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ABSTRACT
Many classes of environmental pollutants, which are found at significant levels in the environment, affect the reproductive functions. The gonadal functions of various animals are regulated by pheromones excreted from mating partners. Pheromones in male urine play essential roles in the sexual maturation of female mice. Pheromones are received by sensory neurons in the vomeronasal organ, which innervate to the accessory olfactory bulb (AOB). The effects of a typical aromatic environmental pollutant (3-methylchoranthrene) on excretion of pheromones from male mice were explored based on neuronal Fos responses of the AOB of female mice. On days 1 and 3 after intraperitoneal administration of 3-methylchoranthrene (3-MC), the density of Fos-immunoreactive (Fos-ir) cells in the AOB of female mice after exposure to urine excreted from the administered males was lower than that after exposure to urine from non administered males. These results suggest that 3-MC blocks chemical communication from male to female mice by reducing pheromonal activities.

Key words: Environment, Pheromone, Toxicology, Male sexual function, testosterone

1. **Introduction**

Many classes of environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), which are found at significant levels in the environment, affect the reproductive functions of males and females. PHAs including 3-methylchoranthrene (3-MC) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are widely distributed in a variety of environmental products such as tobacco and other plants smoke (Konstandi et al., 1997). Exposure of female rats to 3-MC or 4-tert-octylphenol has been found to be associated with a significant increase in estrus cycle length (Blake and Ashiru, 1997; Konstandi et al., 1997). In utero and lactational exposure of male rats to TCDD reduces spermatogenesis and reproductive capability (Mably et al., 1992). As for the reproductive field, environment pollutants affect the endocrine system. Exposure of female rats to TCDD reduces plasma concentration of estrogen and prolactin (Chaffin et al., 1996; Moore et al., 1989). Plasma testosterone concentrations in male rats are reduced by treatment with 3-MC and TCDD (Konstandi et al., 1997; Mably, 1992). Treatment with 3-MC causes substantial increases in metabolism of estradiol in liver microsomes from female rats (Suchar et al., 1996). In human organs, PHAs also modulate endocrine functions. Exposure to 3-MC and benzopyrene increases human chorionic gonadotrophin secretion in human placental explants (Barnea and Shurtz-Swirski, 1992). Most responses to PAHs such as 3-MC and dioxin are mediated by the aryl hydrocarbon receptor (AhR) (Riddick et al., 2003). AhR is present in various tissues of human (Schmidt and Bradfield, 1996). AhR mediates antiestrogenicity in human breast cancer cells (Safe et al., 1998).

The gonadal functions of various animals are regulated by pheromones excreted from mating partners. Pheromones in male urine play essential roles in the sexual maturation of female mice. For example, male urine accelerates puberty and increases the weight of the uterus (Drickamer, 1986; Kaneko et al., 1980). Some volatile and nonvolatile pheromones exhibit a strong dependence on the endocrine status of the animals (Inamura et al., 1997; Knopf et al., 1983; Kuhn et al., 1984; Novotny et al., 1984), suggesting that the production of pheromones is controlled by hormones. We hypothesized that aromatic environmental pollutants such as 3-MC suppress chemical communication between males and females by decreasing pheromonal activities. Pheromones are received by sensory neurons in the vomeronasal organ (VNO), which

innervate to the accessory olfactory bulb (AOB). Urinaly pheromones induces excitatory responses in the vomeronasal sensory neurons (VSNs) (Inamura and Kashiwayanagi, 2000). Immunohistological methods have been used to visualized Fos as a means of identifying neurons that are activated by stimulation (Kaczmarek and Chaudhuri, 1997). We have shown previously that exposure of the VNOs of female rats to male rat urine induces expression of Fos-immunoreactive (Fos-ir) cells in the AOB(Inamura et al., 1999a). The density of Fos-ir cells at the AOB after exposure to urine dilution correlates with pheromonal activities in urine (Yamaguchi et al., 2000). The object of the present study was to determine whether 3-MC interferes with the activity of male urine to stimulate female VSNa as follows. 1) The effects of 3-MC on the urinary excretion of VNO stimulating substances in male mouse urine, assessed by the ability of male urine to induce c-fos expression, as a marker for neural activity, onto female's AOB. 2) Effects of 3-MC on major urinary protein (MUP) content in male urine. 3) Effects of 3-MC on sensitivities of the male VNO to female urine.

2. Materials and methods

All experiments were carried out in accordance with the Guidelines for the Use of Laboratory Animals of the Graduate School of Pharmaceutical Sciences, Hokkaido University. The adult C57BL/6 mice obtained from Sankyo Laboratory, Sapporo, Japan were kept in a room at 22 ± 0.5ºC and 58% relative humidity, with a 12 h light: 12 h dark cycle (light off at 20.00 h). All mice had free access to food and water in the housing cage.

2.1 Urinary stimulation

Fifteen male mice (~50 g) received an intraperitoneal injection of 40 mg/kg 3-MC (98% purity, Sigma-Aldrich Co., St. Louis, MO) dissolved in corn oil (5 mg/ml) as described by Nukaya et al. (2004). Fifteen control mice were injected with corn oil (8 ml/kg) alone. Male urine was collected from more than 10 mice 1 and 3 days after the injection of 3-MC or vehicle using a metabolic cage for one night just before exposure. Female urine was collected from more than 10 mice without any injection. The nose of an adult C57BL/6 female or male mouse with 3-MC dosage was subjected directly to a spray of urine dilutions (20 ml) per animal with a cosmetic atomizer from male or

female mice, respectively, for 30 minutes. Three mice were used under each condition. The mice were freely moving in the small cage during the spray without anesthetic. Fresh urine was diluted twice with the control salt solution, which has a salt composition similar to that of urine. The control salt solution consisted of (mM): 100 NaCl, 200 KCl, 0.7 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-NaOH, pH 7.4. The animals were sacrificed 75 minutes after exposure to the stimulus. The animals were deeply anesthetized with sodium pentobarbital (35 mg/kg) just prior to sacrifice.

2.2 Immunohistochemistry

Immunohistochemical experiments were carried out as described previously (Inamura et al., 1999a). The animals were perfused through the heart with PBS, followed by fixation with 4% paraformaldehyde. The olfactory bulbs, along with the brain, were removed and soaked in the same fixative solution overnight and then cut serially on a vibratome at a thickness of 50 μm. 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) for Fos immunostaining, followed by two washes of PBS. After a 1-hr incubation in 3% normal goat serum, the sections were incubated with c-Fos polyclonal antibody (1:8000, Ab-5; Oncogene Research Products, Cambridge, MA) in PBSx for 24 hr at room temperature. The control section was incubated without c-Fos antibody. The sections were then rinsed in PBSx and incubated with biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA) for 1 hr. The sections were rinsed again in PBSx, 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. The sections were rinsed with water and mounted. All Fos-ir cells in the AOB were counted under a microscope by eye. The sections were analyzed blind by the same person. To estimate the density of Fos-ir cells, the area of the periglomerular cell (PGC) layer, mitral/tufted cell (M/TC) layer and granule cell (GC) layer was measured in photographs of the AOB by SigmaScan Pro (SPSS Inc., Chicago, IL). The densities of Fos-ir cells were compared by two-factor analysis of variance (two-factor ANOVA and three-factor ANOVA) with Scheffe post-hoc testing. Statistical analyses were performed with StatView version 5.0 (SAS Institute Inc., SAS Campus Drive City,

USA). Data are expressed as mean ± SEM.

2.3 SDS-polyacrylamide gel electrophoresis

Urinary proteins were separated on 15% acrylamide gel by SDS-PAGE. Samples (5 μl of crude urine) were subjected to electrophoresis. Proteins were visualized by staining in 0.25% Coomassie blue made in fixative solution.

3. Results

3.1 Fos expression in the AOB after exposure to male urine from mice with 3-MC

Exposure to urine from male mice administered with the vehicle induced Fos expression in the PGC layer, M/TC layer, and GC layer of the AOB of female mice (Fig. 1a), while exposure to urine from males with 3-MC-administration resulted in only a small amount of Fos expression (Fig. 1b). Exposure to urine from males 3 days after the 3-MC-administration induced Fos-ir cells to a similar degree as that in those exposed to urine from males 1 day after administration (Fig. 1c).

In the AOB, the M/TCs directly receive inputs from the VNSs at the glomeruli. Interneurons such as PGCs and GCs provide a lateral inhibition between the M/TCs. The AOB of rodents was divided into $G_{i2\alpha}$-positive rostral and $G_{o\alpha}$-positive caudal regions (Jia and Halpern, 1996). Our previous study indicated that the information detected by the VNSs located in the apical layer of the vomeronasal epithelium and that detected by neurons in the basal layer are transmitted, respectively to the rostral and caudal regions of the AOB (Duchamp et al., 1974; Inamura et al., 1999b; Inamura et al., 1999a). Figure 2 shows the densities of Fos-ir cells (number /mm^2) at the caudal and rostral regions in the PGC layer, M/TC layer and GC layer after exposure to urinary pheromones with or without administration of 3-MC. A three-factor ANOVA found a main effect of with versus without 3-MC administration ($F(3, 48) = 43.45$, $p < 0.0001$), a main effect of layers ($F(2, 48) = 399.241$, $p < 0.0001$), a main effect of rostral region versus caudal region ($F(1, 48) = 131.287$, $p < 0.0001$), a significant administration condition by layers interaction ($F(6, 48) = 15.651$, $p < 0.0001$), and a significant layers by regions interaction ($F(6, 48) = 44.557$, $p < 0.0001$). Fisher's PLSD post-hoc testing indicates that the densities of Fos-ir cells of the mice day 1 ($p < 0.0001$) and days 3 (p<

0.0001) after administration of 3-MC were lower than those day 1 after administration of vehicle.

Next, we focused on the Fos-ir cells at the M/TC layer in further analysis because the activities of the M/TCs well reflect the magnitude of responses at the vomeronasal receptor neurons to pheromones. A two-factor ANOVA found a main effect of with versus without 3-MC administration ($F(3, 16) = 13.063$, $p = 0.0001$), and a main effect of rostral region versus caudal region ($F(1, 16) = 22.332$, $p = 0.0002$). Fisher's PLSD post-hoc testing indicates that the density of Fos-ir cells in the M/TC cell layer of the rostral region of the AOB in female mice after exposure to urine excreted from the administered males was lower than that after exposure to urine from non administered males on day 1 ($p = 0.0089$) and day 3 ($p = 0.0048$) after the intraperitoneal dosage of 3-MC. These results suggest that 3-MC decreases urinary pheromones received by $G_{i2\alpha}$-positive sensory neurons of the vomeronasal organ. In the caudal region, the densities of Fos-ir cells after exposure to urine from mice with 3-MC-administration on day 1 ($p = 0.0258$, Fisher's PLSD) and day 3 ($p = 0.0296$, Fisher's PLSD) were also lower than that without 3-MC-administration, suggesting that 3-MC also inhibits the production of urinary pheromones received by $G_{o\alpha}$-positive VSNs.

The difference in the expression of Fos-ir cells at the M/TC layer between exposure to urine from mice with vehicle-administration and exposure to urine with 3-MC-administration was further analyzed by dividing the area along the lateral-medial axis in the AOB (Fig. 3). Fisher's PLSD testing indicates that the density of Fos-ir cells of the four slices in the rostral region and of three slices in the caudal region after exposure to urine with vehicle was much than that after exposure to control salt solution ($p < 0.05$). The density of Fos-ir cells in the rostral region after exposure to urine with 3-MC-administraiton was less than that after exposure to urine with the vehicle in the three lateral slices ($p < 0.05$). Similarly, the density of Fos-ir cells in the caudal region after exposure to urine with 3-MC-administraiton was less than that after exposure to urine with the vehicle in the two lateral slices (slice number 1, $p < 0.01$; slice number 3, $p < 0.05$).

3.2 Effect of 3-MC on excretion of major urinary proteins

Intraperitoneal administration of 3-MC decreased the levels of mRNA encoding major urinary proteins (MUPs), which are thought to be candidate pheromones themselves or possible carrier proteins of pheromones in urine (Nukaya et al., 2004). Mouse urinary proteins in male mouse urine with and without intraperitoneal dosage of 3-MC were analyzed by polyacrylamide-SDS gel electrophoresis (Fig. 4). The urine was found to contain several protein species; most strongly stained were proteins of ~19 kilodaltons, as reported previously (Norstedt and Palmiter, 1984). SDS analyses were performed on the urine on days 1 and 3 after the 3-MC treatment. The protein levels in the urine from males 1 day after administration were similar to those from non administered mice. The levels of a protein with an apparent molecular mass of 19 kilodaltons in urine from 3 days after the administration were lower than those in the control mice.

3.3 Effect of 3-MC on sensitivity of the vomeronasal system

Pheromones are received by V1R or V2R, which activated phospholipase C via G_i or G_o in the VSN (Halpern and Martinez-Marcos, 2003; Jia and Halpern, 1996; Luo et al., 1994; Matsunami and Buck, 1997; Sasaki et al., 1999). Diacylglycerol or inositol-1,3,5-trisphosphate induces excitation in VSNs via TRPC2 or unidentified cation channels (Inamura et al., 1997; Inamura and Kashiwayanagi, 2000; Liman et al., 1999). There is no significant difference between males and females in these transduction processes in VNOs. The effects of 3-MC on the sensitivity of the vomeronasal system of administered males to female urinary pheromones were examined (Fig. 5). A two-factor ANOVA found a main effect of with versus without urinary stimulation ($F(2, 12) = 5.419$, $p = 0.021$), a main effect of rostral region versus caudal region ($F(1, 12) = 44.864$, $p < 0.0001$), and a significant stimulation condition by regions interaction ($F(2, 12) = 6.156$, $p = 0.0145$). Fisher's PLSD post-hoc testing indicates that the density of Fos-ir cells of males with the 3-MC-administration at the rostral region of the M/TC layer of the AOB after exposure to female urine was slightly lower than that of mice without the administration, although the difference was not significant ($p = 0.2265$). This suggests that 3-MC does not affect pheromonal responses of males under this experimental condition. In the ferret, treatment with testosterone propionate augments the neuronal Fos responses of the main olfactory bulb

to estrous females' pheromones, but the neuronal responses of the AOB remain unchanged (Kelliher et al., 1998). In the present study, the neuronal responses of the AOB were not changed by the 3-MC-administration, which is in agreement with previous observations.

4. Discussion

Two populations of sensory neurons that express different families of pheromone receptors in the vomeronasal organ project information to rostral and caudal regions of the AOB, respectively (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). These subregions receive different pheromonal information from VSNs (Brennan et al., 1999; Inamura et al., 1999b; Inamura et al., 1999a; Matsuoka et al., 1999; Sasaki et al., 1999). The neuronal Fos responses of females not only at the rostral region but also at the caudal region of the AOB to male urine are inhibited by exposure to 3-MC, suggesting that 3-MC inhibits activities of multiple pheromones.

There are protease-sensitive and protease-insensitive pheromonal molecules in rodent urine (Tsujikawa and Kashiwayanagi, 1999). Dialyzed male rat urine (less than 500 Da) or the remaining substances (greater than 500 Da) are also ineffective in inducing Fos expression in female rat AOB, but combining these two fractions restores their effectiveness (Yamaguchi et al., 2000). In mice, MUPs and volatile molecules such as dehydro-*exo*-brevicomin (DHB), *sec*-butyl-dihydrothiazole (SBT), and 6-hydroxy-6-methyl-3-heptanone have been identified as urinary pheromones (Halpern and Martinez-Marcos, 2003; Novotny, 1999). A combination of MUP, DHB and SBT is extremely effective in inducing c-*fos* mRNA expression in the female mice AOB, whereas each pheromone alone is unable to induce one (Guo et al., 1997). These results suggests that the effective chemosignal inducing c-fos expression in the AOB is a combination of high and low molecular weight elements and that loss of either the volatiles of urine (day 1 after 3-MC), the involatile components (e.g. MUPs, day 3 after treatment), or both volatiles and involatiles, might result in a loss of pheromonal activity of urine.

MUPs, whose biological function relates to chemical communication, are important components of male mouse urine. MUP induces puberty acceleration such

as that involved in the increase in uterus weight (Mucignat-Caretta et al., 1995). MUPs also are responsible for binding volatile pheromones and their subsequent release from drying urine (Bocskei et al., 1992). The levels of mRNA encoding MUP mouse liver are differentially regulated by growth hormone, thyroxine, and testosterone (Knopf et al., 1983). Nukaya et al. have shown that the treatment of male mice with 3-MC decreases the levels of mRNA for growth hormone receptors and MUPs in the liver (2004). It is possible that 3-MC reduces pheromonal activities by a decrease in MUPs via growth hormone receptor. However, on 1 day after the 3-MC-treatment, the mRNA levels for growth hormone receptor and MUPs decreased, while those of MUPs in urine were essentially unchanged. These results suggest that 3-MC blocks chemical communication between male and female mice by reducing pheromonal activities without changing the total amount of MUPs in male mice 1 day after administration. Multiple MUP genomic clones, however, have been isolated from mice (Bishop et al., 1982). Therefore, we cannot entirely exclude the possibility that a decrease in the levels of a specific MUP results in reduction of pheromonal activities in urine. It is also possible that 3-MC and/or its metabolites in urine excreted from males inhibits pheromone induced activation in VSNs on 1 day after the 3-MC-treatment. A large amounts of metabolites of an another PAH pollutant, benz[j]aceanthrylene, is excreted in faces and urine (Hegstad et al., 1999).

Exposure of adult male rats to 3-MC is associated with significant reduction in circulating plasma testosterone levels (Konstandi et al., 1997). Attractiveness of male urinary odors to female meadow voles is dependent on plasma testosterone concentration in males (Bigelow and Nebert, 1982). In addition, out of hundreds of compounds present in intact male mouse urine but only 57 compounds in urine of castrated male mice (Lin et al., 2005). Volatile male mouse urinary pheromone, 7-exo-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]-3-octene, is dependent on testosterone levels in the male (Novotny et al., 1984). These suggest that 3-MC results in a decrease in testosterone and its metabolites and that is responsible for decreased pheromones in urine in male mice. At this time, the effects of decreases in pheromonal activities by 3-MC on behaviors are unclear. Female mole rats choose to spend a longer period of time next to males with high blood and urine testosterone levels (Gotterich et al., 2000). It is therefore possible that 3-MC affects preferences of

females for males.

Synchrony of menstrual cycle among a group of women living together is a well established pheromonal effect in human (McClintock, 1971; Stern and McClintock, 1998). Expression of a putative pheromone receptor gene has been shown in human olfactory mucosa (Rodriguez et al., 2000). Therefore, it is possible that environmental pollutants, such as PAHs, interfere with a chemical communication not only between rodents but also other animals including human, which lead to disturb sexual and social behaviors.

Conflict of Interest Statement

There are no conflicts of interest in the present study.

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FIGURE LEGENDS

Figure 1 Sagittal sections of the AOB of female mice stained with antibodies to Fos protein following exposure to urine excreted from mice 1 day after the administration of vehicle (corn oil; a), 1 (b) and 3 (c) days after the administration of 3-MC. The rostral portion is on the left. Scale bars: 200 μm.

Figure 2 The density of Fos-immunoreactive (Fos-ir) cells (number/mm^2) in the PGC layer, M/TC cell layer and GC layer of the AOB without (open column) and with exposure to urine excreted from mice administered both the vehicle and 3-MC (closed column). Vertical bars represent the mean ± SEM (n = 3).

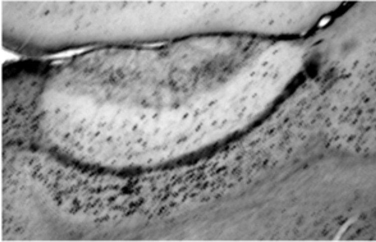
Figure 3 The density of Fos-ir cells in each slice of the M/T cell layer of the AOB after exposure to urine. Vertical points represent the mean ± SEM (n = 3).

Figure 4 SDS-PAGE of urinary proteins excreted from mice without and with 3-MC-administration. The urine sample from mice administered the vehicle was analyzed in lane 1. The gel also demonstrates the time-dependent effect of the 3-MC administration on MUP (lanes 2 and 3). In the left lane, the labeled markers are molecular weight standards. Molecular weights (kilodaltons) are indicated.

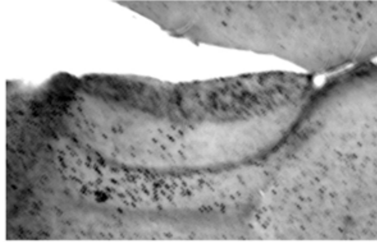
Figure 5 The density of Fos-ir cells (number/mm^2) in the M/TC cell layer of male mice with and without 3-MC-administration after exposure to urine from females. Vertical bars represent the mean ± SEM (n = 3).

Figure 1 Shiraiwa et al.

a) 1 day after vehicle administration



b) 1 day after 3-MC administration



c) 3 days after 3-MC administration

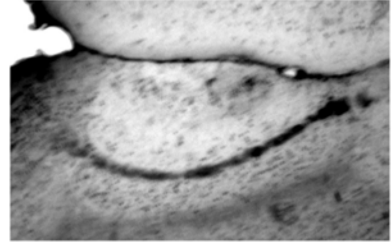


Fig. 2 Shiraiwa et al.

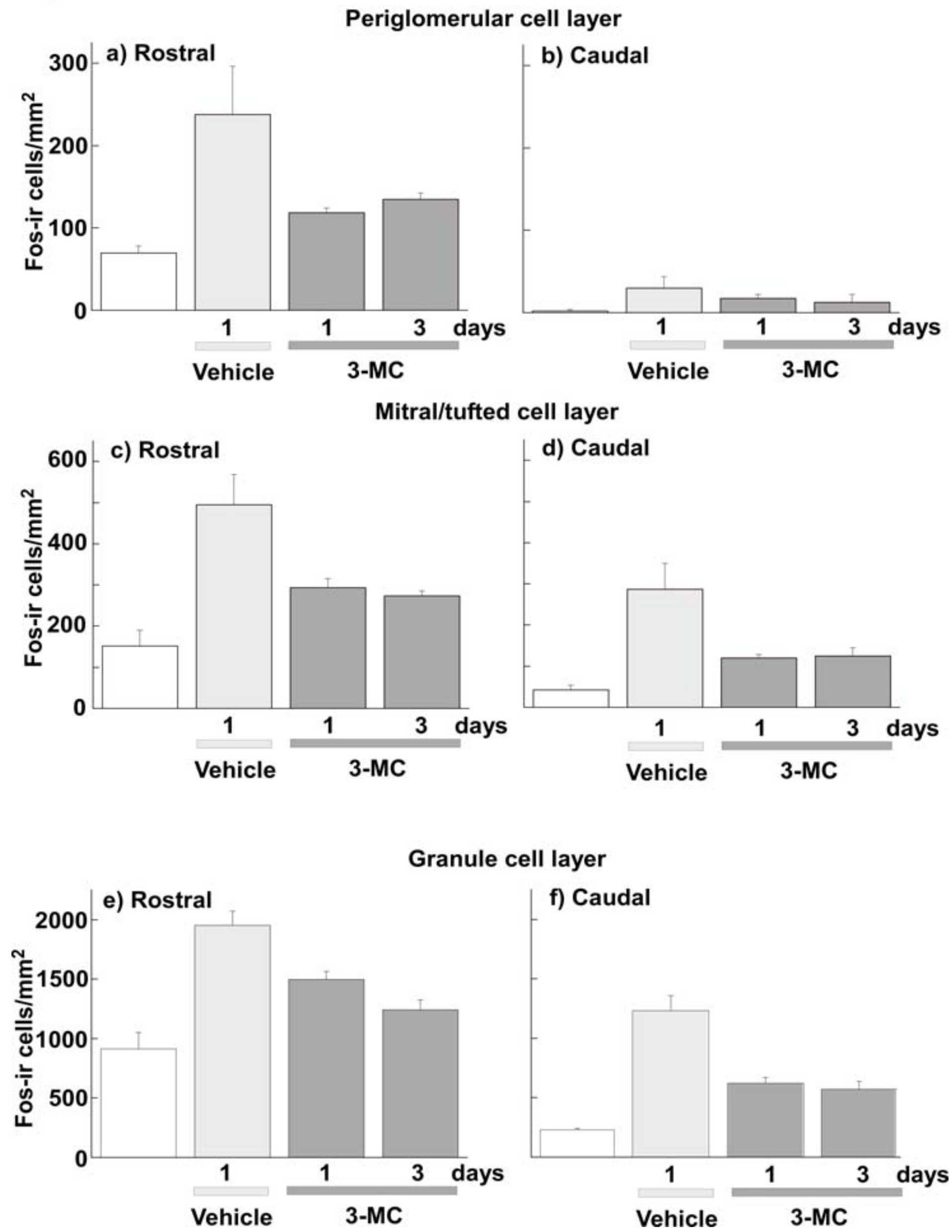


Fig. 3 Shiraiwa et al.

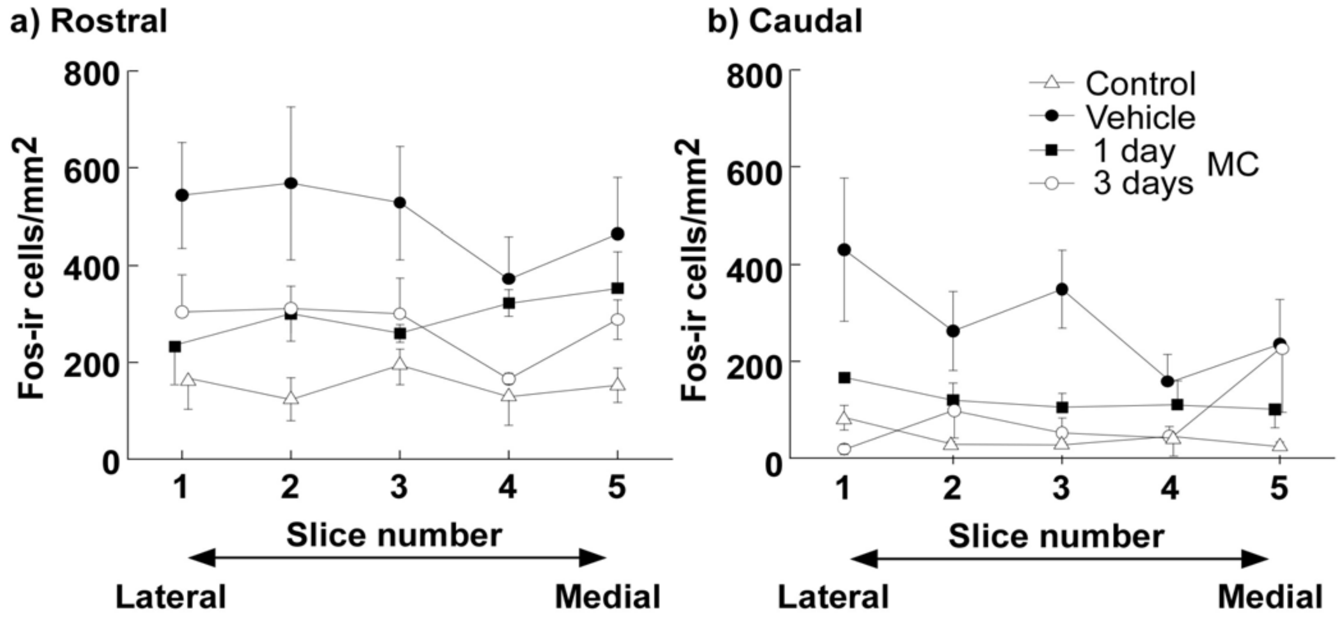


Fig. 4 Shiraiwa et al.

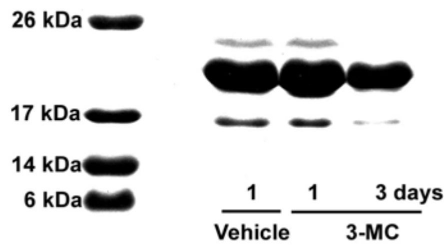


Fig. 4 Shiraiwa et al.

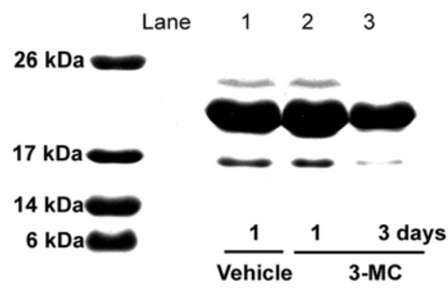


Fig. 5 Shiraiwa et al.