Inhibition of Fos-Immunoreactivity in Response to Urinary Pheromones by β -Adrenergic and Serotonergic Antagonists in the Rat Accessory Olfactory Bulb

Kouhei INAMURA and Makoto KASHIWAYANAGI*

Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita 12-jo, Nishi 6-chome, Kita-ku, Sapporo 060-0812, Japan. Received April 3, 2000; accepted June 5, 2000

The vomeronasal organs of female Wistar rats after the intraperitoneal administration of ketanserin and propranolol prior to sacrifice were exposed to sprayed urine of male Wistar rats. To explore the effects of these antagonists, we studied Fos-immunoreactive (Fos-ir) structures, which correlate with cellular activity, in the accessory olfactory bulb of female rats after the vomeronasal organ was exposed to urine. After the administration of 3 mg/kg ketanserin, the expression of Fos-ir cells in the periglomerular cell layer in response to male Wistar urine was inhibited, while that in the mitral/tufted cell and granule cell layers was not changed. The administration of 20 mg/kg propranolol inhibited the expression of Fos-ir cells in all three layers. These results suggest that serotonin and noradrenaline are likely involved in the modulation of the expression of Fos-ir cells in response to the urine in the accessory olfactory bulb.

Key words urinary pheromone; rat; accessory olfactory bulb; Fos; neurotransmitter

Pheromonal signals, which are primarily received by the vomeronasal organ, provide specific information concerning the identity, gender, and social status of different members of the population and affect the sexual behaviors of conspecific male and female animals *via* the vomeronasal organ in a variety of mammals.¹⁻³⁾ The vomeronasal sensory neurons in the vomeronasal organ project to the accessory olfactory bulb located on the dorso-caudal surface of the main olfactory bulb.⁴⁾ The accessory olfactory bulb has a cellular composition similar to that of the main olfactory bulb.^{2,3)} The main olfactory bulb is composed of three types of neurons, including mitral/tufted cells, periglomerular cells, and granule cells, as is the accessory olfactory bulb. Mitral/tufted cells are the only output neurons of the accessory olfactory bulb, and they send excitatory projections to the anterior as well as the posterior portions of the medial amygdaloid nucleus.⁵⁾ Glutamate acts as an excitatory neurotransmitter between mitral/tufted cells and periglomerular cells, and between mitral/tufted cells and granule cells.^{6,7)} Periglomerular cells and granule cells inhibit the excitation of mitral/tufted cells *via* γ -aminobutyrate (GABA). In addition, serotonergic and adrenergic innervations have been observed in the main olfactory bulb.^{8,9)} The β -adrenergic antagonist propranolol has been observed to induce a suppression of the odor-related patterns of Fos accumulation in the main olfactory bulb, suggesting a physiological significance of β -adrenergic receptors in the main olfactory bulb.¹⁰⁾

The induction of c-fos has been widely used as an assay for studying the excitability of populations of neurons within many different brain regions, including the olfactory bulb.¹¹⁾ Exposure of the vomeronasal organ of the female Wistar rat to male and female Wistar urinary pheromones has been observed to induce the appearance of many Fos-ir cells in the accessory olfactory bulb.^{12,13)} In a separate study, we found that many more Fos-ir cells were expressed in the sexually experienced rats than in inexperienced rats in the localized region of the periglomerular cell layer, suggesting that a sexually experienced male's preference for oestrous urine may be brought about by an increase in excitability at the

periglomerular cell layer in the localized region of the accessory olfactory bulb (submitted for publication). In the present study, we addressed the question of whether noradrenergic and serotonergic innervation play a physiological role in the accessory olfactory bulb during urinary pheromone reception by measuring the effects of antagonists for serotonin and noradrenaline.

MATERIALS AND METHODS

Animals The Wistar rats (more than 3 month old) obtained from Sankyo Laboratory Co., Sapporo, Japan, were housed in same-sex groups of four and kept in a room at $22 \pm 0.5^\circ\text{C}$ and 58% relative humidity, with a 14-h light/10-h dark cycle (light off at 21:00 h). The males and females were housed in the same room. All rats had free access to food and water.

Urinary Stimulation Urinary stimulation was carried out as described by Inamura *et al.*¹²⁾ and Tsujikawa and Kashiwayanagi.¹³⁾ Twenty-five ml of crude urine from male Wistar rats was sprayed on the nares of female rats with commercial cosmetic sprays every 1 min for *ca.* 30 min. Reproductive results were obtained by the present stimulating method.^{12,13)} Rats were gently held by hand during a spray without anesthetic. Urine was collected from four or five rats using a metabolic cage just before exposure. The animals, which were deeply anesthetized with pentobarbital sodium (35 mg/kg), were sacrificed 75 min after exposure to the stimulus.

Pharmacological Treatment Pharmacological treatments were carried out as described by Sallaz and Jourdan.¹⁰⁾ Drugs were given intraperitoneally in volumes of 1 ml normal (0.9%) saline per injection. Female rats received either ketanserin (3 mg/kg) or propranolol (20 mg/kg). Drugs of these concentrations were used in previous studies.^{10,14-18)} Following a 30 min post-injection recovery period,¹⁰⁾ animals were submitted to urine and the Fos procedure, as described below.

Tissue Processing and Immunohistochemistry The an-

* To whom correspondence should be addressed. e-mail: yanagi@hucc.hokudai.ac.jp

Fig. 1. The Density of Fos-ir Cells (Number/mm²) in the Accessory Olfactory Bulb of Rats Stimulated with Male Wistar Urine (A, C and D) or Water (B) Following the Intraperitoneal Administration of 3 mg/kg Ketanserin (C) or 20 mg/kg Propranolol (D)

White and shadowed columns indicate the density of Fos-ir cells in the rostral and caudal regions. Vertical bars represent the mean $\pm$ S.E.M.

imals were perfused through the heart with phosphate-buffered saline (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 μ m. The free-floating sagittal sections were first treated with 0.3% H₂O₂ for 15 min in PBS with 0.4% Triton X-100 (PBSx) or PBS for Fos or G_{i2 α} immunostaining, respectively, followed by two washes of PBS. After a 1-h incubation in 3% normal rabbit serum or 10% normal goat serum, the sections were incubated with c-Fos polyclonal antibody (1:5000, OA-11-823; Cambridge Research Biochemicals) in PBSx for 24 h at 4 °C or with antibody to a synthetic peptide fragment of G_{i2 α} (1:5000; Wako, Osaka, Japan) overnight at room temperature. The control section was incubated without c-Fos antibody. The sections were then rinsed in PBSx or PBS and incubated with biotinylated goat anti-sheep IgG (1:200; Vector, Burlingame, CA) for 1 h or with biotinylated goat anti-rabbit IgG (1:200; Vector), respectively. The sections were rinsed again in PBSx or PBS, incubated with ABC (ABC Elite kit, Vector) for 1 h, and developed with diaminobenzidine (DAB)/H₂O₂ (0.05% DAB and 0.003% H₂O₂ in 0.05 M Tris-HCl buffer) for 12 min or 4 min. The sections were rinsed with water and mounted. All Fos-ir cells

in the accessory olfactory bulb were counted under a microscope. The sections were analyzed blind. To estimate the density of Fos-ir cells, areas of the periglomerular layer, mitral/tufted cell layer, and granule cell layer in photographs of the accessory olfactory bulb were measured. Data are given as mean $\pm$ standard error of the mean.

RESULTS AND DISCUSSION

As shown previously, exposure to urine from male Wistar rats induced Fos expression in the accessory olfactory bulb of the female Wistar rat.^{12,13} Figure 1A shows the density of Fos-ir cells (number/mm²) in sagittal sections (lateral and medial regions) of the accessory olfactory bulb of female Wistar rats after exposure to male Wistar urine. There are two distinct regions in the accessory olfactory bulb.^{19,20} To estimate the density of Fos-ir cells in these two regions, we divided the accessory olfactory bulb into rostral and caudal regions using immunostaining with anti-G_{i2 α} antibody to identify the boundary between rostral and caudal (data not shown). Fos-ir cells were found in the mitral/tufted cell layer, granule cell layer, and periglomerular cell layer. The greatest density of labeled cells was observed in the granule cell layer. Exposure to water resulted in no appearance of Fos-ir

cells in any layer of the accessory olfactory bulb (Fig. 1B).

The administration of 3 mg/kg ketanserin did not change the density of Fos-ir cells in response to male urine in either the rostral or caudal regions of the mitral/tufted cell and granule cell layer (Fig. 1C). However, the administration of ketanserin significantly suppressed the expression of Fos-ir cells in both regions of the periglomerular cell layer ($p < 0.05$, repeated analysis of variance (ANOVA)). Afferent serotonergic neurons project to the accessory olfactory bulb.⁸⁾ Antidepressant drugs suppress copulation in sexually active animals,²¹⁾ suggesting that serotonin is related to sexual behaviors.²²⁾ Therefore, it is possible that pheromonal information via serotonin at the accessory olfactory bulb may play a role in sexual behaviors.

The administration of propranolol reduced the density of Fos-ir cells after exposure to urine in the mitral/tufted cell and granule cell layers (Fig. 1D; $p < 0.05$ and $p < 0.005$, repeated ANOVA). In the periglomerular cell layer, there were no Fos-ir cells in response to urine after the administration of propranolol ($p < 0.1$, repeated ANOVA). It is known that the ewe learns the odor of her lamb, which later allows for distinguishing between her offspring and alien lambs.²³⁾ The activation of β -adrenergic receptors in the main olfactory bulb during the period surrounding parturition is required to establish olfactory recognition of the lamb.²³⁾ In rat pups, noradrenaline is necessary for the acquisition of learned olfactory behavior and main olfactory bulb responses.¹⁴⁾ The distribution of noradrenergic fibers in the accessory olfactory bulb is as similar to that in the main olfactory bulb,⁹⁾ suggesting that β -adrenergic receptors may play a physiological role in the accessory olfactory bulb.

The $G_{i2\alpha}$ -expressing neurons in the vomeronasal epithelium project to the rostral portion of the accessory olfactory bulb and the $G_{o\alpha}$ -expressing neurons project to the caudal portion.^{19,20)} Recent studies have demonstrated that these two anatomically segregated pathways are functionally different in mice and rats.^{12,13,24-27)} However, there was no significant difference in the degree of inhibition by these antagonists between the rostral and caudal regions.

Acknowledgements We gratefully thank Prof. Kenzo Kurihara for his support. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan. This work

was also supported by the Akiyama Foundation. K. I. is a research fellow of the Japan Society for the Promotion of Science.

REFERENCES

- 1) Keverne E. B., Murphy C. L., Silver W. L., Wysocki C. J., Meredith M., *Chem. Senses*, **11**, 119—133 (1986).
- 2) Wysocki C. J., Meredith M., "Neurobiology of Taste and Smell," ed. by Finger T. E., Silver W. L., Wiley, New York, 1987, pp. 125—150.
- 3) Halpern M., *Ann. Rev. Neurosci.*, **10**, 325—362 (1987).
- 4) Barber P. C., Raisman G., *Brain Res.*, **81**, 21—30 (1974).
- 5) Martinez-Marcos A., Halpern M., *Eur. J. Neurosci.*, **11**, 3789—3799 (1999).
- 6) Brennan P., Kaba H., Keverne E. B., *Science*, **250**, 1223—1226 (1990).
- 7) Nakanishi S., *Trends Neurosci.*, **18**, 359—364 (1995).
- 8) McLean J. H., Shipley M. T., *J. Neurosci.*, **7**, 3016—3028 (1987).
- 9) McLean J. H., Shipley M. T., Nickell W. T., Aston-Jones G., Reyher C. K., *J. Comp. Neurol.*, **285**, 339—349 (1989).
- 10) Sallaz M., Jourdan F., *Brain Res.*, **721**, 66—75 (1996).
- 11) Schoppa N. E., Kinzie J. M., Sahara Y., Segerson T. P., Westbrook G. L., *J. Neurosci.*, **18**, 6790—6802 (1998).
- 12) Inamura K., Kashiwayanagi M., Kurihara K., *Eur. J. Neurosci.*, **11**, 2254—2260 (1999).
- 13) Tsujikawa K., Kashiwayanagi M., *Biochem. Biophys. Res. Commun.*, **260**, 222—224 (1999).
- 14) Sullivan R. M., Wilson D. A., Leon M., *J. Neurosci.*, **9**, 3998—4006 (1989).
- 15) Chojnacka-Wojcik E., Tatarczynska E., Golembiowska K., Przegalinski E., *Neuropharmacol.*, **30**, 711—717 (1991).
- 16) Klisch J., Bode-Greuel K. M., *Brain Res.*, **578**, 1—7 (1992).
- 17) Grignaschi G., Mantelli B., Samanin R., *Neurosci. Lett.*, **152**, 103—106 (1993).
- 18) Gue M., Alary C., Rio-Lacheze C. D., Junien J. L., Bueno L., *Eur. J. Pharmacol.*, **233**, 193—199 (1993).
- 19) Halpern M., Shapiro L. S., Jia C., *Brain Res.*, **677**, 157—161 (1995).
- 20) Jia C., Halpern M., *Brain Res.*, **719**, 117—128 (1996).
- 21) Yells D. P., Prendergast M. A., Hendricks S. E., Nakamura M., *Pharmacol. Biochem. Behav.*, **49**, 121—127 (1994).
- 22) Taylor G., Bardgett M., Csernansky J., Early T., Haller J., Scherrer J., Womack S., *Physiol. Behav.*, **59**, 479—485 (1996).
- 23) Levy F., Gervais R., Kindermann U., Orgeur P., Piketty V., *Behav. Neurosci.*, **104**, 464—469 (1990).
- 24) Inamura K., Matsumoto Y., Kashiwayanagi M., Kurihara K., *J. Physiol. (London)*, **517**, 731—739 (1999).
- 25) Brennan P. A., Schellinck H. M., Keverne K. B., *Neuroscience*, **90**, 1463—1470 (1999).
- 26) Dudley C. A., Moss R. L., *Neuroscience*, **91**, 1549—1556 (1999).
- 27) Halem H. A., Cherry J. A., Baum M. J., *J. Neurobiol.*, **39**, 249—263 (1999).