Induction of c-Fos Expression in Mouse Vomeronasal Neurons by Sex-specific Non-volatile Pheromone(s)

Hiroko Kimoto and Kazushige Touhara
Department of Integrated Biosciences, University of Tokyo, Chiba 277-8562, Japan

Correspondence to be sent to: Kazushige Touhara, e-mail: touhara@k.u-tokyo.ac.jp

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Introduction

Many mammalian species utilize chemical signals, commonly termed pheromone, for social and sexual communication between the same species via the vomeronasal organ (VNO). VNO-ablated or genetically modified mice showed behavioral impairments, providing strong evidence that pheromones are detected by VNO (Wysoki and Lepri, 1991; Del Punta et al., 2002; Loconto et al., 2003; Norlin et al., 2003). The vomeronasal neuroepithelium can be divided in two separate zones, characterized by specific pheromone receptors and G proteins: the apical layer that expresses the V1R class of receptors and Gαi2 and the basal layer that expresses the V2R class of receptors and Gαo (Dulac and Torello, 2003). Calcium imaging and electrophysiological recording revealed that V1R/Gαo-neurons responded to volatile chemicals including 2-heptanone, farnesene and dimethylpyrazine (Leinders-Zufall et al., 2000) and that V1rb2 was shown to be one of 2-heptanone receptors (Boschat et al., 2002). V2R/Gαo-neurons have been suggested to respond large non-volatile compounds such as peptide and protein (Krieger et al., 1999), although no ligand pair with V2R has been reported. To elucidate unambiguously the molecular mechanisms underlying pheromone communication, we need to identify receptors and their ligands in combination with behavioral and neuroendocrinological assays. In this report, using c-Fos as a marker for neuronal activation in the vomeronasal system (Halem et al., 1999, 2001), we provide evidence that non-volatile pheromone(s) in soiled bedding, not originated from urine, activate V2R/Gαo-neurons in a sex-specific manner.

Materials and methods

BALB/c mice (SLC, Shizuoka, Japan) were housed under a 12 h light/dark cycle (light on at 8:00 a.m.). Adult male mice were placed individually in clean bedding. After 2 days, the soiled bedding was collected and utilized for assay. Urine was collected from BALB/c adult males by holding the tail and waiting for natural discharge. Collected urine was immediately frozen and stored at –80°C until use. Mice were exposed to clean bedding or soiled male bedding and were killed after continuous exposure for 90 min. The stimulation was carried out between 8:00 a.m. and 11:00 a.m. After exposure, mice were perfused intracardially with ice-cold 4% paraformaldehyde (PFA) in PBS. Snouts were removed and post-fixed in 4% PFA at 4°C. The sample was placed in a 30% sucrose solution in PBS for 20 h at 4°C and embedded in OCT (Sakura, Tokyo, Japan). Every third cryosection (15 µm each) was collected and mounted on a MAS-coated glass slide (Matunami Glass Ind. Ltd, Japan). Slides were treated with 1% H2O2 for 30 min in TBS containing 0.1% Triton X-100 (TBST), followed by incubation with a blocking solution including 3% bovine serum albumin in TBST. The VNO sections were incubated for 60–70 h at 4°C with a 1:5000 dilution of anti-c-Fos polyclonal antibody (Ab-5; Oncogene, San Diego, CA) in the blocking solution and then incubated with the biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Staining was performed using ABC kit (Vector Laboratories) and DAB (Sigma). The stained sections were then incubated with a 1:500 dilution of anti-Gαo antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in TBST for 24 h at 4°C. Under a confocal microscope, the locations of c-Fos positive neurons were determined using Alexa488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, OR).

Results and discussion

Soiled bedding of adult BALB/c male elicited c-Fos expression in VNO of 10-week-old virgin female BALB/c mice (Figure 1A). Exposure to clean bedding did not induce c-Fos expression. c-Fos positive neurons were shown to be localized mainly in the basal layer by double staining of c-Fos and Gαo, indicating that the responses to some substance(s) in soiled bedding were mediated by V2Rs. We quantified the number of c-Fos positive neurons in VNO from anterior to posterior and found that induction of c-Fos expression was observed consistently in V2R/Gαo-neurons (i.e. 8.3 ± 1.0 per slice in...
the basal layer and 0.2 ± 0.05 in the apical layer; Figure 1B). Exposure of soiled male bedding to male mice did not elicit c-Fos expression (Figure 1C). Conversely, male mice responded to soiled female bedding, suggesting that c-Fos induction was sex-specific.

We next investigated whether a compound(s) inducing c-Fos expression was volatile or non-volatile. When mice were placed on a wire mesh barrier without direct interaction with soiled bedding, induction of c-Fos expression was not observed (data not shown), suggesting that the putative pheromone(s) was non-volatile. When virgin male and female mice were free to interact each other, c-Fos expression was observed in both male and female (data not shown). These results were consistent with the observation that activation of accessory olfactory bulb (AOB) neurons required direct contact between the snout of test mice and stimulus conspecifics (Luo et al., 2003).

Previous studies have demonstrated that urine alone was effective in inducing c-Fos expression in AOB neurons (Guo et al., 1997; Inamura et al., 1999; Yamaguchi et al., 2000). It is, therefore, conceivable that adult male urine contains compounds inducing c-Fos expression in female VNO. Unexpectedly, urine induced weak c-Fos immunoreactivity in only a few neurons (data not shown). These results suggest that a main effective pheromonal component(s) in soiled male bedding is not originated from urine. Since numerous studies have described that urine or urine-derived compounds cause several pheromonal effects including male–male aggression, puberty acceleration and pregnancy block (Dulac and Torello, 2003; Brennan and Keverne, 2004), our observation that the component(s) in the accessory olfactory bulb (AOB) neurons required direct contact between the snout of test mice and stimulus conspecifics (Luo et al., 2003).

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References