-	0 - 4	
	4/8	0/3	4/12	4/8	0/3	3/12	

a: Mean ± SEM, b: Range of the data,
c: Number of mice that showed the behavior/total number of mice tested
*: $p<0.05$ vs WT, +: $p=0.06$ vs WT
EB: Estradiol benzoate, WT: Wild type mice, KO: Neuron specific ERa knockout mice, HZ: Heterozygous mice

([$F(2,20)=0.70$, n.s.]; Figure 2-B), and the number of transitions between light and dark compartments ([$F(2,20)=1.44$, n.s.]; Figure 2-C) , as compared to WT and HZ mice.

Social Behaviors: The mutant mice failed to show any component i.e. mount, intromission and ejaculation, of male sexual behaviors (Table 1). In contrast, 75% of WT and 66% of HZ male mice showed male typical sexual behaviors and 40% of them ejaculated at least once in three 30 min tests. The Mann Whitney statistical test reveals significant differences among three genotypes of mice in the latency to the first mount [$H[2]=6.880$, $p<0.05$] and marginally significant differences in the number of mounts and intromissions ($H[2]=5.597$, $p=0.0609$). These results suggest that ERα in neurons is necessary for male mice to display any component of male type sexual behavior.

In addition, none of the gonadally intact KO mice showed any aggressive behavior in the resident-intruder paradigm while 60% of WT and 25% of HZ mice showed aggressive behavior at least once in three 15min tests (Table 1). There were significant differences among three genotypes of mice in the cumulative duration of aggressive bouts [$H[2]=6.772$, $p<0.05$]. After GDX and EB treatment, though 60% of WT and 50% of HZ mice showed aggressive behaviors, none of KO mice showed any aggressive behavior although no statistically significant differences in the cumulative duration of aggressive bouts [$H[2]=2.807$, n.s.]. In addition, some WT and HZ mice both as gonadally intact and after GDX and EB treatment showed male type sexual behavior toward a male intruder mouse during aggressive behavior tests, whereas none of KO mice showed this type of behavior either.

Body Weight: As an average, gonadally intact KO mice were 3g heavier than WT mice although there were not statistically significant differences (Figure 3-A). At 2weeks after gonadectomy, body weight of mice of all three genotypes equally decreased ([genotype: $F[2,20]=2.954$, $p=0.075$; gonadal state: $F[1,20]=21.185$, $p<0.001$; genotype x gonadal state: $F[2,20]=1.933$, n.s.]; Figure 3-B).

ERα Immunostaining: We examined distribution of

ERα positive cells throughout hypothalamic and limbic brain areas and compared among three genotypes. As expected, ERα immunoreactive cells were highly concentrated in a number of brain regions such as the medial preoptic area and hypothalamic ventromedial nucleus in both WT and HZ mice (Figure 4). In contrast, we did not find any nuclear staining for ERα in KO mice although we could detect fibrillary staining specific for ERα in the medial preoptic area and ventromedial nucleus (Figure 4).

Discussion

In the present study, we have found that deletion of ERα selectively in neurons completely abolishes sexual and aggressive behavior in male mice. In our previous studies using αERKO mice in which ERα was absent in every cell including neurons, glial cells, and peripheral cells, both sexual and aggressive behaviors were also greatly reduced in male mice (Ogawa et al., 1997, 1998, 2000; Scordalakes & Rissman, 2003). In these studies, although it was assumed less likely, we could not completely rule out the possibility that alterations induced by ERα deletion in peripheral gonadal cells might contribute behavioral changes via ERα-independent mechanisms in the brain. For instance, testosterone levels known to be slightly higher in αERKO mice compared to WT mice. Therefore, testosterone, after being aromatized to estradiol, might further suppress the ex-

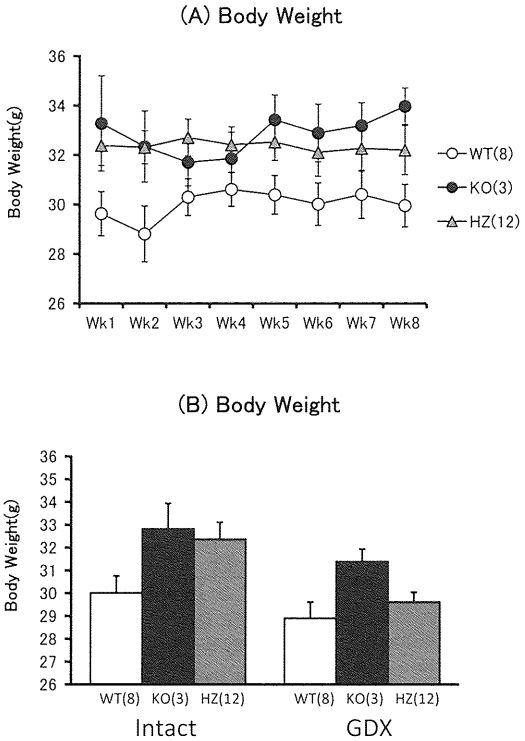

Figure 3. Changes of body weight during weekly measurements as gonadally intact (A), and comparison between gonadally intact and gonadectomized conditions (B). WT: wild type, KO: neuron specific ERα knockout, HZ: heterozygous mice. Numbers in parentheses indicate the number of mice used in each genotype group. Data presented as Mean ± SEM.

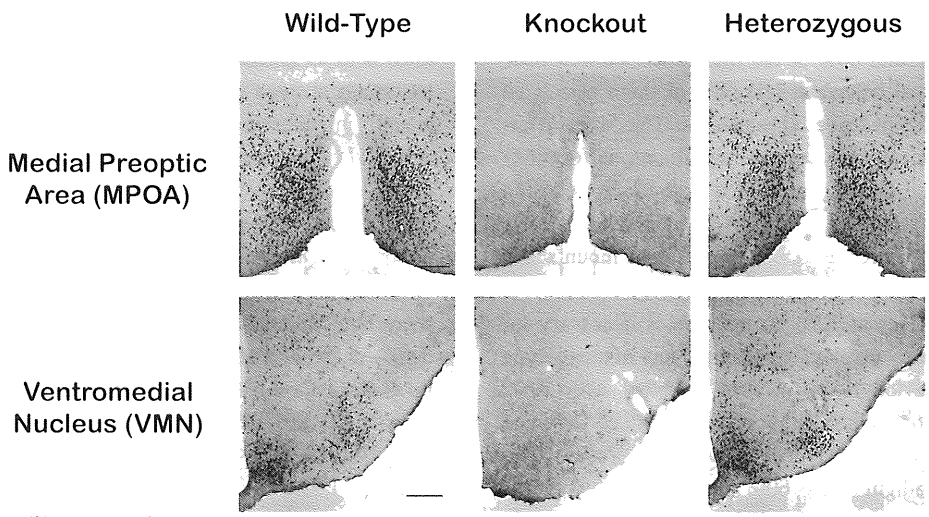

Figure 4. Representative photomicrographs of ERα immunoreactive cells in the medial preoptic area and ventromedial nucleus of the hypothalamus. Scale bar represents 200μm.

pression of aggressive behavior by acting through ERβ, which is known to have an inhibitory role for the induction of this behavior (Ogawa, Chan, Chester, Gustafsson, Korach, & Pfaff, 1999; Nomura, Durbak, Chan, Gustafsson, Smithies, Korach, Pfaff, & Ogawa, 2003). In addition, recent studies have demonstrated that ERα is expressed in many types of cells other than neurons in the brain, including astrocytes, microglia, and endothelial cells. In the present study, we actually found fibrillary staining of ERα in neuron specific ERα KO mouse brain. Therefore, central effects of ERα mediated action of testosterone may not be solely dependent on neuronal ERα. Against all these facts, our findings in the present study clearly demonstrate that ERα expressed in neurons plays a critical role in the expression of male-type social behaviors.

Detailed analysis of sexual and aggressive behavior revealed that behavioral alterations found in neuron specific ERα KO mice in the present study and those previously found in αERKO mice were not completely identical. In present study, we found that all components of sexual and aggressive behavior were completely abolished in the KO mice. On the other hand, it was reported that αERKO mice showed reduced but still low number of mounting behavior although they never showed ejaculation (Ogawa et al., 1997, 1998, 2000). Similarly, although aggressive behavior accompanied with vigorous lateral attacks was never observed, αERKO mice often showed mild type of aggressive behavior (Ogawa et al., 1997, 1998, 2000). Since genetic background are not exactly the same between two types of ERα knockout mice and we have not tested them side-by-side at the same time, it is not possible to directly compare their behavioral characteristics. Nevertheless, it should be noted that more severe behavioral alterations were found in neuron specific ERα KO mice. This fact leads the possibility that sexual and aggressive behavior might be inhibitory regulated by non-neuronal ERα mediated action of gonad steroids. To further investigate the involvement of glial ERα in these behaviors, a comparative behavioral analysis of mice that lack glial ERα is necessary.

In contrast to the stark effect on social behaviors, the selective deletion of ERα in neurons did not alter novel environment activity or anxiety related behaviors of male mice. We only found a marginally significant increase of total distance in the open field test. αERKO males that lack ERα in all types of cells showed a feminized pattern of locomotor activity and lower anxiety in the open field test (Ogawa et al., 1997). Therefore, it is possible that the presence of ERα in glial or peripheral cells preserved masculinization. One of target regions for sexually dimorphic anxiety traits may be the amygdala where estrogen in the neonatal period increases the number of proliferating glia in the basolateral amydala (BLA), solely in the male rat (Dmitar, Slobodan, Bojan, Cvetkovic & Lozance, 1995). It remains to be examined if the glial number in the αERKO male BLA is lower than the WT male and similar to the WT female. In addition, as with the αERKO mice, the non-temporal nature of the gene deletion does not allow us to distinguish organizational versus activational effects in the present study.

Finally, we found a small increase in body weight in ERα KO mice compared to WT mice in the present study. It has been reported that αERKO mice, particularly female αERKO mice, are significantly heavier than WT mice (Heine, Taylor, Iwamoto, Lubahn, & Cooke, 2000; Manrique, Lastra, Habibi, Mugerfeld, Garro, & Sowers, 2012). Relatively smaller effects of neuron specific ERα deletion on body weight suggest glial or peripheral ERα contribution to this trait.

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(Received Sep. 28 : Accepted Oct. 30)