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Changes in Fos expression in the accessory olfactory bulb of sexually experienced male rats after exposure to female urinary pheromones

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Abstract

We studied Fos-immunoreactive (Fos-ir) structures in the accessory olfactory bulb (AOB) of rats after the vomeronasal organ was exposed to urine. Exposure of the vomeronasal organ of male Wistar rats to oestrous and dioestrous female Wistar urine led to the appearance of many Fos-ir cells in the rostral region of the periglomerular cell (PGC) layer, but induced few Fos-ir cells in the caudal region. These results suggest that the regionalization of Fos-ir cells after exposure to female urine is remarkable in the PGC layer of the AOB. Sexually experienced male rats have been shown to prefer oestrous to dioestrous female urine, while sexually inexperienced males do not exhibit these preferences. In the present study, we compared the expression of Fos-ir cells in the AOB of sexually experienced and sexually inexperienced male rats following exposure to oestrous and dioestrous urine. In the localized region (lateral and rostral sectors) of the PGC layer, many more Fos-ir cells were expressed in the sexually experienced rats than in the inexperienced rats. These results suggest that sexual experience in males enhances the transmission of reproductively salient information concerning potential oestrous status to a specific PGC region of the AOB.

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Introduction

Chemical signals excreted from animals affect the sexual behavior of conspecific male and female animals.   For example, sexually experienced male rats and mice prefer reception female odor over non-receptive female odor, but inexperienced males show no preference (Carr et al., 1965; Hayashi and Kimura, 1974).   Information regarding the females' endocrine state is transmitted to males by means of urinary pheromones.   Sexually experienced male rats prefer oestrous to dioestrous urine odor (Pfaff & Pfaffmann, 1969;Lydell & Doty, 1972).   Sexually inexperienced males do not exhibit these preferences, indicating that male rats develop the ability to process odor information regarding a female's state of sexual receptivity after sexual experience. The sexual behavior and olfactory interest in female odors of male mice carrying a knockout of the imprinted gene Peg3 does not change with sexual experience, suggesting that sexual experience-dependent olfactory learning is mediated by genomic imprinting (Swaney et al., 2007).

The vomeronasal organ detects pheromones related to sexual behaviors (reviewed in (Keverne et al., 1986;Wysocki & Meredith, 1987;Halpern & Martinez-Marcos, 2003)).   The sensory neurons of rats and mice possess cell bodies located in the apical and basal layers of the vomeronasal sensory epithelium, and these cell bodies express the GTP-binding proteins $G_{i2\alpha}$ and $G_{o\alpha}$, respectively (Jia & Halpern, 1996).   Apically and basally situated sensory neurons have been described as respectively projecting to the rostral and caudal regions of the accessory olfactory bulb (AOB) (Jia & Halpern, 1996).   Pheromonal information transmitted via mitral/tufted cells (MTC) is modified by GABA-immunoreactive interneurons at the periglomerular cell (PGC) layer, and by granule cells (GCs) at the MTC layer (Keverne et al. 1986;Wysocki & Meredith,

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1987;Halpern & Martinez-Marcos, 2003).

The modulation of gonadal function by the smell of urine has been well established in rodent vomeronasal organs (Keverne et al., 1986;Wysocki & Meredith, 1987;Halpern & Martinez-Marcos, 2003).　Attraction thresholds of female mice for volatile odors from male and estrous female urine are lower than that of males, indicating a sex dimorphism in the detection and/or processing of odor information in urine by the olfactory system (Baum and Keverne, 2002).　In a previous study, we demonstrated that the sensory neurons of female rats, which respond to male Wistar urine, are localized in the apical layer of the epithelium, where $G_{i2\alpha}$ is selectively expressed (Inamura et al., 1999b, Fig. 1).　Exposure of the vomeronasal organ of the female Wistar rat to male Wistar urine induces c-Fos expression, which is correlated with cellular activity, primarily in the rostral region of the AOB (Inamura et al., 1999a). In the present study, we first addressed the question of whether neurons in the rostral region of the AOB of male rats preferentially respond to female or male urine.　We then compared the expression of Fos-immunoreactive (Fos-ir) cells of sexually experienced and inexperienced males after exposure to oestrous or dioestrous urine in order to explore changes in cellular responses to urinary pheromones as related to sexual experience.

Materials and Methods

All experiments were carried out in accordance with Guidelines for the Use of Laboratory Animals of the Graduate School of Pharmaceutical Sciences, Hokkaido University.

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Animals

The Wistar rats obtained from Sankyo Laboratory Co., Sapporo, Japan, were housed in same-sex groups of four and were kept in a room at 22 ± 0.5°C and 58% relative humidity on a 14-hr light/10-hr dark cycle (light off at 21:00).   The males and females were housed in the same room.   All rats had free access to food and water.

Stimulation with urine

The noses of forty adult male Wistar rats more than 6 months old with or without sexual experience were subjected to a spray of fresh urine from female Wistar rats or a control salt solution for 30 minutes.   The rats were gently held by hand during the spraying phase of the experiment, and no anesthetics were used.   The control salt solution consisted of the following (in mM): 100 NaCl, 200 KCl, 0.7 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-NaOH, pH 7.4.   Urine was collected from four or five rats using a metabolic cage just prior to exposure.   To obtain oestrous and dioestrous urine, the oestrous cycle was determined based on the observation of fresh vaginal smears of female Wistar rats (less than 6 months old).   The animals were deeply anesthetized with pentobarbital sodium (35 mg/kg) 75 minutes after exposure to the stimulus.

Tissue processing and immunohistochemistry

The animals were perfused through the heart with phosphate-buffered saline (PBS), followed by fixation with 4% paraformaldehyde.   The olfactory bulbs, still connected to the brain, were removed; the tissue samples were soaked in identical fixative solution overnight, and then were sliced in a serial manner on a vibratome at a thickness of 50 μm.   We typically obtained approximately 11 sagittal sections

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containing the PGC, MTC, and GC layers.   Of these, the third and ninth sections along the medial-lateral axis were selected for $G_{i2\alpha}$ immunostaining.   The sixth section was selected as a control for Fos immunostaining.   The remaining sections were used for Fos immunostaining.   The free-floating sagittal sections were first treated with 0.3% H_2O_2 for 15 min in PBS with 0.4% Triton X-100 (PBSx) or PBS for Fos or $G_{i2\alpha}$ immunostaining, respectively, followed by two washes of PBS.   After 1-hr incubation in 3% normal goat serum, the sections were incubated overnight at room temperature with c-Fos polyclonal antibody (1:8000, Ab-5; Oncogene Research Products, Cambridge, MA) in PBSx for 24 hr with or without antibody to a synthetic peptide fragment of $G_{i2\alpha}$ (1:5000; Wako, Osaka, Japan).   The control section was incubated without c-Fos antibody.   All sections treated in this manner were then rinsed in PBSx or PBS and incubated with biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA) for 1 hr, respectively.   The sections were rinsed again in PBSx or PBS, incubated with ABC (ABC Elite kit, Vector) for 1 hr, and developed with DAB/H_2O_2 (0.05% DAB and 0.003% H_2O_2 in 0.05 M Tris-HCl buffer) for 12 min or 4 min.   The sections were rinsed with water and mounted.   All of the counting of Fos-immunostained cells was carried out using a microscope, and Fos-ir cells in the AOB were counted by a person blinded to the experimental protocol.   The area of the PGC layer, the MTC layer, and the GC layer in the photographs of all stained sections of the AOB were measured using SigmaScan Pro software (SPSS Inc. Chicago, IL).   Differences in cellular responses as a function of source of urine, sexual experience, and region of the AOB were analyzed using a repeated-measures analysis of variance (ANOVA) with Fisher's PLSD post-hoc test.   Data are given as mean ± standard error of the mean.

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Results

Fos-immunoreactivity in the AOB after exposure to urine

Figure 2a shows a schematic drawing of the AOB. Exposure of the vomeronasal organ of sexually experienced male Wistar rats to urine from oestrous female Wistar rats induced Fos-immunoreactivity in various cells of the AOB (Fig. 2b). Fos-ir cells were found in the PGC layer, the MTC layer, and the GC layer. The greatest density of labeled cells was observed in the GC layer. Fos-immunoreactivity was not uniform in the AOB along the rostral-caudal axis. For example, a remarkable number of Fos-ir cells appeared in the rostral region of the PGC layer, whereas few were observed in the caudal region. Exposure to dioestrous urine also preferentially induced the expression of Fos-ir cells in the rostral region of the AOB (Fig. 2c). In the sexually inexperienced males, exposure to oestrous or dioestrous urine induced expression of Fos-ir cells in a pattern similar to that observed when sexually experienced males were exposed to dioestrous urine (Fig. 2d and e).

Increases in Fos-immunoreactivity in response to urine

Figure 3 shows the density of Fos-ir cells (number/mm^2) in serial sagittal sections of the AOB of sexually experienced and inexperienced males after exposure to oestrous female urine and the control salt solution. The boundary between the rostral and caudal regions of the AOB was identified using immunostaining with anti-$G_{i2\alpha}$ antibody (data not shown). The data from each group were cast into a four-factor ANOVA as follows: with or without urine stimulation, layers (PGC, MTC, and GC), region (rostral or caudal), and slice number (medial to lateral). In males with sexual experience, this analysis revealed a main effect of control salt solution versus oestrous urine (F(1, 612) =

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127.027, p < 0.0001).   Fisher's PLSD post-hoc testing indicated that the density of Fos-ir cells in rats after exposure to oestrous urine was higher than that after exposure of the rats to the control salt solution (p < 0.0001).   A four-factor ANOVA also found a main effect of control salt solution versus urine preparations in sexually experienced males (to dioestrous urine: $F(1, 426) = 29.002$, p < 0.0001) and in sexually inexperienced males (to oestrous urine: $F(1, 378) = 121.041$, p < 0.0001; to dioestrous urine: $F(1, 384) = 89.97$, p < 0.0001).   Fisher's PLSD post-hoc testing indicated that the Fos-ir cell density in rats with or without sexual experience, and after exposure to either oestrous or dioestrous urine, was higher than that of rats that had been exposed to the control salt solution (p < 0.0001).

Moreover, a main effect of layers and an interaction between stimulation and layers were found to be significant (experienced male exposed to oestrous urine: $F(2, 612) = 80.655$, p < 0.0001: $F(2, 612) = 20.74$, p < 0.0001; experienced male exposed to dioestrous urine: $F(2, 426) = 29.038$, p < 0.0001: $F(2, 426) = 11.187$, p < 0.0001; inexperienced male exposed to oestrous urine: $F(2, 378) = 166.144$, p < 0.0001: $F(2, 378) = 57.744$, p < 0.0001; inexperienced male exposed to dioestrous urine: $F(2, 384) = 136.552$, p < 0.0001: $F(2, 384) = 43.961$, p < 0.0001).

Differential expression of Fos-immunoreactivity in the rostral and caudal regions of the AOB

Next, we focused on the differential expression of Fos-immunoreactivity in sexually experienced and inexperienced males after their exposure to urine from oestrous or dioestrous females.   The Fos-ir cell densities were cast into a five-factor ANOVA as follows: with or without sexual experience, type of urine (oestrous or

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dioestrous), layers (PGC, MTC, and GC), region (rostral or caudal), and slice number (medial to lateral).   This analysis revealed a main effect of rostral-caudal regions (F1, 1416) = 332.823, p < 0.0001).   Fisher's PLSD post-hoc testing indicated that the density of Fos-ir cells in the rostral region of the AOB after exposure to urine was higher than that in the caudal region (p < 0.0001).   The analysis also revealed a main effect of layers (F(2, 1368) = 425.616, p < 0.0001) and an interaction between rostral-caudal region and layers (F(2, 1416) = 185.473, p < 0.0001).   We further analyzed differences in Fos-immunoreactivity in the rostral and caudal regions in the PGC, MTC, and GC layers.   Four-factor ANOVA revealed main effects of the rostral-caudal region in the PGC layer (F(1, 472) = 547.9, p < 0.0001), MTC layer (F(1, 472) = 120.266, p < 0.0001), and GC layers (F(1, 472) = 164.5051, p < 0.0001), indicating significant regional differences in Fos-ir cell density in the PGC, MTC, and GC layers of the rostral and caudal regions.

Interactions between layers, sexual experience, and types of urine

Sexually experienced male rats have been shown to prefer oestrous to dioestrous urine, whereas sexually inexperienced males do not exhibit these preferences (Pfaff & Pfaffmann, 1969;Lydell & Doty, 1972).   Five-factor ANOVA revealed no main effect of sexual experience (F(1, 1416) = 1.788, p = 0.182) and type of urine (F(1, 1416) = 0.612, p = 0.434), although there were significant interactions between layers, sexual experience, and types of urine (F(2, 1416) = 4.116, p = 0.0165), and between rostral-caudal regions, layers, sexual experience, and types of urine (F(2, 1416) = 3.078, p = 0.0464).   We compared the expression of Fos-ir cells in sexually experienced and inexperienced males after exposure to oestrous or dioestrous urine in the PGC, MTC,

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and GC layers.

Differential expression of Fos-immunoreactivity in the PGC layer of the AOB of sexually experienced and inexperienced males

In the present study, we demonstrated higher levels of expression of Fos-ir cells in the rostral than in the caudal region of the male AOB after exposure to female urine. The difference in the density of Fos-ir cells after exposure to urine was most striking in the PGC layer (Fig. 2).   In sexually experienced male rats, **a colony of** Fos-ir cells appeared in the rostral region of the PGC layer (arrowheads) after exposure to oestrous urine (Fig. 2b).   **The number of Fos-ir cells observed in the PGC layer after the exposure of sexually experienced males to dioestrous urine or after the exposure of sexually inexperienced males to oestrous or dioestrous urine was less than that after the exposure of sexually experienced males to oestrous urine** (Fig. 2).   **Thus, t**he difference in Fos immunoreactivity of the AOB of sexually experienced males after exposure to oestrous and dioestrous urine was significant in the PGC layer.   Therefore, we focused primarily on the expression of Fos-ir cells in the PGC layer.

Differences in the expression of Fos-ir cells in the PGC layer of sexually experienced and inexperienced males exposed to either dioestrous or oestrous urine were further analyzed by dividing the area along the rostral-caudal axis in the AOB into four regions (Fig. 4, inset).   The density of Fos-ir cells in the PGC layer of different groups was cast into a three-factor ANOVA as follows: animals with or without sexual experience, type of urine (oestrous or dioestrous), and 32 locations (4 regions along the rostral-caudal axis in slices 1 to 8).   This analysis revealed a main effect of types of stimuli ($F_{(1, 944)} = 8.101$, $p = 0.0045$), location ($F_{(31, 944)} = 53.442$, $p < 0.0001$), and

an interaction between sexual experience and type of urine ($F(1, 944) = 25.683$, $p <$ 0.0001).   Fisher's PLSD post-hoc testing indicated that the density of Fos-ir cells in the sexually experienced males was greater after exposure to oestrous urine than after exposure to dioestrous urine ($p < 0.0001$).   The density of Fos-ir cells after exposure to oestrous urine in the sexually experienced males was also higher than that of Fos-ir cells in sexually inexperienced males after exposure to oestrous ($p < 0.0001$) or dioestrous ($p < 0.0001$) urine.

The pattern of expression of Fos-ir cells in sexually experienced males after exposure to oestrous urine differed from that of **the** same group after exposure to dioestrous urine ($p < 0.0001$, Fisher's PLSD), and also differed from that of sexually inexperienced males after exposure to oestrous ($p < 0.0001$, Fisher's PLSD) or dioestrous ($p = 0.0006$, Fisher's PLSD) urine.   In the first region at the rostral-caudal axis, the density of Fos-ir cells in the second ($p = 0.0081$) and fourth ($p = 0.0463$) slices at the medial-lateral axis of sexually experienced males after exposure to dioestrous urine was more than three times that after exposure to dioestrous urine (Fisher's PLSD). Most Fos-ir cell density in these sectors of sexually inexperienced males after exposure to oestrous urine (Fig. 4d) was either similar to the Fos-ir cell density observed after exposure of the animals to dioestrous urine (Fig. 4c).   In the first region at the rostral-caudal axis, the density of For-ir cells in the first ($p = 0.0046$) and second ($p = 0.0431$) slices at the medial-lateral axis of sexually experienced males after exposure to oestrous urine was larger than that of sexually inexperienced males (Fisher's PLSD).

Figure 5 shows differences in the expression of Fos-ir cells in the PGC layer in animals exposed to either dioestrous or oestrous urine.   In sexually experienced males, the Fos-ir cell density in the lateral-rostral sectors after exposure to oestrous urine was

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much higher than that after exposure to dioestrous urine (Fig. 5a).   However, in

sexually inexperienced males, Fos-ir cell density in these sectors after exposure to

oestrous urine was similar to that after exposure to dioestrous urine (Fig. **5c**).   Thus,

the expression of Fos-ir cells in the rostral-caudal sectors after exposure to oestrous

urine differed markedly between sexually experienced and inexperienced males.

Differential expression of Fos-immunoreactivity in the MTC layer of sexually

experienced and inexperienced males

The density of Fos-ir cells in the MTC layer of sexually experienced or

inexperienced males exposed to dioestrous or oestrous urine was analyzed by dividing

the area along the rostral-caudal axis in the AOB into four regions (Fig. 6, inset); this

series was carried out according to methods similar to those used for examination of the

PGC layer.   Three-factor ANOVA analysis revealed a main effect of type of stimulus

$(F(1, 926) = 8.736, p = 0.0032)$, sexual experience $(F(1, 926) = 26.317, p < 0.0001)$, and

location $(F(1, 926) = 12.545, p < 0.0001)$.   The **density** of Fos-ir cells in sexually

experienced males after exposure to oestrous urine **was less than** that of the same group

after exposure to dioestrous urine $(p = 0.0042$, Fisher's PLSD).   In addition, different

Fos-ir cell expression patterns were observed between the experienced and sexually

inexperienced males after exposure to oestrous urine $(p < 0.0001$, Fisher's PLSD) or

dioestrous urine $(p < 0.0001$, Fisher's PLSD).   However, significant differences were

not observed in individual sectors between sexually experienced and inexperienced

males after exposure to oestrous and dioestrous urine.

Expression of Fos-immunoreactivityin the GC layer of sexually experienced and

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inexperienced males

Differences in the expression of Fos-ir cells in the GC layer of sexually experienced and inexperienced males exposed to either dioestrous or oestrous urine were analyzed by dividing the area along the rostral-caudal axis in the AOB into two regions.   Three-factor ANOVA analysis revealed no main effect of type of stimulus ($F_{(1, 472)} = 0.299$, $p = 0.5847$) or sexual experience ($F_{(1, 472)} = 0.54$, $p = 0.4628$). Fisher's PLSD post-hoc testing indicated no difference between patterns of expression of Fos-ir cells in sexually experienced and inexperienced males after exposure to oestrous or dioestrous urine.

Discussion

The number of Fos-ir cells in the male rat rostral AOB after exposure to female rat urine was higher than that in the caudal AOB.   The density of Fos-ir cells in the PGC layer of sexually experienced males after exposure to oestrous urine was much higher than that after exposure to dioestrous urine.   In the lateral and rostral sectors of the PGC, many more Fos-ir cells were observed in the sexually experienced rats than in the inexperienced rats.   Only a small difference in Fos-ir cell density was observed between the MTC and GC layers of the AOB of sexually experienced males after exposure to oestrous and dioestrous urine in these region.   In the sexually inexperienced males, the density of Fos-ir cells after exposure to oestrous urine was similar to that after exposure to dioestrous urine in all three layers.

Rostral and caudal differences in the patterns of Fos-immunoreactivity of male rats in response to female urine

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We first addressed the question of whether or not neurons in the rostral or caudal region of the AOB of male rats preferentially respond to female urine. Two families of G-protein coupled receptors (vomeronasal GPCRs) unrelated to olfactory GPCRs have been cloned from the rat and mouse vomeronasal epithelium (Dulac & Axel, 1995; Matsunami & Buck, 1997;Ryba & Tirindelli, 1997; Herrada & Dulac, 1997). It is worthwhile to discuss the present results in the context of GPCRs. One family, V1R, consists of sensory neurons expressing $G_{i2\alpha}$ in the upper layer of the epithelium (Dulac & Axel, 1995), and another family, V2R, consists of neurons expressing G_o (Herrada & Dulac, 1997; Ryba & Tirindelli, 1997; Matsunami & Buck, 1997). No differences in hybridization patterns have been observed for the latter type of vomeronasal GPCRs between male and female rats in terms of the laminar distribution of G_i- and G_o-positive VNO neurons (Ryba & Tirindelli, 1997).

The vomeronasal sensory neurons that respond to male or female Wistar rat urine are localized in the apical or basal layer of the sensory epithelium, respectively (Inamura et al., 1999b). The application of male rat urine preferentially induces the expression of Fos-ir cells in the rostral region of the female rat AOB (Inamura et al., 1999a). These results suggest that the response to male Wistar urine is induced via the former type of GPCR (i.e., V1R), whereas responses to female Wistar urine and male Donryu urine are induced via the latter type of GPCR (i.e., V2R). It is expected that female rat urine also preferentially induces Fos-ir cells in the caudal region of the male rat AOB. However, the present study revealed a higher number of Fos-ir cells in the rostral AOB of the male rat after exposure to female rat urine than that in the caudal AOB.

There is good evidence that volatile pheromones activate V1Rs and non

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volatiles activate V2Rs. Thus the VSN of the mutant mice lacking two of the 12 V1R gene families does not respond **to** some volatile pheromones (Punta et al., 2002), while application of a volatile pheromone, 2-heptanone, induces an inward current in isolated V1Rb2 expressing VSNs (Boschat et al., 2002). VSNs expressing a V2R respond to nine-amino-acid peptide pheromones (Leinders-Zufall et al., 2004) and a male-specific 7-kDa peptide (ESP1) secreted from the extra orbital lacrimal gland stimulates V2R-expressing VSNs (Kiminoto et al., 2005). These results suggest that volatile and non-volatile pheromones are received by **V1R- and V2R-expressing neurons** of mice, respectively. In rats, the activity of the component in male urine **that** induce**s** expression of Fos-immunoreactivity in the rostral region as well as in the caudal region of the AOB of females is abolished by pronase treatment (Tsujikawa and Kashiwayanagi, 1999). Exposure of the female rate VNO to either **a** dialyzed urine preparation (<500 Da) or the remaining **constituents** (>500 Da) of male **rat urine** does not induce expression of Fos-ir cells in the rostral and caudal regions of the AOB, whereas exposure to a mixture of these preparations did not induce expression (Yamaguchi et al., 2000). These **findings suggest** that a combination of low and high molecular weight substances is necessary for the activation of **V1R- and V2R-expressing neurons**, and/or the increases in Fos-immunoreactivity in the rostral and caudal regions of AOB of rats. Expression of VR1, a pheromone receptor of the V2R family which is expressed in the basal layer of the vomeronasal sensory epithelium, is higher in gonadectomized male mice than in gonadectomized females (Alekseyenko et al., 2006). These results suggest that unidentified vomeronasal GPCRs, which receive urinary pheromones of combination of low and high molecular weight substances, are expressed differentially **in** male and female rats.

Changes in endocrine state as a consequence of mating

Mating itself and/or odors (pheromones) of mating partners induce(s) changes in levels of gonadal hormones such as luteinizing hormone, prolactin, and testosterone in male rats, mice and hamsters (Frankel, 1984;Fowler et al., 2003; Kamel et al., 1977;Macrides et al., 1974 and 1975).   Endogenous changes in endocrine state can affect various sexual behaviors.   Plasma testosterone levels are higher in old mating rats than in old non-mating rats (Smith et al., 1992;Frankel, 1984).   Testosterone regulates the mating behavior of male hamsters by maintaining the responsiveness of magnocellular division of the medial preoptic nucleus to vaginal secretions from female hamsters (Swann, 1997).   In gonadectomized ferret males and females, the application of testosterone propionate augments the MOB's neuronal Fos responses to pheromones from estrous females.   Androgen receptor-immunoreactivity is present in the GC layer of the MOB of male and female ferrets, suggesting that testosterone acts directly on these cells to augment their responsiveness to pheromones (Kelliher et al., 1998).

In the mammalian brain, adult neurogenesis has been found to occur primarily in the forebrain subventricular zone and dentate gyrus of the hippocampus throughout the entire lifespan (Fowler et al., 2002).   Testosterone also upregulates cell survival, but not cell proliferation in the DG of adult male rats (Galea et al., 2006).   Neurogenesis occurs in female mice that mate with males, implying that forebrain olfactory neurogenesis may contribute to adaptive behaviors in mating and pregnancy (Shingo et al., 2003).   Gonadal steroid hormones also modulate the dendrites of neurons in the adult rat central nervous system.   Testosterone induces an elongation of dendrites of the medial nucleus of the amygdala (Cooke & Woolley, 2005).   Androgen receptor-ir

cells have been observed in the MTC layer and GC layer of the AOB (Portillo et al., 2006), suggesting that changes in plasma testosterone levels could be associated with alterations in sexual behaviour, as well as with partner and olfactory preference for receptive, as perceived via the AOB.   The increase in testosterone induced by mating may affect the receptor distribution/ expression in the AOB.

   As described above, the expression of VR1 is higher in gonadectomized male mice than in gonadectomized females (Alekseyenko et al., 2006).   Estradiol-treated gonadectomized males have lower VR1 expression levels than those of untreated males, suggesting that gonadal hormones also affect the response of vomeronasal organ neurons to chemosignals by altering receptor levels (Alekseyenko et al., 2006).

Changes in responses to oestrous urine in the PGC layer of sexually experienced male rats

Testosterone levels are known to increase with mating in sexually experienced male rats, whereas they are unaffected in sexually naïve rats (Kamel et al., 1977). Sexually experienced male rats prefer oestrous to dioestrous urine, while sexually inexperienced males do not exhibit these preferences (Pfaff & Pfaffmann, 1969;Lydell & Doty, 1972) .   In sexually experienced male rats, the Fos-ir cell density in the lateral-rostral sectors after exposure to oestrous urine is much higher than that after exposure to dioestrous urine (Fig. 4a).   In sexually inexperienced males, exposure to oestrous urine is not associated with an increase in Fos-ir cell density in these sectors (Fig. 4b).

Although **male-soiled bedding** do not attract 'chemically inexperienced female mice, they become attractive after repeated exposure to male soiled bedding, which

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contains non-volatile attractive pheromones (Moncho-Bogani et al., 2002).   Exposure to male bedding directly induces expression of Fos-ir cells in the MTC and GC layers of the rostral region of the AOB of chemically inexperienced female mice.   Exposure to male-derived volatiles, however, does not induce Fos-ir cells in the AOB of chemically inexperienced and experienced females (Moncho-Bogani et al., 2005), indicating that the preference of chemically experienced female mice for male-derived volatiles is not mediated by the vomeronasal system.   As shown in the present study, oestrous urine induces more Fos-immunoreactivity in the PGC layer of the rostral region of the AOB in sexually experienced male rats.   Therefore, it is possible that male-derived volatiles induce Fos-ir cells in the PGC layer of the AOB of chemically experienced male female mice.

In pregnant mice, exposure to urine from males that belong to different strains than those with whom they have mated induces implantation failure (Bruce, 1959). The infusion of an agonist for a metabotropic glutamate receptor (mGluR2) prevents implant failure, suggesting that mGluR2 is involved in the formation of a memory of a mating partner (Kaba et al., 1994).   The size of asymmetrical excitatory synapses (MTCs to GCs) of the reciprocal synapses is larger in the group of female mice exposed to stud male pheromones with mating than that without mating, suggesting that olfactory memory formation is associated with a synaptic structure in the MTC layer of the AOB (Matsuoka et al., 1997).   It is possible that similar changes occurring in the male mouse may well underlie the change in c-fos expression and behavioural output that occurs following mating.   However, mGluR2 may not be directly involved in the enhancement of Fos-expression in the PGC layer of sexually experienced male rats, because mGluR2 expression is restricted to GCs in the rat AOB (Hayashi et al., 1993).

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The preference for oestrous urine in sexually experienced male mice may be supported by changes (or efficiency of transmission) in a number of distributed structures **including** the nucleus accumbens (Swaney et al., 2007).

The mechanism by which sexual experience induces an augmentation of Fos-immunoreactivity to oestrous urine in the PGC layer in a localized region of the male AOB, which indeed is a kind of memory, remains obscure. Also unclear is whether or not the augmentation of Fos-immunoreactivity at the PGC layer is directly related to the preference for oestrous urine among sexually experienced males. The present results suggest that the preference of sexually experienced males for oestrous urine may be brought about by an enhancement of the transmission of information regarding oestrous status to the vomeronasal system, including the PGC in the localized region of the AOB and/or the nucleus accumbens.

Abbreviations

ANOVA , analysis of variance; AOB, accessory olfactory bulb; Fos-ir, Fos-immunoreactive; GPCR. G-protein coupled receptor; GC, granule cell; LH, luteinizing hormone; mGluR, metabotropic glutamate receptor, MTC, mitral/tufted cell; PGC, periglomerular cell; PBS, phosphate-buffered saline; PBSx, PBS with 0.4% Triton

X-100; VSN, vomeronasal nerves

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Figure legends

Figure 1   Schematic drawing of the zonal organization of the vomeronasal sensory

epithelium and accessory olfactory bulb of female rats.   Vomeronasal sensory neurons

in the apical ($G_{i2\alpha}$-positive) and basal ($G_{o\alpha}$-positive) layer of the vomeronasal

epithelium project their axons to the rostral ($G_{i2\alpha}$-positive) and caudal ($G_{o\alpha}$-positive)

regions of the accessory olfactory bulb (AOB), respectively.   Our previous studies

(Inamura et al., 1999a and b) have shown that the vomeronasal sensory neurons of

female Wistar rats that respond to male Wistar urine are localized in the apical layer of

the vomeronasal epithelium, and Fos-ir cells have been shown to appear in the rostral

region of the male AOB after exposure to female urine.   AOB, accessory olfactory

bulb; GC, granule cell layer; MOB, main olfactory bulb; MTC, mitral/tufted cell layer;

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PG, periglomerular cell layer; VNN, vomeronasal nerves.

Figure 2   Fos-ir cells in the male rat AOB after exposure to female urine.   Schematic drawing of the AOB (a).   Sagittal sections of the AOB of sexually experienced males (b and c), and inexperienced males (d and e) after exposure to oestrous urine (b and d) or dioestrous urine (c and e).   Arrowheads indicate the PGC layer in the rostral region. GC, granule cell layer; MTC, mitral/tufted cell layer; PG, periglomerular cell layer.
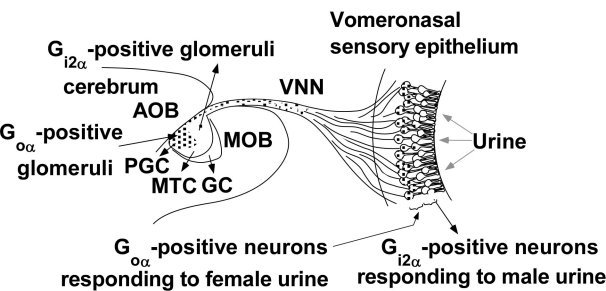
Figure 3   The density of Fos-ir cells (number/mm^2) in the AOB of sexually experienced and inexperienced males after exposure to oestrous urine, dioestrous urine, or control salt solution.   White columns and shadowed columns indicate the density of Fos-ir cells in the rostral and caudal regions of the AOB, respectively.   Vertical bars represent the mean ± standard error of the mean.

Figure 4   Three-dimensional expression of the density of Fos-ir cells in the PGC layer of the AOB of sexually experienced (upper) and inexperienced (lower) males after exposure to oestrous or dioestrous urine.   Inset, the AOB was divided into four regions along the rostral-caudal axis.   The rostral and caudal halves of the $G_{i2\alpha}$-positive region were defined as regions 1 and 2, respectively.   The rostral and caudal halves of the $G_{i2\alpha}$-negative region were defined as regions 3 and 4, respectively.   Vertical bars represent the mean.
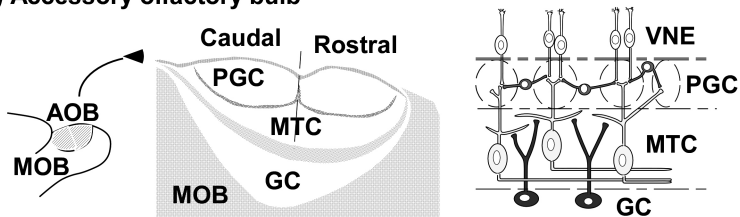
Figure 5   Localized difference in the expression of Fos-ir cells in the PGC layer when sexually experienced males (a) and inexperienced males (b) were stimulated with

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dioestrous or oestrous urine.　　The vertical axis indicates a subtraction of the mean number of Fos-ir cells/mm^2 obtained from three animals after exposure to dioestrous urine from that after exposure of to oestrous urine.
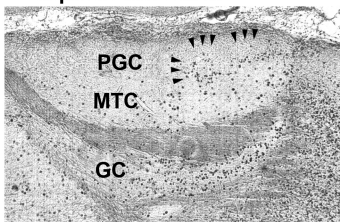
Figure 6　　Three-dimensional expression of the density of Fos-ir cells in the MTC layer of the AOB of sexually experienced (upper) and inexperienced (lower) males after exposure to oestrous or dioestrous urine.　　Inset, the AOB was divided into four regions along the rostral-caudal axis.　　The rostral and caudal halves of the $G_{i2\alpha}$-positive region were defined as regions 1 and 2, respectively.　　The rostral and caudal halves of the $G_{i2\alpha}$-negative region were defined as regions 3 and 4, respectively.　　Vertical bars represent the mean.

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Vomeronasal sensory epithelium

$G_{i2\alpha}$-positive glomeruli

cerebrum

$G_{o\alpha}$-positive glomeruli

AOB

VNN

Urine

MOB

PGC

MTC GC

$G_{o\alpha}$-positive neurons responding to female urine

$G_{i2\alpha}$-positive neurons responding to male urine

a) Accessory olfactory bulb

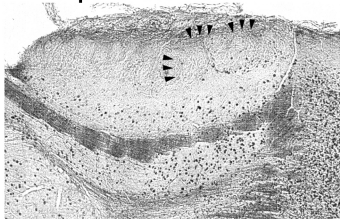Caudal · Rostral
PGC
MTC
GC
AOB
MOB
MOB

VNE
PGC
MTC
GC

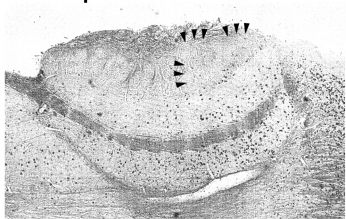**b) Oestrous urine in sexually experienced males**

PGC
MTC
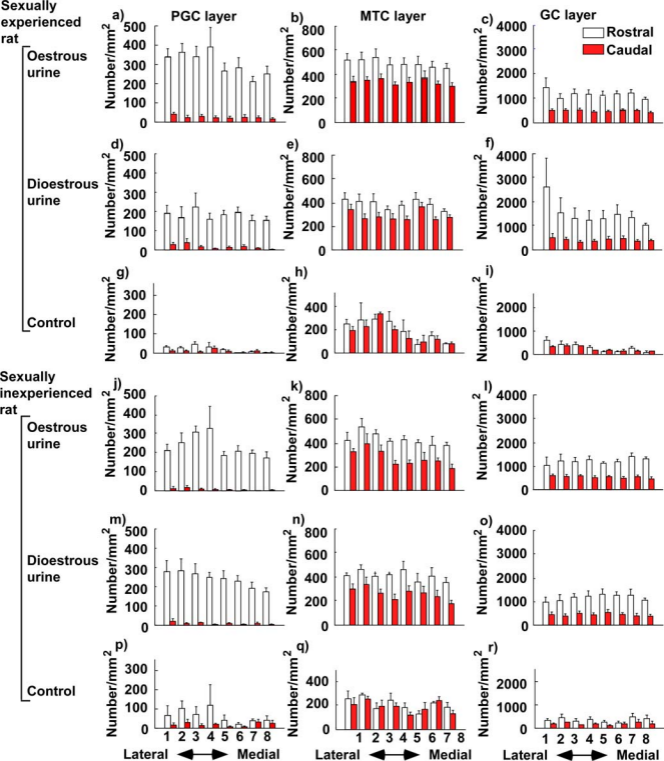GC

c) Dioestrous urine in sexually experienced males
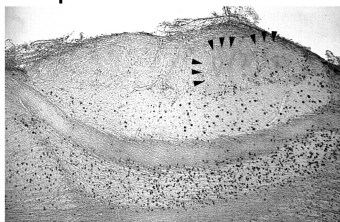
d) Oestrous urine in sexually inexperienced males

e) Dioestrous urine in sexually inexperienced males
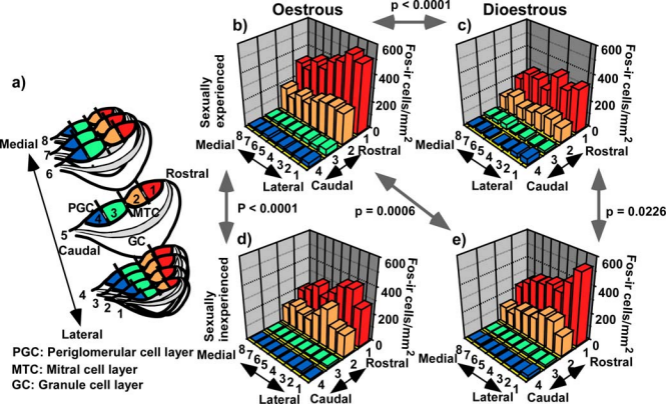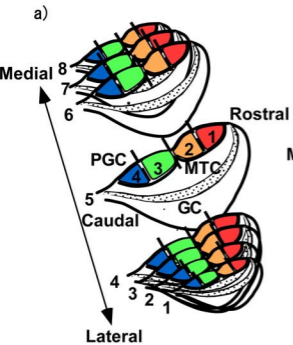
a)

Medial

PGC 3 MTC

GC

Caudal

Lateral

PGC: Periglomerular cell layer
MTC: Mitral cell layer
GC: Granule cell layer

b) Oestrous

p < 0.0001

c) Dioestrous

Sexually experienced

Fos-ir cells/mm²

Medial

Lateral Caudal Rostral

d) Sexually inexperienced

P < 0.0001

p = 0.0006

e)

p = 0.0226

Fos-ir cells/mm²

b) Sexually experienced males

c) Sexually inexperienced males

a)

Medial

Rostral

PGC

MTC

Caudal

GC

Lateral

PGC: Periglomerular cell layer
MTC: Mitral cell layer
GC: Granule cell layer

Oestrous

Dioestrous

p = 0.0042

a)

b) Sexually experienced

c)

P < 0.0001

p < 0.0001

p = 0.0018

d) Sexually inexperienced

e)

PGC: Periglomerular cell layer
MTC: Mitral cell layer
GC: Granule cell layer