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Title

Female mice avoid male odor from the same strain via the vomeronasal system in an estrogen-dependent manner.

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Abstract

Inbreeding avoidance is essential to providing offspring with genetic diversity. Females' mate choice is more crucial than males' for successful reproduction because of the high cost of producing gametes and limited chances to mate. However, the mechanism of female inbreeding avoidance is still unclear. To elucidate the mechanism underlying inbreeding avoidance by females, we conducted Y-maze behavioral assays using BALB/c and C57BL/6 female mice. In both strains, the avoidance of male urine from the same strain was lower in the low estrogen phase than in the high estrogen phase. The estrous cycle-dependent avoidance was completely prevented by vomeronasal organ removal. To assess the regulation of the vomeronasal system by estrogen, the neural excitability was evaluated by immunohistochemistry of the immediate early gene products. While estrogen did not affect neural excitability in the VNO, estrogen enhanced the neural excitability of the mitral cell layer in the AOB induced by urine from the cognate males. These results suggest that female mice avoid odor from genetically similar males in an estrogen-dependent manner via the vomeronasal system and the excitability of the mitral cells in the AOB is presumed to be regulated by estrogen.

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31

32 **Keywords**

33 accessory olfactory bulb, estradiol, inbreeding avoidance, mouse, pheromone

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Introduction

In many species, females choose their mating partners. For successful reproduction, mate choice by females is more crucial than that by males because the cost of producing gametes is higher and the chance of mating for each breeding season is more limited in females than in males (Janetos 1980; Uy et al. 2001). In choosing a mate, females respond to various sensory cues, including body color, sex ornaments, songs, and odor signals, as is commonly found in fish, amphibians, birds, and mammals (Asaba et al. 2014; Kavaliers et al. 2004; Milinski et al. 2005; Mossman and Drickamer 1996; Schwartz et al. 2001; Sherborne et al. 2007; Wedekind et al. 1995). Such cues can provide females with diverse information about potential mates, including physical condition, ability to reproduce, and genetic compatibility (Hamilton and Zuk 1982; Kennedy et al. 1987; Wedekind et al. 1995). Mating with males with a better physical condition and reproductive ability enables females to obtain offspring with vigorousness and high fertility. An alternative significance is inbreeding avoidance, which results in genetic diversity of offspring, potential adaption to various environments, and protection from recessive diseases (Consuegra and Garcia de Leaniz 2008; Penn et al. 2002). Most of the studies on mammalian inbreeding avoidance have been conducted in mice. Studies on mice under seminatural conditions provided evidence indicating the existence of inbreeding avoidance (Potts et al. 1991; Sherborne et al. 2007). A variety of cues present in odor affect intraspecific communication. For instance, by sniffing odor originated in the urine, male mice can detect only slight differences in females' genetic

backgrounds (Yamazaki et al. 1979) and avoid the odor from genetically similar females (Beauchamp et al. 1988; Yamazaki et al. 1979). In female mice, despite some supporting evidence of inbreeding avoidance (Potts et al. 1991; Robert and Gosling 2003; Sherborne et al. 2007), only a limited line of evidence has suggested that odor is a key component associated with inbreeding avoidance. Our previous report demonstrated that BALB/c females stayed for shorter duration near the urine from BALB/c males compared with that from C57BL/6 males, suggesting their avoidance of urine from males with an identical genetic background (Yano et al. 2012). However, because BALB/c mice were used as subjects in this study, it seems unclear whether genetic difference is a relevant factor or not. The possibility that other unidentified factors make BALB/c mice less attractive should not be excluded, and this question should be addressed.

The vomeronasal system plays important roles in odor communication for reproduction (Munger et al. 2009). The vomeronasal system consists of the vomeronasal organ (VNO), the accessory olfactory bulb (AOB), and subcortical areas such as the medial amygdala, the bed nucleus of the stria terminalis, and the medial preoptic area. Odors bind to specific receptors expressed in vomeronasal neurons of the VNO and the generated signals formed as action potentials are conveyed to mitral cells and granule cells in the AOB. The signals then reach the central regions that are involved in behavioral and endocrine regulation. Lesions of the vomeronasal system impair pheromone-induced behavioral and endocrine responses including sick conspecific-avoidance (Boillat et al. 2015), lordosis behaviors (Haga et al.

2010; Keller et al. 2006) and the induction of estrus by male odor (Oboti et al. 2011). If odor signals induce females' inbreeding avoidance, it is assumed that the vomeronasal system mediates the aforementioned intraspecific communication. In the VNO, 2 types of sensory neurons are identified according to a class of vomeronasal receptors or G proteins (Munger et al. 2009). The neurons with V1R (coupled with $G_{\alpha i2}$), which has a high affinity to volatile pheromones, are distributed to the apical layer of the VNO (Del Punta et al. 2002; Munger et al. 2009), whereas the ones with V2R (coupled with $G_{\alpha o}$), which has an affinity to nonvolatile chemicals, such as peptides and proteins, are localized to the basal region (Haga et al. 2010; Leinders-Zufall et al. 2004; Munger et al. 2009). These neurons innervate the distinct areas of the AOB; the neurons with V1R innervate the anterior AOB, whereas the ones with V2R innervate the posterior AOB. These morphological characteristics indicate that the 2 types of vomeronasal neurons share separate roles in the detection of chemosignals with different chemical natures. The function of the vomeronasal system is affected by steroid hormones, including estrogen (Cherian et al. 2014; Halem et al. 2001). The administration of estrogen enhances the excitability of vomeronasal neurons prestimulated by a male-originated odor (Halem et al. 2001). Interestingly, a few reports are available suggesting that female odor choice is regulated by estrogen (Mossman and Drickamer 1996; Yano et al. 2012). Mossman and Drickamer (1996) reported that the female preference for dominant males was evident only during the mating phase, at which their estrogen level is assumed to be high. As we previously found that BALB/c females' avoidance of BALB/c males was dependent on

estrogen (Yano et al. 2012), this phenomenon was also assumed to be regulated by estrogen.

Therefore, the aim of the present study was to elucidate the possible mechanisms underlying the avoidance of urine from males of the same strain by females. First, whether female mice avoid odor from genetically similar males was examined using a behavioral assay using females of 2 different strains as subjects. Second, possible involvement of the vomeronasal system in odor avoidance was investigated in females from which the VNO had been removed. Finally, potential regulation by estrogen was assessed through behavioral assay and immunostaining of the immediate early gene products in the vomeronasal system. The present study sought to provide novel insight into the reproductive strategy of females.

Materials and methods

Animals

All of the experiments described in this study conformed to the guidelines on the ethical use of animals set by the U.S. National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University. Every effort was made to minimize animal suffering and to reduce the number of animals used. BALB/cByJJcl (BALB/c) and C57BL/6JJcl (C57BL/6) mice were purchased from Clea Japan. Naive females and males in reproduction were kept separately and used for the experiments at the age of 10 to 37 weeks old. The animals were kept at 22°C under a 12:12 hr light/dark cycle with lights on at 0700 with access to food and water ad libitum.

Behavioral assays

Each group consisted of 6-12 BALB/c or C57BL/6 adult females, which were used repeatedly. The correlation between the number of repetitions and avoidance by females was not detected by regression analyses (Yano et al. 2012). The experiments were carried out as described previously (Yano et al. 2012). Briefly, the behavioral assays were conducted with a Y-maze apparatus (50-cm length x 10-cm width x 10-cm height x 3 arms). Cotton impregnated with urine collected by abdominal massage of age-matched BALB/c or C57BL/6 males was placed on each end of the two arms of the Y-maze. The cotton was

separated from the free-moving area by placing mesh fences to prevent direct contacts with urine by females. A female subject was released from the other arm and the behavior was video-recorded for 10 min in the dark. The total duration of the stay in the urine-adjacent areas (within 20 cm from urine) was evaluated. Referring to the protocol of Boillat et al. (2015), D_{ratio} was calculated as an index of avoidance as below:

$$D_{ratio} = \log_2 \frac{D_{dif}}{D_{same}},$$

where D_{dif} is the time of stay near the male urine from the different strain, and D_{same} is that of the same strain. When $D_{ratio} = 0$, it indicates that the time of stay is equal between the 2 urines. $D_{ratio} > 0$ indicates that females stay longer near the male urine from the different strain than the same strain, while $D_{ratio} < 0$ indicates that they stay longer near the male urine from the same strain than the different strain. For example, when $D_{ratio} = 0.3$, the time of stay near the male urine from the different strain is $2^{0.3}$ times ($\doteq 1.23$ times) as long as that of the same strain. All of the behavioral assays were conducted from 1300 to 1800. Following the assays, the estrous cycle was categorized into proestrus, estrus, metestrus, and diestrus stages via the inspection of giemsa-stained vaginal smears. The vaginal smears in proestrus were composed of round epithelial cells and cornified cells. In estrus, the majority of cells were cornified cells without nuclei, and in metestrus, leukocyte appeared in addition to cornified cells. The vaginal smears in diestrus contain large amount of leukocytes and a few epithelial cells.

Vomeronasal organ removal

Twelve BALB/c female mice were divided into 2 groups, a group with vomeronasal organ removal (VNX) and a sham-operated (sham) group. Vomeronasal organ removal was carried out by the midline incision of the palate (Keller et al. 2006) under anesthesia with ketamine (Fujita; 75 mg/kg b.w., i.p.) and medetomidine (Domitor, Zenoaq; 1 mg/kg b.w., i.p.). A midline incision was made in the soft palate extending from the first palatal ridge to the incisors, and the underlying bone was exposed. In the sham group, the incision was closed without any treatment. In the VNX group, the rostral end of the VNO was exposed by drilling, the caudal end of the vomer bone was cut off, and the VNO was excised by gentle twists. The incision was closed with sutures. Atipamezole (Antisedan, Zenoaq; 1 mg/kg b.w. s.c.) was postoperatively administered to the females. The females were treated with carprofen (Rimadyle, Pfizer; 4 mg/kg b.w./day, s.c.) to alleviate pain and ampicillin (Sankyo; 5 mg/kg b.w. /day, s.c.) was given for 3 days; the animals were carefully inspected for possible bleeding and breathing difficulties. Behavioral assays were carried out 3 weeks after the operation.

When the series of the behavioral assays were finished, a histological examination was performed to assess the accomplishment of vomeronasal organ removal by staining with horseradish peroxidase conjugated soybean agglutinin (SBA-HRP). SBA binds to axons of vomeronasal neurons, which project to the glomerular layer of the AOB. This approach is reported to be useful and reliable for detecting intact vomeronasal neurons in mice (Keller et al. 2006; Key and Giorgi 1986). The lack of SBA-HRP staining in the AOB therefore

indicates that the VNO was successfully removed. Transcardial perfusion with PBS was followed by fixation with 4% paraformaldehyde (Wako) in PBS in anesthetized females. The olfactory bulb was postfixed with 4% paraformaldehyde overnight and immersed in 10% and 20% sucrose in PBS overnight successively. Brains were frozen in OCT compound (Funakoshi) and stored at -80°C until sectioning; 20- μ m-thick slices were cut sagittally and mounted on MAS-coated glass slides (Matsunami). The sections were treated with 3% normal goat serum (NGS, Sigma)/1% H₂O₂ in PBS for 2 h, incubated in SBA-HRP (Sigma; 15 μ g/ml) in PBS for 40 min, and treated with diaminobenzidine (Sigma; 0.5 mg/ml)/0.1% H₂O₂ in PBS for 5 min. After every step, the sections were washed 3 times with PBS under gentle agitation. The specimens were examined under an optical microscope.

Estradiol administration

BALB/c females were ovariectomized under anesthesia and subcutaneously implanted with silicon adhesive-sealed silicon tubes (inner diameter: 1.5 mm, outer diameter: 2.5 mm, full length: 14 mm) filled with 15 μ l of 0 or 270 μ g/ml β -estradiol (Wako) dissolved in sesame oil (Dubal et al. 2001; Yano et al. 2012). After ovariectomy, the mice were treated with the drugs as stated above for postoperative care. The serum concentration of estradiol was measured after the treatment with β -estradiol or sesame oil (n = 3 per group) using an Estradiol EIA Kit (Cayman). 4 days after each treatment, blood was collected from the left ventricle. Sera were frozen at -80°C until the assay.

184

185 **Exposure to male urine**

186 Four days after ovariectomy and treatment with estradiol or sesame oil, free-moving
187 BALB/c females were exposed to water or male urine collected from BALB/c and C57BL/6,
188 which was poured over cotton placed in tea balls to prevent direct contact by females. After
189 90 min of exposure, the brains and the VNOs were collected, fixed, cryoprotected, and frozen
190 as described above. The VNOs were decalcified with 5% EDTA/7% sucrose in PBS (pH 7.4)
191 for 3 days followed by cryoprotection.

192

193 **Immunohistochemistry**

194 The VNOs were coronally sectioned at a 20- μ m thickness in a cryostat and mounted on
195 MAS-coated glass slides. Every 20th slice was collected. The total number of the slices per
196 mouse was 6-7 and the sections were used bilaterally. These sections were washed 3 times for
197 10 min with PBS, pre-treated with 7.5% NGS in 0.1% Triton X-100/PBS (PBSx) for 2 h, and
198 incubated with an antibody cocktail, made of anti-Egr-1 rabbit IgG (sc-189, Santa Cruz;
199 1:250) and anti-G $_{\alpha i2}$ mouse monoclonal antibody (05-1403, Merck; 1:800), in 3% NGS in
200 PBSx overnight at 4°C. The sections were incubated with a second antibody mix, Rhodamine
201 Red-conjugated donkey antibody anti-rabbit IgG (711-296-152, Wako; 1:200) and
202 FITC-conjugated goat antibody anti-mouse IgG (sc-2010, Santa Cruz; 1:200), in 3% NGS in
203 PBSx for 30 min and then incubated with DAPI (KPL; 1:2000) in PBS. The AOBs were

sagittally sectioned at a 20- μ m thickness in a cryostat. Every fifth slice was collected and immersed in PBSx. The total number of the slices from a hemisphere was 3-4. The free-floating sections were washed 3 times with PBSx for 10 min and then blocked with 7.5% NGS in PBSx for 2 h, followed by incubation with the anti-c-Fos rabbit polyclonal antibody (Ab5, Merck; 1:16000)/3%NGS in PBSx overnight at 4°C. The sections were incubated with biotinylated goat antibody anti-rabbit IgG (ABC Elite kit, Vector; 1:200)/3% NGS in PBSx for 30 min, sensitized by incubation with avidin-biotin-peroxidase complex solution (ABC Elite kit, Vector) for 1 h, and reacted with diaminobenzidine for 4 min. These sections were then incubated with anti-G_{ai2} mouse monoclonal antibody (05-1403, Merck; 1:1600)/3% NGS in PBSx for 2 h and then FITC-conjugated goat antibody anti-mouse IgG (sc-2010, Santa Cruz; 1:200)/3% NGS in PBSx for 30 min. The slices were mounted on glass slides. After every step except for post blocking, the sections were washed 3 times with PBS or PBSx under gentle agitation for 5 min. The specimens were imaged using an all-in-one microscope (BZ-9000, Keyence). The number of c-Fos- or Egr-1- positive cells was blindly counted and normalized by a unit of area.

Statistical analysis

Data were analyzed using R ver. 2.12.0. In the behavioral assays, D_{ratio} was expressed as the mean $\pm$ S.E.M. For the comparison between 2 groups in the behavioral assays, Welch's *t*-test was used as the sample size was different. In immunohistological studies, all data were

224 expressed as the mean $\pm$ S.E.M. For the comparison between 2 groups, Student's *t*-test was
225 used. A value of $P < 0.05$ was considered to be significant.

226

Results

Estrous cycle-dependent avoidance of male odor.

Our previous studies demonstrated that BALB/c females avoided the male odor from their same strain in metestrus, diestrus, and proestrus, but not in estrus, which is just after the termination of the mating phase (Yano et al. 2012). In the present study, we conducted a counter experiment using C57BL/6 females as subjects and urine from C57BL/6 and BALB/c males as odor stimuli. As shown in Figure 1A, the avoidance of male odor from the same strain varied according to the stage of the estrous cycle. The duration of the stay near male urine from the same strain was not shorter than near that from the different strain, even in metestrus, diestrus, and proestrus. However, D_{ratio} tended to be smaller in estrus than in the other stages, which was similar to the tendency of BALB/c females. As it was reported that the level of estrogen is lower in estrus than in the other stages in the estrous cycle (Walmer et al. 1992), the data obtained were reanalyzed by dividing into 2 groups, the “low estrogen phase” and the “high estrogen phase,” which corresponded with estrus and the other stages. In C57BL/6 females, the avoidance of the same strain was found to be significantly lower in the low estrogen phase than in the high estrogen phase (D_{ratio} ; low estrogen phase: -0.36 ± 0.14 , high estrogen phase: -0.01 ± 0.07 , $p < 0.05$) (Figure 1B). Similarly, in BALB/c females, the avoidance of the same strain in the low estrogen phase was also lower than that in the high estrogen phase (D_{ratio} ; low estrogen phase: -0.03 ± 0.10 , high estrogen phase: 0.25 ± 0.05 , $p < 0.05$) (Figure 1B).

Involvement of the vomeronasal system in the periodic avoidance of male odor from the same strain.

The glomerular layer completely disappeared in all females in the VNX group (Figure 2A, D), assuring that the VNO was successfully removed. In the sham group, the trends of avoidance were similar to those in intact females (Figure 1, 2B-C), and the D_{ratio} was significantly lower in the low estrogen phase than in the high estrogen phase (D_{ratio} ; low estrogen phase: 0.03 ± 0.10 , high estrogen phase: 0.28 ± 0.07 , $p < 0.05$) (Figure 2C). In contrast, in the VNX group, no significant difference was found between the low estrogen phase and the high estrogen phase (Figure 2E-F).

Estradiol increased c-Fos-positive cells in the AOB in response to male odor from the same strain.

To clarify which area in the vomeronasal system is affected by estrogen, the expression of Egr-1 and c-Fos was investigated in β -estradiol- or sesame oil-treated ovariectomized females. Estradiol did not affect the number of Egr-1-positive neurons in the G_{ai2}^+ or G_{ai2}^- area in the VNO (Figure 3). In the AOB, in contrast, the number of c-Fos-positive cells exposed to male urine from the same strain increased in estradiol-treated females compared with oil-treated females (Figure 4C, F, H-K). Estradiol treatment alone had no effect on the number of c-Fos-positive cells (Figure 4A-B, E, H-K). A significant increase in the number of

267 c-Fos-positive cells was found in the mitral cell layer both in the anterior AOB
268 (c-Fos-positive cells/0.1 mm²; estradiol: 23.6 ± 4.2, oil: 50.1 ± 9.7, $p < 0.05$) and in the
269 posterior AOB (c-Fos-positive cells/0.1 mm²; estradiol: 18.9 ± 1.7, oil: 33.7 ± 3.5, $p < 0.05$)
270 (Figure 4H-I). In the granular cell layer, the number of c-Fos-positive cells was increased by
271 the administration of estradiol, but it was not significant (Figure 4J-K). In contrast, in females
272 exposed to male urine from the different strain, the administration of estradiol did not
273 increase the number of c-Fos positive cells (Figure 4D, G, H-K). On the fourth day after the
274 administration of estradiol, the blood concentration of estradiol was 48.6 ± 4.7 pg/ml. In
275 contrast, after the treatment with sesame oil, the estrogen level dropped to 7.4 ± 2.5 pg/ml,
276 which corresponded to the level in estrus (Walmer et al. 1992; Nelson et al. 1981).

277

Discussion

The avoidance of male odor from the same strain was observed in similar manners in both BALB/c and C57BL/6 females (Figure 1A). This result suggests that odor is a key component in inducing avoidance. The present finding that lesions of the vomeronasal organ completely prevented the female avoidance of male odor from the same strain (Figure 2B-C, E-F) suggests that the vomeronasal system mediates the avoidance of odors from genetically similar males. Some types of chemosignals in mouse urine are reported to represent the degree of genetic similarity (Hurst et al. 2001). The factors that are most likely to represent genetic compatibility are major urinary proteins (MUPs) and major histocompatibility complex (MHC) class-1 molecules because both of them exhibit high polymorphism (Hurst et al. 2001; Roberts and Gosling 2003). MUPs play roles as carriers of volatile ligands, and polymorphism of MUPs contributes to the diversity of volatile chemicals in the urine (Hurst and Beynon 2004). MUPs themselves could also be possible cues to genetic similarity. It is reported that MUPs stimulate the vomeronasal sensory neurons (Chamero et al. 2007). MHC class-1 molecules affect urinary odors containing volatile small molecules and MHC class-1 ligand peptides, and these compounds also could be cues conveying information about genetic compatibility (Wyatt 2014). The potential detection of slight differences in MHC peptides through the vomeronasal system has been demonstrated (Leinders-Zufall et al. 2004; Sturm et al. 2013). Such chemosignals could be involved in the avoidance.

The female avoidance of male urine from the same strain varied between the high estrogen

phase and the low estrogen phase (Figure 1B). Our previous study, which demonstrated that estrogen enhances the BALB/c females' avoidance of male odor from the same strain (Yano et al. 2012), suggests that estrogen regulates female avoidance depending on genetic similarity. Estradiol may not solely regulate the estrous cycle-dependent avoidance because the difference of estradiol level between metestrus/diestrus and estrus is not critical (Walmer et al. 1992; Nelson et al. 1981). Dey et al. (2015) recently reported that progesterone silences MUP-detecting sensory neurons in VNOs and changes behaviors in female mice. Progesterone and other possible estrous cycle-dependent hormones may contribute inbreeding avoidance. However, potential involvement of progesterone has to be carefully interpreted in our present study as inhibition of direct nasal contact may prevent involatile compound including MUP from reaching the vomeronasal sensory neurons. Avoidance of a certain male in the high estrogen phase would reduce the chance of mating with the male because ovulation and mating occur in a dark phase just after proestrus, the last stage in the high estrogen phase. Here, we have to emphasize that "estrus" in this study does not indicate a mating phase but denotes after a mating phase, because we collected vaginal smear in a light phase of a light/dark cycle. Ovulation of female mice occurs in the dark phase after "proestrus". Avoidance of genetically similar males occurs not only in proestrus but also in metestrus and diestrus, which would keep females away from the males and prevent females from mating with the males.

Unexpectedly, in C57BL/6 females, D_{ratio} was almost zero, even in the high estrogen

phase (Figure 1B). This may be due to possible disturbances of the response by unknown components contained in the male urine. Male urine is a cocktail of a variety of compounds. Some of them are attractive or repellent for females (Brechtbühl et al. 2008; Roberts et al. 2010). The urinary composition is highly variable depending on the strain (Roberts et al. 2010). Certain chemicals that are attractive for female mice have been found in urine from C57BL/6 males but not in that from BALB/c males (Roberts et al. 2010). Such chemicals may potentiate the attractive effect of odor from C57BL/6 males, and the avoidance of C57BL/6 males by females from the same strain can become ambiguous.

In the AOB, neural excitability induced by the male urine was found to be enhanced by the administration of estradiol (Figure 4H-I), though estradiol had no effects on the VNO (Figure 3). These results suggest that estrogen affects neural excitability at the level of the AOB, but not the VNO. Conversely, enhancement of neural excitability in the VNO has been reported by administering estradiol to ovariectomized female mice (Halem et al. 1999). A clear interpretation for this discrepancy is unknown, but the possibility that such a difference could be due to differences in the chemosignals used as odor stimuli should not be excluded. In these experiments, male bedding was used as an odor stimulus, while urine was used in the present study. Bedding would contain not only urine but also other odor sources such as feces, hairs, sweat, and tears, some of which can stimulate the VNO (Haga et al. 2010).

In the present study, neural excitability was strongly enhanced by estradiol, particularly in the mitral cell layer in the AOB (Figure 4H-I). The mitral cells form synapses with the

central nervous system (Baum and Kelliher 2009). The enhancement of the neural excitability in the mitral cells can affect that in the central nervous system, affecting behaviors and endocrine status (Ichikawa 2003). How estrogen regulates the neural excitability of the mitral cells in the AOB and whether estrogen has a direct effect on the AOB is unknown. When estrogen can affect the AOB directly, there should be some receptors for estrogen in the AOB. Estrogen activates estrogen receptors such as ER- α and ER- β , but they have not been found in the AOB (Mitra et al. 2003). Recently, novel estradiol receptors have been identified. G-protein coupled receptor 30 (GPR30), which induces acute effects by binding estrogen, is reported to exist on cells in the brain, kidney, ovary, and VNO (Revankar et al. 2005; Cherian et al. 2014). Such a novel estrogen receptor may be involved in the enhancement of neural excitability in the AOB. When estrogen could affect the AOB indirectly, the AOB may be regulated by neurons projecting from other areas, as the AOB forms neural connections with the amygdala and the hypothalamus, which express estrogen receptors (Baum and Kelliher 2009). These mechanisms would regulate both the anterior and posterior areas in the AOB (Figure 4H-I).

Avoidance can be the inverse of preference. However, estradiol enhanced the neural excitability in the AOB only when females were exposed to the male urine from the same strain, but not when they were exposed to water or the male urine from a different strain (Figure 4H-I). These results led us to conclude that females avoid odor from genetically similar males and that estrogen promotes the repelling effect of the odor. Considering the

pattern of the enhancement by estradiol, this idea may be more appropriate to represent the mechanism of inbreeding avoidance than another possible theory that females prefer odors from genetically dissimilar males, though more detailed studies are needed.

The present study demonstrated that female mice avoided odor from genetically similar males in an estrogen-dependent manner via the vomeronasal system, and the excitability of the mitral cells in the AOB was presumed to be regulated by estrogen. This machinery may induce the female inbreeding avoidance of genetically similar males.

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- 485

Figure legends

Figure 1. The ratio of the duration of the stay near male urine in BALB/c females and C57BL/6 females.

In Y-maze assays, the duration of the stay near urine from BALB/c males or C57BL/6 males was evaluated. As an index of avoidance, D_{ratio} was calculated and expressed as the mean $\pm$ S.E.M. The data of BALB/c females were recalculated from Yano et al. (2012). (A) D_{ratio} in each stage of the estrous cycle. (B) D_{ratio} in the high estrogen phase (from metestrus to proestrus) and low estrogen phase (estrus). M: metestrus, D: diestrus, P: proestrus, E: estrus. The numerals near the markers indicate the number of trials. “*” denotes that D_{ratio} was significantly different between the high estrogen phase and the low estrogen phase ($p < 0.05$, Welch’s t -test).

Figure 2. The ratio of the duration of the stay near male urine in VNX and sham females.

Y-maze assays were conducted using BALB/c females whose VNO was removed (VNX) and sham-operated females (sham). (A, D) The removal of the VNO was confirmed by disappearance of the glomerular layer in the AOB, using staining by SBA-HRP. a: anterior, p: posterior, Gl: glomerular layer, Mit: mitral cell layer, Gr: granular cell layer. Scale bars indicate 100 μm . (B-C, E-F) D_{ratio} is given as the mean $\pm$ S.E.M. D_{ratio} in sham females was shown in (B-C) and that of VNX females was shown in (E, F). (B, E) indicate the D_{ratio} in

each stage of the estrous cycle, and (C, F) indicate the D_{ratio} in the high estrogen phase and the low estrogen phase. M: metestrus, D: diestrus, P: proestrus, E: estrus. The numerals near the markers indicate the number of trials. “*” denotes that D_{ratio} was significantly different between the high estrogen phase and the low estrogen phase ($p < 0.05$, Welch’s *t*-test).

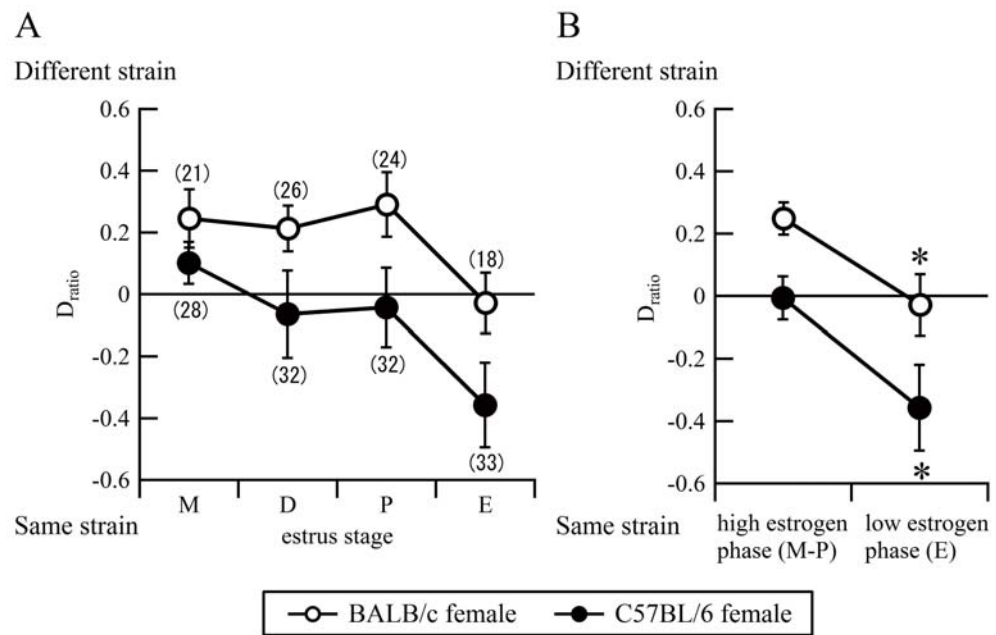
Figure 3. The number of Egr-1-positive cells in the VNO of BALB/c females.

Ovariectomized BALB/c females were treated with estradiol or sesame oil and then exposed to water, BALB/c males’ urine or C57BL/6 males’ urine. (A-C) The VNOs were stained with anti-Egr-1 antibody (A) and DAPI (B). (C) A merged image showing Egr-1 (green) and DAPI (blue). Arrows indicate an Egr-1-positive cell. The scale bar represents 50 μ m. Columns show the mean number of Egr-1-positive cells per 1 mm² in the $G_{\alpha i2+}$ layer (D) and $G_{\alpha i2-}$ layer (E) in the VNO of the females. Columns indicate average values (mean $\pm$ S.E.M.) from 4 or 5 individuals. Filled columns show the means obtained from females treated with estradiol and white columns denote those of sesame oil. Each group contains 4 or 5 individuals.

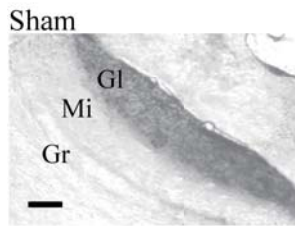
Figure 4. The number of c-Fos-positive cells in the AOB in BALB/c females.

Ovariectomized BALB/c females were treated with estradiol or sesame oil, and then exposed to water, BALB/c males’ urine or C57BL/6 males’ urine. (A) Immunostaining of c-Fos on sagittal sections of the AOB from the females treated with sesame oil and exposed

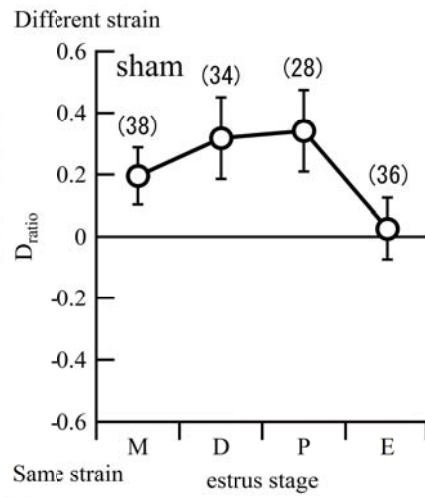
526 to water. a: anterior, p: posterior, Gl: glomerular layer, Mit: mitral cell layer, Gr: granular cell
527 layer. The black box indicates the region magnified in (B-G). (B-D) is the anterior mitral cell
528 layer of oil-treated females, while (E-G) is that of estradiol-treated females. (B, E), (C, F) and
529 (D, G) are from females exposed to water, BALB/c males' urine and C57BL/6 males' urine,
530 respectively. The scale bars represent 100 μm . (H-K) show the number of c-Fos-positive cells
531 per 0.1 mm^2 in the anterior mitral cell layer (H), the posterior mitral cell layer (I), the anterior
532 granular cell layer (J), and the posterior granular cell layer (K). Columns indicate the average
533 values (mean $\pm$ S.E.M.) from 4 or 5 individuals. Filled columns show the means obtained
534 from females treated with estradiol and white columns denote those of sesame oil. $*p < 0.05$,
535 Student's *t*-test.



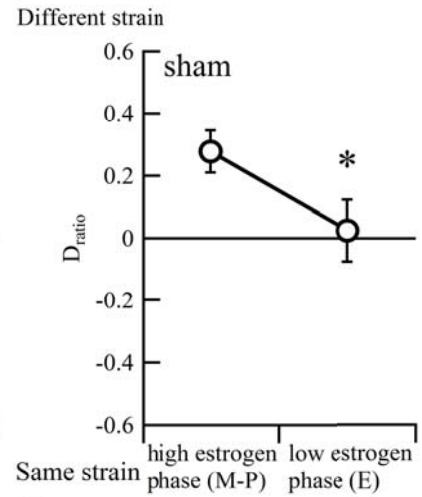
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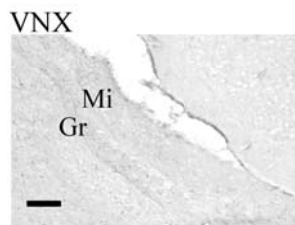
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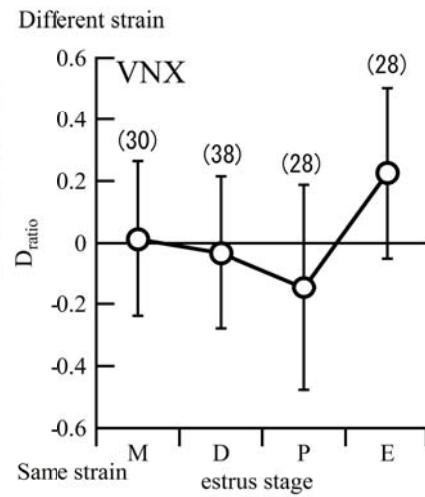
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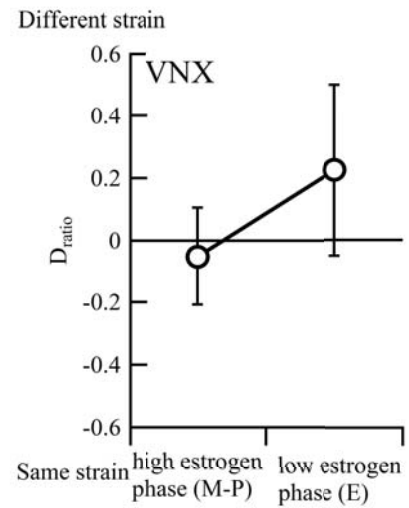
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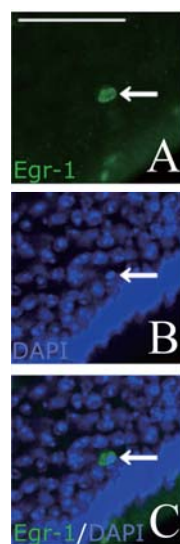


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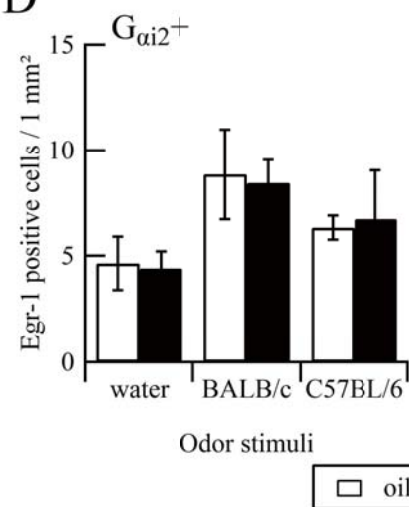


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D



E

