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

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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 (Wysocki 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 $G\alpha_{i2}$ and the basal layer that expresses the V2R class of receptors and $G\alpha_o$ (Dulac and Torello, 2003). Calcium imaging and electrophysiological recording revealed that V1R/$G\alpha_{i2}$-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/$G\alpha_o$-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/$G\alpha_o$-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 in PBS for 4 h at 4°C and then incubated in 0.5 M EDTA for 48 h 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 an MAS-coated glass slide (Matunami Glass Ind. Ltd, Japan). Slides were treated with 1% H_2O_2 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-$G\alpha_o$ 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 $G\alpha_o$, 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/$G\alpha_o$-neurons (i.e. 8.3 ± 1.0 per slice in

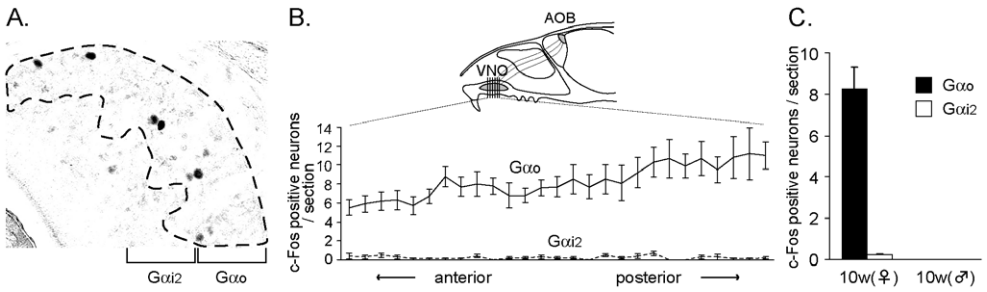

Figure 1 Induction of c-Fos expression by conspecific soiled bedding in V2R/$G\alpha_o$-neurons **(A)** c-Fos immunostaining of BALB/c female VNO stimulated with BALB/c male soiled bedding. The $G\alpha_o$ layer is shown by dotted line. **(B)** Quantification of the numbers of c-Fos positive neurons from anterior to posterior of VNO. **(C)** The numbers of c-Fos positive neurons in the VNO of 10-week-old male or female mice exposed to adult male soiled bedding.

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 stimulant(s) is non-volatile and not urine-originated is likely associated with other sociosexual behaviors. The final goal of this project is to identify the sex-specific compound(s) inducing c-Fos expression, conceivably the ligand(s) of a V2R and to prove that it is indeed an authentic pheromone(s). We are currently characterizing the substance(s) and its/their receptors in order to explore molecular mechanisms underlying pheromonal communication in mice.

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References

Boschat, C., Pelofi, C., Randin, O., Roppolo, D., Luscher, C., Broillet, M.C. and **Rodriguez, I.** (2002) *Pheromone detection mediated by a V1r vomeronasal receptor.* Nat. Neurosci., 5, 1261–1262.

Brennan, P.A. and **Keverne, E.B.** (2004) *Something in the air? New insights into mammalian pheromones.* Curr. Biol., 14, R81–R89.

Del Punta, K., Leinders-Zufall, T., Rodriguez, I., Jukam, D., Wysocki, C.J., Ogawa, S., Zufall, F. and **Mombaerts, P.** (2002) *Deficient pheromone responses in mice lacking a cluster of vomeronasal receptor genes.* Nature, 419, 70–74.

Dulac, C. and **Torello, A.T.** (2003) *Molecular detection of pheromone signals in mammals: from genes to behaviour*. Nat. Rev. Neurosci., 4, 551–562.

Guo, J., Zhou, A. and **Moss, R.L.** (1997) *Urine and urine-derived compounds induce c-fos mRNA expression in accessory olfactory bulb*. Neuroreport, 8, 1679–1683.

Halem, H.A., Cherry, J.A. and **Baum, M.J.** (1999) *Vomeronasal neuroepithelium and forebrain Fos responses to male pheromones in male and female mice*. J. Neurobiol., 39, 249–263.

Halem, H.A., Baum, M.J. and **Cherry, J.A.** (2001) *Sex difference and steroid modulation of pheromone-induced immediate early genes in the two zones of the mouse accessory olfactory system*. J. Neurosci., 21, 2474–2480.

Inamura, K., Kashiwayanagi, M. and **Kurihara, K.** (1999) *Regionalization of Fos immunostaining in rat accessory olfactory bulb when the vomeronasal organ was exposed to urine*. Eur. J. Neurosci., 11, 2254–2260.

Krieger, J., Schmitt, A., Lobel, D., Gudermann, T., Schultz, G., Breer, H. and **Boekhoff, I.** (1999) *Selective activation of G protein subtypes in the vomeronasal organ upon stimulation with urine-derived compounds.* J. Biol. Chem., 274, 4655–4662.

Leinders-Zufall, T., Lane, A.P., Puche, A.C., Ma, W., Novotny, M.V., Shipley, M.T. and **Zufall, F.** (2000) *Ultrasensitive pheromone detection by mammalian vomeronasal neurons.* Nature, 405, 792–796.

Loconto, J., Papes, F., Chang, E., Stowers, L., Jones, E.P., Takada, T., Kumanovics, A., Fischer Lindahl, K. and **Dulac, C.** (2003) *Functional expression of murine V2R pheromone receptors involves selective association with the M10 and M1 families of MHC class Ib molecules.* Cell, 112, 607–618.

Luo, M., Fee, M.S. and **Katz, L.C.** (2003) *Encoding pheromonal signals in the accessory olfactory bulb of behaving mice*. Science, 299, 1196–1201.

Norlin, E.M., Gussing, F. and **Berghard, A.** (2003) *Vomeronasal phenotype and behavioral alterations in $G\alpha_{i2}$ mutant mice.* Curr. Biol., 13, 1214–1219.

Wysocki, C.J. and **Lepri, J.J.** (1991) *Consequences of removing the vomeronasal organ.* J. Steroid Biochem. Mol. Biol., 39, 661–669.

Yamaguchi, T., Inamura, K. and **Kashiwayanagi, M.** (2000) *Increases in Fos-immunoreactivity after exposure to a combination of two male urinary components in the accessory olfactory bulb of the female rat*. Brain Res., 876, 211–214.