Volatile female odors activate the accessory olfactory system of male mice without physical contact

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Abbreviations:

AOB; accessory olfactory bulb

AOS; accessory olfactory system

GCL; granule cell layers

GL; glomerular cell layer

MCL; mitral cell layers

MOS; major olfactory system

OVX; ovariectomized

VNO; vomeronasal organ

VNOi; received sham-operation of removal of the vomeronasal organ

VNOx; vomeronasal organ-removed

Abstract

We previously reported that male mice are more attracted to volatile odors from intact female mice than from ovariectomized female mice. In the present study, we investigated male attraction to volatile odors from soiled bedding collected from the cages of estrous or ovariectomized female mice. There was no difference in the total time spent sniffing volatile odors from estrous and ovariectomized female mice, suggesting that female mice emit volatile odors which are not excreted into bedding. To test this possibility, we investigated c-Fos expression in the mitral cell layer (MCL) and granule cell layer (GCL) of the accessory olfactory bulb 60 min after exposure of male mice to volatile odors without physical contact. Volatile odors from an estrous female mouse significantly increased the total number of c-Fos positive cells in each of the rostral and caudal GCL, but not in the MCL. After exposure to volatile odors from estrous bedding, the total number of c-Fos positive cells did not increase. Volatile odors from a male mouse did not increase the total number of c-Fos positive cells. Volatile odors from an ovariectomized female mouse increased c-Fos expression only in the caudal GCL. These results suggest that female mice emit specific volatile odors which are not excreted into bedding, and that the volatile odors activate the accessory olfactory system of male mice without physical contact. To characterize the female-specific volatile odors, we conducted habituation-dishabituation tests. Whereas sham-operated male mice discriminated between volatile odors of estrous and ovariectomized female mice, vomeronasal organ-removed male mice did not. These results suggest that male mice discriminated whether or not female mice were ovariectomized, by volatile odors via the accessory olfactory system, and that the female-specific volatile odors are involved in reproduction.

Keywords:
Accessory olfactory bulb, vomeronasal organ, pheromone, c-Fos, habituation-dishabituation test, sexual attraction

Many animals have two olfactory systems, the major olfactory system (MOS) and the accessory olfactory system (AOS) (Firestein, 2001). The MOS detects general odors (i.e., food, predator, and prey) (Firestein, 2001) and some pheromones, i.e., chemicals conveying information among individuals of the same species (Brennan and Keverne, 2004; Hudson and Distel, 1986; Meredith M, 1986; Restrepo D et al., 2004). The AOS is also important for detecting pheromones (Rodriguez, 2004). In the AOS, receptor neurons in the vomeronasal organ (VNO), vomeronasal neurons, send their axons to the accessory olfactory bulb (AOB). In the AOB, the vomeronasal neurons synapse with dendrites of mitral cells, which project their axons to the medial nucleus of the amygdala and the posteromedial cortical amygdaloid nucleus (Guillamon and Segovia, 2004).

Pheromones are classified into two types, volatile and non-volatile pheromones. Aphrodisin, non-volatile pheromone in the vaginal secretions of estrous hamsters, attracts males and promotes copulatory activity (Flower DR, 1996). Major urinary proteins, proteins in rodent urine, convey information about the individual recognition (Hurst JL et al., 2001). These non-volatile pheromones also act as transporters and reservoirs of volatile pheromones (Brennan and Keverne, 2004). The binding with these proteins support the transport and the remaining of volatile pheromones. Thus, volatile pheromones reach animals directly or via these carrier proteins. Because volatile pheromones reach animals from a distance, volatile pheromones should activate the AOS without physical contact with pheromonal source. Luo M et al. reported that activation of the AOS requires physical contact with pheromonal sources (Luo M et al., 2003), suggesting that volatile pheromones may not activate the AOS. Volatile urinary components of male mice, such as alpha- and beta-farnesene and 2-heptanone, however, activate the AOS *in vivo* (Trinh and Storm, 2005) and *in vitro* (Leinders-Zufall

et al., 2000). Some volatile pheromones may activate the AOS without physical contact with pheromonal source.

The AOS has an important role in male sexual behaviors (kevern, 2004). Many studies of the involvement of the AOS in male sexual behavior have been conducted under conditions in which the male mice make physical contact with the odor sources (Halem et al., 2001; Meredith and Westberry, 2004; Pankevich et al., 2004; Paredes et al., 1998; Wersinger and Rissman, 2000), suggesting that male mice could detect non-volatile and volatile pheromones. In these studies, urine (Meredith and Westberry, 2004; Pankevich et al., 2004) and soiled bedding (Halem et al., 2001; Paredes et al., 1998; Wersinger and Rissman, 2000) are used as odor sources. Although female mouse urine contains sex attractants (Davies and Bellamy, 1972), some animals emit pheromones from salivary (Booth and Baldwin, 1980) or sebaceous glands (Iwata et al., 2000). Other animals might also emit odorants which are not excreted into urine and soiled bedding.

In order to study males detecting volatile female odors via the AOS restrictively, our experiments were conducted using conscious female mice under the condition that tactile contacts were prevented.

EXPERIMENTAL PROCEDURES

Animals

Sexually naive male and female *ddY* mice (7-15 wk old; Clea Japan, Tokyo) were used. Each mouse was individually housed in a plastic cage (182 x 260 x 128 mm; l x w x h) until the tests. A pellet diet (Clea Japan) and water were available *ad libitum*. The temperature (22 ± 2 ˚C) and humidity (35 ± 5 %) in the laboratory were kept constant and a 12:12 h light:dark cycle was maintained with lights on at 0600 h.

Odor sources

Female mice were either bilaterally ovariectomized (OVX) (*n*=18) or received a sham operation (*n*=30) under avertin anesthesia (360 mg/kg) at least 10 d before the tests and were individually housed until testing. To determine the stages of the estrous cycle in sham-operated female mice, vaginal smears were collected between 1600 and 1800 h. According to the criteria of Nothnick (Nothnick, 2000), we used only estrous sham-operated females. Male mice (*n*=12) as odor sources were also maintained in individual cages until the test. To collect soiled bedding from a female mouse housing cage, a sham-operated (estrous bedding) or OVX female (OVX bedding) was kept in the cage with 20 g of wood shavings for 24 h. A sham-operated female was housed in the cage from proestrous to estrous. Soiled bedding was collected (10 g) just before each test. To avoid the effects of individual features, animals as odor sources were used only once in a series of tests.

Test apparatus

We used the handmade transparent acrylic box (test box) described in our previous report (Muroi Y et al., 2006). The test box (250 x 572 x 200 mm; l x w x h) was enclosed and divided equally by two partitions into three compartments; the upwind,

middle, and downwind compartments. Each partition had two opaque panels, 15 mm apart, with a 30 x 30-mm square hole covered with wire mesh. Filtered air (temperature: 22 ± 2˚C, humidity: 35 ± 5%) was drawn into the test box from outside the laboratory through a flexible duct attached to the top of the upwind compartment. A fan attached to the top of the downwind compartment directed the airflow from the upwind compartment to the downwind compartment via the middle compartment. Airflow was directed out from the downwind compartment (approximately 0.035 m^3/min). The holes in the panels were centered at a different height. The holes in the panels (starting with the upwind panel) were located at the bottom, 7 cm from the bottom (upwind window), at the bottom, and at the top. The air from the upwind compartment flowed into the middle compartment only through the upwind window. This test box allows the subject in the middle compartment to be exposed to volatile odors from odor sources in the upwind compartment without visual or tactile contact with the odor sources.

Measurement of male attraction to volatile odors from soiled bedding

The procedures used were described our previous report with a few modifications (Muroi Y et al., 2006). Each male was tested for three consecutive days (*n*=6). In each daily test, each male was placed in the middle compartment for 30 min. After 10 g of soiled bedding was transferred into the upwind compartment, the total time spent touching the upwind window was measured for 30 min. Only the test on the first day was performed without transferring soiled bedding into the upwind compartment (control). In the tests on the second and third days, we used soiled bedding collected from an estrous or an OVX female mouse housing cage. When one type of soiled bedding was used on the second day, the other type of soiled bedding was used on the third day. We defined sniffing behavior as

touching the upwind window.

Presentation of odors

Each subject was exposed to volatile odors from an estrous female mouse (*n*=7), an OVX female mouse (*n*=6), a male mouse (*n*=6), or 10 g of estrous bedding (*n*=6). After a subject was housed in the middle compartment of the test box for 30 min, each odor source was transferred into the upwind compartment. As a control, the same procedures were performed without transferring any odor sources (*n*=5). When a subject was directly exposed to estrous bedding, 10g of estrous bedding was transferred into the subjects' home cage (*n*=6). After 60 min of each exposure, the subject was decapitated under ether anesthesia. The brain was immediately dissected from the skull and immersed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 1 week.

Immunohistochemistry

Brains were sectioned sagittally at 40 μm (Microslicer, DTK-1000, DOSAKAEM, Kyoto, Japan). Each section was washed with PBS, and then treated with 0.5% Triton X-100/PBS (PBST) for 60 min at room temperature. After incubation in 1% H_2O_2/PBS for 20 min, the sections were placed in 2% goat serum /PBST for 1 h at room temperature. For detection of c-Fos, the sections were incubated in rabbit polyclonal c-Fos antibody (Ab-2; Calbiochem, La Jolla, CA) diluted 1:5000 in PBST for 24 h at 4˚C, and then placed into biotinylated goat anti-rabbit IgG solution (1:200) (VECTASTAIN elite ABC kit; Vector Laboratories, Burlingame, CA) for 2 h, followed by incubation in avidin-biotin peroxidase complex solution (1:200) for 2 h. We selected two sections 600 μm to 720 μm inside from lateral side of one of the olfactory bulbs. For evaluation of c-Fos expression in the AOB, we averaged the number of c-Fos positive cells in four sections obtained from both olfactory bulbs per subject. C-Fos positive cells were

counted at x100 with a microscope under the condition that given treatments of the subjects were concealed from the investigator. The total number of c-Fos positive cells in the MCL and GCL was evaluated in the rostral and caudal parts. The rostral and caudal parts of the AOB were divided by the midline from both sides of the AOB.

Removal of the VNO

Removal of the VNO (VNOx, *n*=12) or sham-operation (VNOi, *n*=12) was performed under avertin anesthesia 2 wk before each test began as described in Saito *et al.* (Saito and Moltz, 1986). The half of each group and the remaining were subjected to the staining of c-Fos and the habituation-dishabituation test, respectively. The total number of c-Fos positive cells in the MCL and GCL was evaluated without division of the rostral and caudal parts. Soybean agglutinin binds with the vomeronasal neurons selectively (Key and Giorgi, 1986). To ensure successful operations (Pankevich et al., 2004), the brains were stained with biotinylated soybean agglutinin (1:10000) (J-OIL MILLS, JAPAN) with similar procedures to c-Fos stain after all the tests. Only mice in which the staining was totally absent in the glomerular cell layer (GL) of both AOB were used for data analysis of VNOx male mice (Fig. 4C and D), because the vomeronasal neurons synapse with mitral cells in the GL.

Habituation-dishabituation tests

To evaluate whether subjects could discriminate two different odors, we conducted habituation-dishabituation tests. As odor sources, estrous female mice, 10 g of estrous bedding, OVX female mice, and male mice were used. The procedures in a previous report (Pankevich et al., 2004) were modified to be suitable for our experiments using our test box. To avoid contaminating the upwind compartment with odors,

odor sources were transferred in a cylindrical case (100 x 110 mm; diameter x height). Because the top of the case was covered with wire mesh and the wall contained 12 holes (diameter: 5 mm) at a height of 5 cm, the odors leak out of the case. After a subject was housed alone for 30 min in the middle compartment of the test box, an odor source was transferred into the upwind compartment as described below. The subject received three 2-min presentations with an empty case at 1-min intervals, followed by three 2-min presentations with one odor source at 1-min intervals, and finally by three 2-min presentations with the other odor source at 1-min intervals. On a separate day, a similar test was given with the reverse sequence of presentation with the two odors. We used the average of both tests for data analysis.

Statistical analysis

To evaluate male attraction to soiled bedding, data were analyzed by repeated measures one-way analysis of variance (ANOVA) after Bartlett test. Tukey-Kramer test was used as a *post hoc* test. To compare the total number of c-Fos positive cells in the rostral or caudal part of the AOB among the groups, data were analyzed by one-factor ANOVA after Bartlett test. Tukey-Kramer test was used as a *post hoc* test. To compare c-Fos expression between the rostral and caudal parts of the AOB in the group presented with an odor, data were analyzed by Student's *t*-test after F test. To compare c-Fos expression between VNOi and VNOx males, we analyzed the MCL data using Student's *t*-test after F test. The GCL data were analyzed with Welch's *t*-test, because F test showed the different variance between the GCL data of VNOi and VNOx males. For analysis of habituation-dishabituation tests, Student's *t*-test was used after F test. A *P* value of less than 0.05 was considered statistically significant.

Animal care and ethical standards

All procedures for care and use of animals were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996.

Results

Male attraction to volatile odors from soiled bedding

When soiled bedding was upwind, the total time spent touching the window significantly increased compared with when soiled bedding was absent (Fig. 1). ANOVA showed significant differences between the cases of them [each n=6, F(2, 15)=12.94; P<0.01]. A *post hoc test* revealed that the total time spent touching the window was significantly more when estrous or OVX bedding was upwind than when soiled bedding was absent (P<0.01). There was no difference in the total time spent touching the window between the presentation with estrous bedding or OVX bedding (Fig. 1).

Female-specific volatile odors

There was a significant difference in the total number of c-Fos positive cells in each of the rostral and caudal part of the MCL among the groups [rostral, F(5, 30)=7.97; caudal, F(5, 30)=27.53; each P<0.01]. When male mice sniffed estrous bedding with physical contact, the total number of c-Fos positive cells significantly increased in each of the rostral and caudal MCL, compared with when male mice were not presented with any odors (Fig. 2A and 3A, C). When male mice sniffed volatile odors from an estrous female, estrous bedding, an OVX female or a male without physical contact, the total number of c-Fos positive cells in the MCL did not increase. (Fig. 2A and 3A, B, D, E, F).

There was a significant difference in the total number of c-Fos positive cells in each of the rostral and caudal of the GCL among the groups [rostral, F(5, 30)=33.51; caudal, F(5, 30)=16.82; each P<0.01]. When male mice sniffed estrous bedding with physical contact, the total number of c-Fos positive cells in each of the rostral and caudal GCL

significantly increased, whereas did not increase when male mice sniffed estrous bedding without physical contact (Fig. 2B and 3A, C, D). Though physical contact was not allowed, the total number of c-Fos positive cells in each of the rostral and caudal GCL significantly increased, when male mice sniffed volatile odors from an estrous female mouse (Fig. 2B and 3B). When male mice sniffed volatile odors from an OVX female, the total number of c-Fos positive cells significantly increased in the caudal GCL, but not in the rostral GCL (Fig. 2B and 3E). When male mice sniffed volatile odors from a male mouse, the total number of c-Fos positive cells did not increase in either the rostral or caudal GCL (Figs. 2B and 3F).

There was a significant difference in the total number of c-Fos positive cells between the rostral and caudal part of the GCL, but not the MCL, in some groups (Fig. 2A, B). When male mice sniffed estrous bedding with physical contact, the total number of c-Fos positive cells was more in the rostral GCL than in the caudal GCL (Fig. 2B, 3C). In contrast, when male mice sniffed volatile odors from an estrous or an OVX female mouse, the total number of c-Fos positive cells was more in the caudal GCL than in the rostral GCL (Fig. 2B, 3B, 3E).

When VNOx male mice sniffed volatile odors from an estrous female mouse, c-Fos positive cells in the GCL were scarcely detected, in contrast to VNOi male mice (Fig. 4B, E, F). There was no difference in the total number of c-Fos positive cells in the MCL between VNOi and VNOx males (Fig. 4A, E, F).

Habituation-dishabituation tests

In all habituation-dishabituation tests, there was a significant increase in the total time males spent touching the upwind window at the initial presentation with the first odor, compared with that at the final presentation with the

vacant case (P<0.05; Fig. 5A, B, C).

First, we conducted the tests using an estrous female mouse and estrous bedding from its home cage. The total time spent touching the upwind window at the initial presentation with the second odor significantly increased in both VNOi and VNOx males, compared with that at the final presentation with the first odor (Fig. 5A).

Second, we conducted the tests using an estrous female mouse and a male mouse. The total time spent touching the upwind window at the initial presentation with the second odor significantly increased in VNOi and VNOx males, compared with that at the final presentation with the first odor (Fig. 5B).

Finally, we conducted the tests using an estrous and an OVX female mouse. The total time touching the upwind window at the initial presentation with the second odor significantly increased in VNOi males, compared with that at the final presentation with the first odor (Fig. 5C). VNOx males showed no difference in the total time spent touching the upwind between at the initial presentation with the second odor and at the final presentation with the first odor (Fig. 5C).

Discussion

We previously reported that male mice are more attracted by volatile odors from intact female mice than ovariectomized female mice under the condition that physical contact with female mice was prevented (Muroi Y et al., 2006). When bedding from estrous and OVX mice were presented under the condition allowing physical contact, similar results were obtained (Paredes et al., 1998). In the present study, when estrous and OVX bedding were presented without physical contact, male mice were not more attracted by volatile odors from estrous bedding than OVX bedding, suggesting that female mice emit volatile odors which are not excreted into the bedding.

When male mice sniffed volatile odors from an estrous female mouse, the total number of c-Fos positive cells in each of the rostral and caudal GCL increased significantly. Presentation of volatile odors from estrous bedding did not increase the total number of c-Fos positive cells, suggesting that female mice emit volatile odors which are not excreted into the bedding. Removal of the VNO abolished c-Fos expression in the GCL upon exposure to volatile odors from an estrous female mouse, suggesting that male mice detect volatile female odors via the VNO. In contrast to our results, Luo M et al. reported that activation of the AOS requires physical contact with the odor sources (Luo M et al., 2003). They recorded the activity of the AOB with the microelectrodes which tips were positioned in the MCL. In their experiments, mitral cells of male mice were not activated under the condition that male mice could detect volatile female odors without physical contact. Because mitral cells make reciprocal synapses with granule cells (Taniguchi and Kaba, 2001), we expected that c-Fos expression in the MCL would parallel that in the GCL. In agreement with the results of Luo M et al., the total number of c-Fos positive cells did not significantly increase in the MCL, when male mice sniffed

volatile odors from an estrous female. Mitral cells were activated when male mice contacted physically with female mice (Luo M et al., 2003). When male mice sniffed estrous bedding with physical contact, the total number of c-Fos positive cells significantly increased in the MCL. These results suggest that activation of mitral cells rather than the AOB may require physical contact with the odor sources. Similarly to our results, exposure to alarm pheromone for 60 min increased c-Fos positive cells in the GCL, but not in the MCL of rats (Kiyokawa et al., 2005). Exposure to alarm pheromone for 30 min increased c-Fos expression in the MCL (Kikusui et al., 2001). In the present study, shorter exposure to volatile female odors (15 and 30 min) did not increase the total number of c-Fos positive cells in the GCL as well as in the MCL (data not shown). These results suggest that there may be unknown mechanisms in which volatile female odors activate granule cells with bypassing mitral cells, and that the AOS is activated by volatile odors from estrous female mice.

Differently from volatile odors from an estrous female mouse, volatile odors from an OVX female mouse increased the total number of c-Fos positive cells in the caudal, but not rostral GCL, suggesting that volatile female odors detected via the AOS of male mice might be emitted with partial dependence on ovarian function. Volatile odors from a male mouse did not increase the total number of c-Fos positive cells in either of the rostral or caudal GCL. When male mice were exposed to male soiled bedding, or when male mice interacted with male mice, c-Fos expression in the VNO also did not increase (Kimoto H et al., 2005), suggesting that male mice might not emit odors activating the AOS of male mice. In contrast, there is a possibility that male mice might detect male odors via the VNO, because VNO-removed male mice decreased the display of inter-male aggressive behavior (Clancy et al., 1984). C-Fos expression declines with long exposure to the same odor

(Kimoto H et al., 2005). Because animals are always exposed to their own odors, neuronal response to odors from other animals of the same gender might not be reported by c-Fos expression.

After exposure to volatile odors from an estrous female mouse, there was no difference in the total number of c-Fos positive cells in the MCL between VNOi and VNOx, in contrast to that in the GCL. After male rats without a VNO mated with female rats, there was no difference in the total number of c-Fos positive cells in the MCL between VNOi and VNOx (Kondo et al., 2003). Noradrenergic neurons in the locus coeruleus project to the AOB and synapse with mitral cells (McLean et al., 1989). Inputs from these noradrenergic neurons to the AOB are required for mating-induced memory formation in female mice (Rosser and Keverne, 1985). In male mice, noradrenergic neurons might also regulate neuronal activity in the AOB.

Receptor neurons in the VNO express two types of pheromone receptors, V1R (Dulac and Axel, 1995) and V2R (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Receptor neurons in the apical neuronal epithelium express V1R (Ryba and Tirindelli, 1997), whereas receptor neurons in the basal neuronal epithelium express V2R (Herrada and Dulac, 1997). V1R-expressing apical neurons and V2R-expressing basal neurons send their axons to the rostral and caudal part of the AOB, respectively (Herrada and Dulac, 1997). Similarly, rostrally located mitral cells synapse with vomeronasal neuron terminals in the rostral part, whereas caudally located mitral cells synapse with those in the caudal part (Jia and Halpern, 1997). In agreement with a previous report (Halem et al., 2001), when male mice sniffed estrous bedding with physical contact, the total number of c-Fos positive cells was higher in the rostral part than in the caudal part of the GCL. In contrast, when male mice sniffed volatile odors from an estrous or an OVX female mouse without

physical contact, the total number of c-Fos positive cells was higher in the caudal part than in the rostral part of the GCL. Volatile female odors might be different from those from estrous bedding in terms of the receptors activated (V1R or V2R), and in volatile components of their emitting odors, even though both are sexually attractive to male mice (Davies and Bellamy, 1972; Muroi Y et al., 2006). Volatile odors from an OVX female mouse did not increase the total number of c-Fos positive cells in the rostral GCL, in contrast to volatile odors from an estrous female mouse, suggesting that estrous and OVX female mice might be also different in terms of the receptors activated, and in volatile components.

In habituation-dishabituation tests, if a subject discriminates between familiar and novel odors, the total time spent sniffing odors significantly increases at first presentation with a novel odor after final presentation with a familiar odor (Pankevich et al., 2004). Both VNOi and VNOx males discriminated an estrous female mouse with bedding from its home cage by volatile odors, suggesting that this discrimination is via the MOS, not the AOS. Olfaction has an important role in identifying individuals (Brennan, 2004). Because urine includes chemicals about individual identity (Singh, 2001), estrous bedding conveys information about the female mouse living on it. In the present study, male mice recognized that volatile odors from an estrous female mouse were different from those from the bedding of her home cage. These results suggest that volatile female odors detected via the male mouse AOS are not involved in conveying individual information. This discrimination might be due to some odors included in estrous bedding (i.e., urinary and fecal odors unrelated to the individual’s information).

Olfaction is involved in sex discrimination (Keverne, 2004). Both VNOi and VNOx males discriminated an estrous female mouse from a male mouse by volatile odors, suggesting that this discrimination is via the MOS, not the AOS, consistent with

a previous report (Pankevich et al., 2004), and that volatile female odors detected via the AOS of male mice might not be involved in sex discrimination.

VNOi males discriminated an estrous female mouse from an OVX female mouse by volatile odors, whereas VNOx males did not discriminate them, suggesting that this discrimination is via the AOS but not the MOS. We previously reported that male mice are more attracted by volatile odors from intact female mice than ovariectomized female mice (Muroi Y et al., 2006), indicating that male mice discriminate intact female mice with OVX female mice by volatile odors. Male attraction to volatile female odors might be dependent on inputs from the AOS. Volatile odors from an OVX female mouse did not increase the total number of c-Fos positive cells in the rostral GCL, in contrast to volatile odors from an estrous female mouse. These results suggest that volatile female odors detected via the AOS of male mice might convey information about the reproductive ability of female mice.

Non-volatile pheromones such as aphrodisin and major urinary proteins act as transporters and reservoirs of volatile pheromones (Brennan and Keverne, 2004). Volatile odors from estrous bedding did not increase the total number of c-Fos positive cells, suggesting that volatile female odors detected via the AOS of male mice might reach male mice not via non-volatile pheromones functioning as transporters and reservoirs. Because the emission of volatile odors not via transporters and reservoirs allows a female mouse to provide male mice with her present information from a distance, volatile female odors detected via the AOS are very useful for appealing timely for female mice to male mice and attracting male mice.

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Figure legends

Figure 1

Total time male mice spent touching the upwind window. When estrous bedding (est) or OVX bedding (OVX) was in the upwind compartment or absent (control), total time spent touching the upwind window was measured for 30 min (each n=6). Each of three odors was presented on a separate day. Values are means ± SD of percentage of total time spent touching the upwind window per test of total time touching the upwind window in three tests. ** P<0.01 (vs control)

Figure 2

Total number of c-Fos positive cells in the rostral and caudal parts of the MCL (A) and GCL (B). Each group of male mice was exposed to the following odors without physical contact; no odors (control, n=5), an estrous female (female, n=7), an OVX female (OVX, n=6), and male mice (male, n=6). Estrous bedding was presented under the condition that physical contact was allowed (contact, n=6) or not (non-contact, n=6). Values are means ± SEM of the total number of c-Fos positive cells in four sections per subject. Data were compared between the rostral and caudal parts in each group (* P<0.05, ** P<0.01), between the rostral parts of the control group and the other groups (# P<0.05, ## P<0.01), and between the caudal parts of the control group and the other groups (+ P<0.05, ++ P<0.01).

Figure 3

Representative photomicrographs showing c-Fos immunoreactivity in the AOB. Each group of male mice was exposed to the following odors without physical contact; no odor (A), estrous female mouse (B), OVX female mouse (E), and male mouse (F). Estrous bedding was presented under the condition that physical contact was allowed (C) or not (D).

Scale bar = 100 μm

Figure 4

Sham-operated (VNOi) and VNO-removed (VNOx) males were exposed to volatile odors from an estrous female mouse. Each graph shows total number of c-Fos positive cells in the MCL (A) and GCL (B) (each n=6). Values are means ± SEM of the total number of c-Fos positive cells in four sections per subject. ** P<0.01 (vs VNOi). Representative photomicrographs show the AOB sections of VNOi (C) and VNOx males (D) strained with biotinylated soybean agglutinin, and c-Fos immunoreactivity in the AOB of VNOi (E) and VNOx males (F). Scale bar = 100 μm

Figure 5

In each habituation-dishabituation test, various sets of odor sources were presented: an estrous female mouse and estrous bedding of its home cage (A), an estrous female mouse and a male mouse (B), and an estrous female mouse and an OVX female mouse (C) (each n=6). Data are represented by the percentage of total time spent touching the upwind window per trial of total time touching the upwind window in nine trials. Because each odor source of a set was presented twice in the reverse order on a separate day, values are the mean ± SEM of the two tests. Data were compared between third and fourth, or sixth and seventh trials. * P<0.05, ** P<0.01 (VNOi), # P<0.05, ## P<0.01 (VNOx)

Figure 1

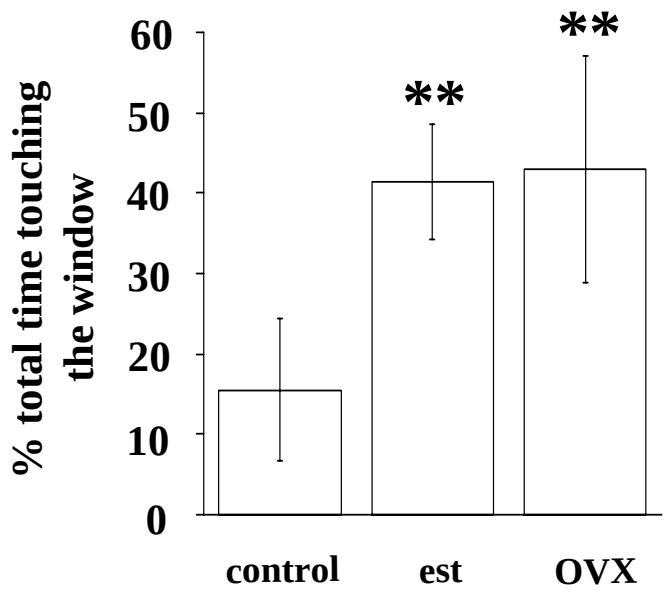

% total time touching the window
60
50
40
30
20
10
0
**
**
control
est
OVX

Figure 2

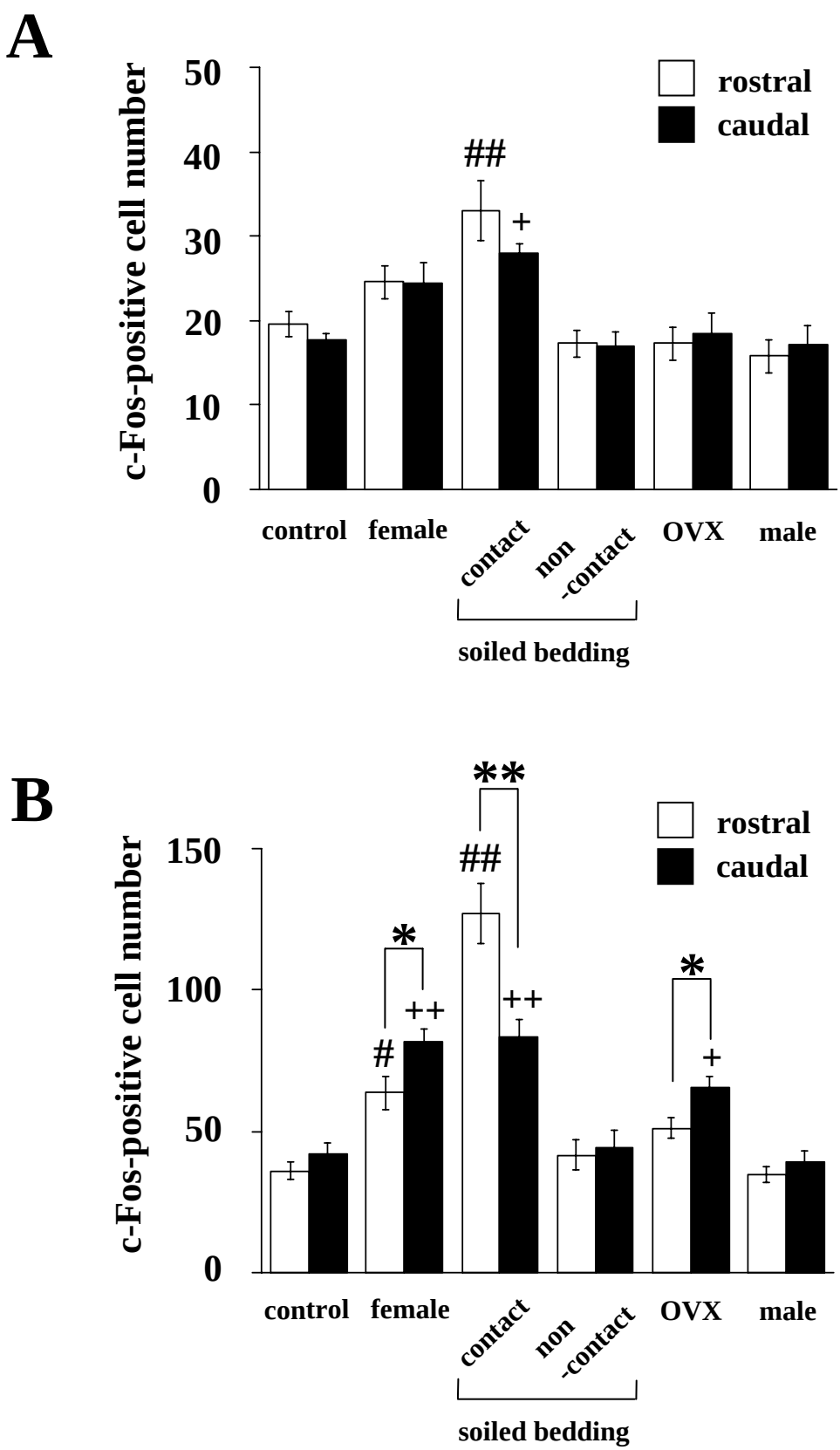

A
c-Fos-positive cell number
50
40
30
20
10
0
rostral
caudal
##
+
control
female
contact
non
-contact
OVX
male
soiled bedding
B
c-Fos-positive cell number
150
100
50
0
**
##
*
++
#
++
*
+
rostral
caudal
control
female
contact
non
-contact
OVX
male
soiled bedding

Figure 3

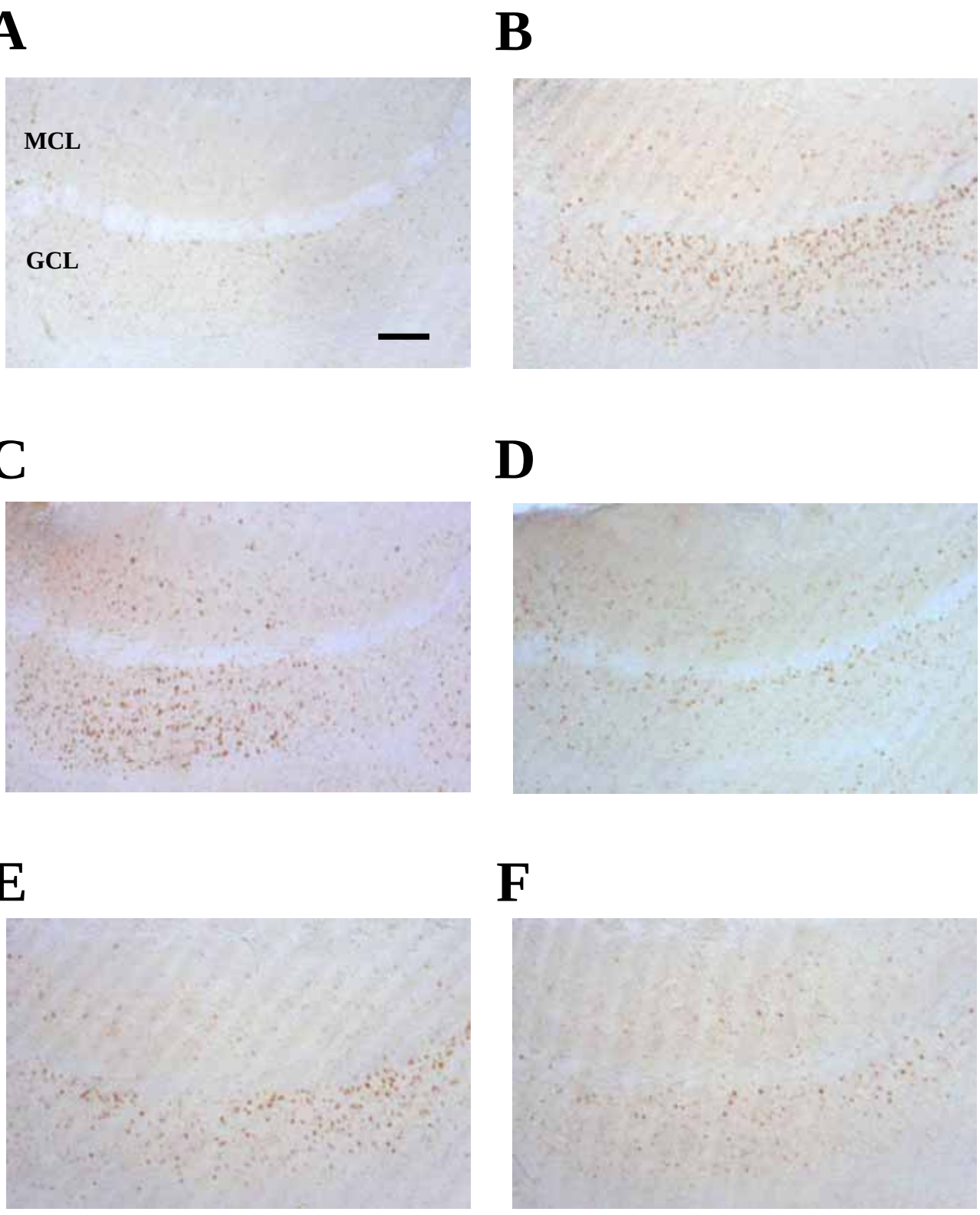


Figure 4

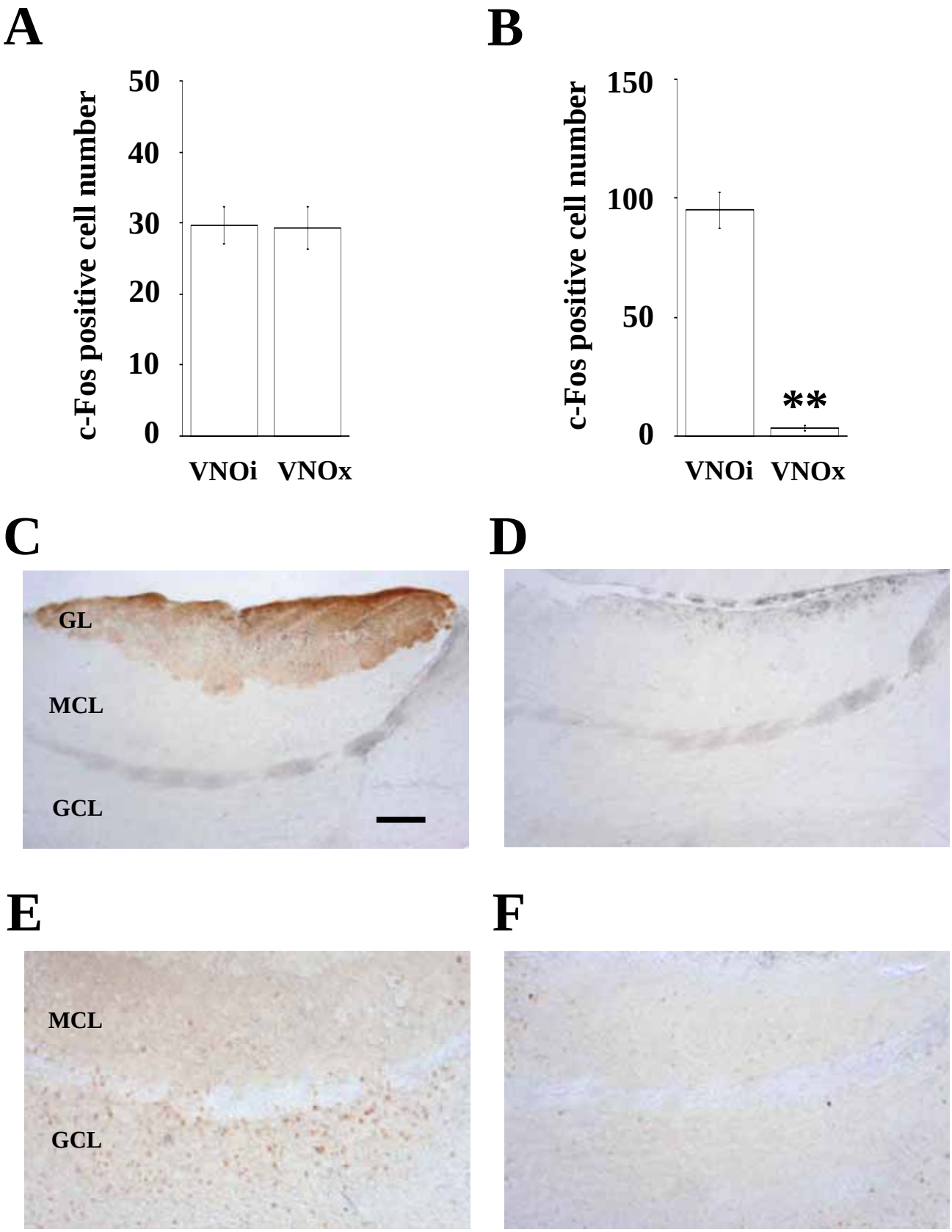
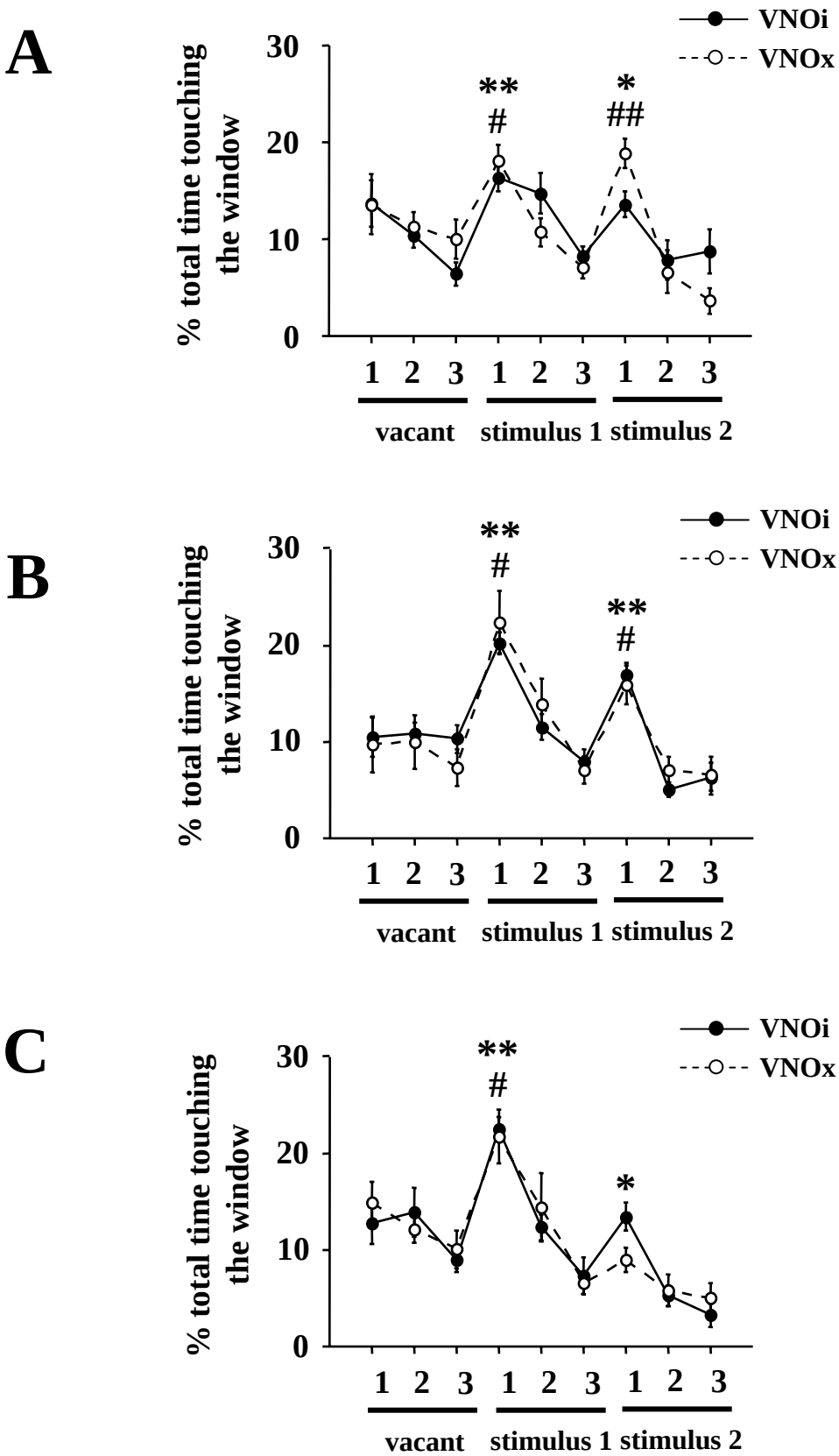

A
B
c-Fos positive cell number
50
40
30
20
10
0
VNOi
VNOx
150
100
50
0
**
C
D
GL
MCL
GCL
E
F
MCL
GCL

Figure 5


A
B
C
VNOi
VNOx
% total time touching the window
30
20
10
0
**
#
*
##
1 2 3 1 2 3 1 2 3
vacant
stimulus 1
stimulus 2