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Protease-Sensitive Urinary Pheromones Induce Region-Specific Fos-Expression in Rat Accessory Olfactory Bulb

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Vomeronasal organs of female Wistar rats were exposed with sprayed urine preparations of male Wistar rats prior to sacrifice. Exposure to crude urine and ultrafiltrated urine preparation (<5000 Da) induced significant Fos expression, which is correlated with cellular activity, in the mitral/tufted cell layer of the accessory olfactory bulb (AOB), while exposure to the remaining substances after ultrafiltration (>5000 Da) and control salt solution did not. Exposure to urine preparation treated with papain induced expression of Fos-immunoreactive cells in the rostral region of the AOB, but did not induce such expression in the caudal region. Exposure to urine preparation treated with pronase induced urine-specific Fos immunoreactivity neither in the rostral nor in the caudal region. These results suggest that at least two different peptides carrying pheromonal activities are contained in male Wistar rat urine. © 1999 Academic Press

The vomeronasal organ exists in many vertebrates for receiving pheromones related to sexual and social behavior (1–3). In female rats, pheromones in urine excreted from males and females induce various changes in gonadal functions, such as reflex ovulation (4) and a reduction in the estrous cycle of female rats (5). These results suggest that urine contains plural pheromones. Biochemical and electrophysiological studies have provided evidence that pheromonal responses in rats are mediated by IP_3 generated via the G-protein linked pathway in the vomeronasal sensory neuron (6–12). However, no study has been carried out to explore the molecular properties of pheromones contained in rat urine.

We showed previously that exposure of the vomeronasal organs of female rats to male rat urine induced expression of Fos-immunoreactive (Fos-ir) cells, which is correlated with cellular activity, in the accessory olfactory bulb (AOB) (13). In the present study, in order to characterize active components stimulating the rat vomeronasal organ, male urine preparation was subjected to ultrafiltration and protease-treatment, and its effects on induction of Fos-ir cells in the AOB were examined.

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MATERIALS AND METHODS

Preparation of crude urine and ultrafiltrated urine. The crude urine collected from adult male Wistar rats in metabolic cage (crude urine) was filtered through a polysulfone disk filter (pore size, 0.45 μm; EB-DISK 25, Kanto Chemical Co., Ltd., Tokyo, Japan). The filtrated urine was ultrafiltrated through a filter (Ultrafree-15, Millipore corporation, Bedford, MO) at 3,500 rpm for 1.5–2.5 h (ultrafiltrated urine). The remaining substances were dissolved in control solution (140 mM NaCl, 284 mM KCl, 1.5 mM $CaCl_2$, and 0.8 mM $MgCl_2$) with the same volume of the urine before ultrafiltration.

Urine preparations treated with papain or pronase. The filtrated urine was treated with an anion and cation exchanger (AG501-X8, Bio-Rad, Hercules, CA) for 60 min at 4°C. The deionized urine with 1 mM cysteine and 100 mM Na_2HPO_4-NaH_2PO_4 (pH 6.5) was treated with 0.1 mg/ml papain for 60 min at 30°C and was ultrafiltrated. The deionized urine with 50 mM Tris–HCl (pH 7.0) was treated with 0.3 mg/ml pronase (type XIV, Sigma, St. Louis, MO) for 60 min at 37°C and was ultrafiltrated.

Immunohistochemistry. Immunohistochemical experiments were carried out as described previously (13). Each animal was sacrificed after exposure to the stimulus sprayed on the nares of female rats with commercial cosmetic atomizers. The sagittal sections fixed 4% paraformaldehyde were incubated with c-Fos polyclonal antibody (1:5000, Cambridge Research Biochemicals; OA-11-823), and then incubated with biotinylated rabbit anti-sheep IgG (1:200, Vector, CA). The sections were further incubated with ABC (ABC Elite kit, Vector), and visualized with DAB. Fos-ir cells were counted under a microscope.

RESULTS

In the AOB, the mitral/tufted (M/T) cells directly receive inputs from the vomeronasal neurons at the glomeruli. Exposure to crude urine from male Wistar rats induced Fos expression in the M/T cell layer of the AOB of female Wistar rats, while exposure to control salt solution having a salt composition similar to that of urine resulted in only a small amount of Fos expression (Figs. 1A and 1B). Exposure to urine preparations

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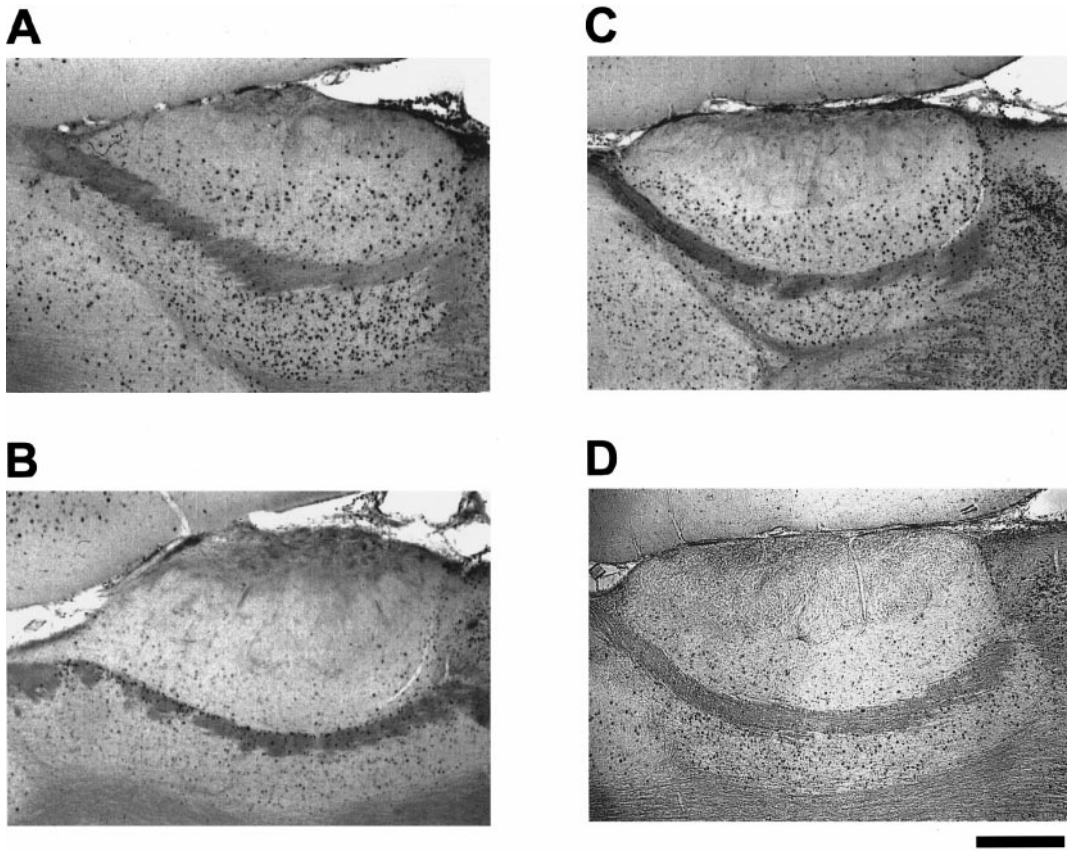

FIG. 1. Sagittal sections of the AOB of female rats stained with antibodies to Fos protein after exposure to control salt solution (A), crude urine (B), ultrafiltrated urine preparation (C) and the remaining component after ultrafiltration (D). The rostral portion is on the right. Scale bars, 200 μm.

ultrafiltrated (<5000 Da) induced Fos-ir cells to a degree similar to that by exposure to crude urine (Fig. 1C). However, the remaining substances after ultrafiltration (>5000 Da) induced only a small number of Fos-ir cells (Fig. 1D).

Figure 2 shows the density of Fos-ir cells in the M/T cell layer of the AOB after exposure to these urine preparations. The density of Fos-ir cells after exposure to ultrafiltrated urine preparation was similar to that after exposure to crude urine, while the density of Fos-ir cells after exposure to the remaining substances after ultrafiltration was similar to that after exposure to control salt solution.

Figure 3A shows the density of Fos-ir cells of the M/T cell layer after exposure to urine preparations with and without papain treatment. Previous studies on marsupials and rodents have shown that the AOB was divided into $G_{i2\alpha}$ positive rostral and the $G_{o\alpha}$ positive caudal regions (14, 15). In rats exposed to urine without papain treatment, Fos-ir cells were found in both the caudal and the rostral regions. The density of Fos-ir cells in the caudal region after exposure to urine preparation treated with papain was reduced to the level after exposure to control salt solution. Figure 3B shows the density of Fos-ir cells of the M/T cell layer after exposure to urine preparations with and without pronase treatment. The density of Fos-ir cells after exposure to urine preparations treated with pronase both in the rostral and caudal regions was similar to that after exposure to control salt solution.

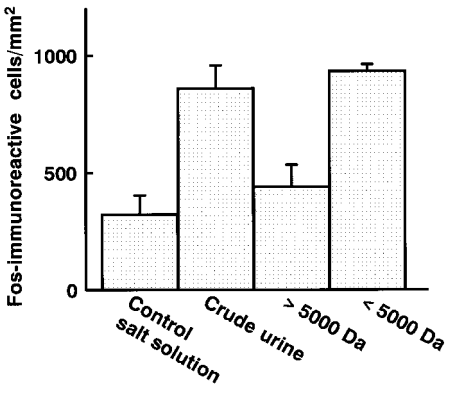

FIG. 2. The density of Fos-ir cells (number/mm^2) in the mitral/tufted cell layer of AOB after exposure to salt solution, crude urine, ultrafiltrated urine and the remaining component after ultrafiltration. Vertical bars represent means ± SEM (n = 3).

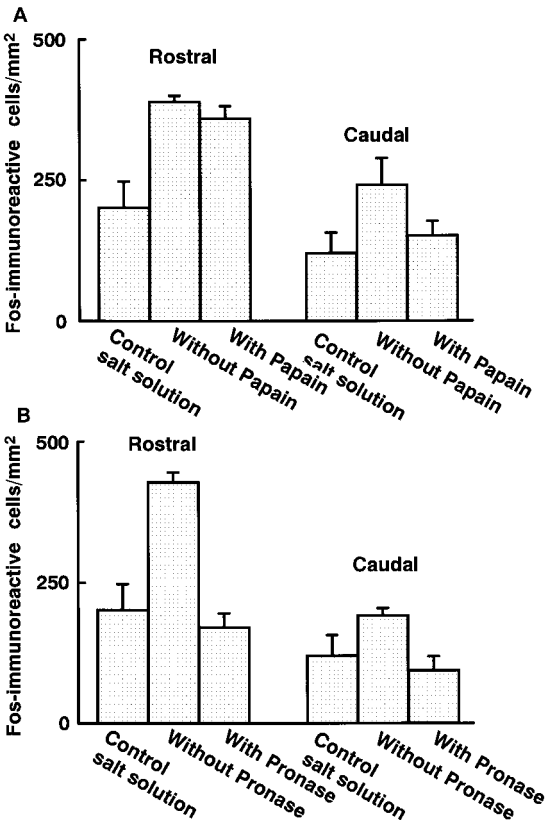

FIG. 3. The density of Fos-ir cells (number/mm^2) in the mitral/tufted cell layer after exposure to urine preparations treated with and without papain (A), and with and without treatment of pronase (B). Vertical bars represent means ± SEM (n = 3).

DISCUSSION

Immunohistological methods have been used to visualize Fos as a means of identifying neurons which are activated by stimulation. In the present study, we showed that exposure to urine preparation ultrafiltrated (<5000 Da) induced Fos-ir cells in the AOB of female Wistar rats, while the remaining substances after the ultrafiltration (>5000 Da) did not induce a remarkable number of Fos-ir cells. These results suggest that molecular weights of components carrying activity to induce Fos-ir cells in the rat AOB are below 5000 Da.

Papain cleaves only certain peptide bonds such as arginine-X bond, while pronase nonspecifically cleaves many types of peptides. The activity of the component in male urine to induce expression of Fos-immunoreactivity in the caudal region of the AOB was abolished by the papain treatment, while that in the rostral region was not. The pronase treatment abolished the activity to induce expression of immunoreactivity in the rostral region as well as that in the caudal region. These results suggest that at least two urinary peptides (papain-sensitive and -insensitive ones) carrying the activity to stimulate the vomeronasal organ of female rats are contained in male Wistar rat urine.

It has been demonstrated that the vomeronasal pump sucks pheromones dissolved in fluid into the vomeronasal organ (2, 3). For example, compounds of low volatility such as major urinary protein complex and aphrodisin, which belongs to a family of extracellular proteins (lipocalin), induce acceleration of puberty onset in mice and copulatory behavior in male hamsters via the vomeronasal organ, respectively (16–18). It is likely that female rats receive peptides in male rat urine as pheromones via the vomeronasal system.

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