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Sex-specific Responses to Urinary Chemicals by the Mouse Vomeronasal Organ

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Abstract

Social behaviors of most mammals are affected by chemical signals, pheromones, exchanged between conspecifics. Previous experiments have shown that behavioral responses to the same pheromone differ depending on the sex and endocrine status of the respondent. Although the exact mechanism of this dimorphism is not known, one possible contributor may be due to sexually dimorphic receptors or due to differences in central processing within the brain. In order to investigate the differences in response between male and female mice to the same pheromonal stimulus two urinary compounds (2-heptanone and 2,5-dimethylpyrazine) were used to stimulate the production of Inositol (1,4,5)-trisphosphate (IP_3) in microvillar membrane preparations of the vomeronasal organ as an indirect measurement of pheromonal stimulation. Incubation of such membranes from prepubertal mice with urine from the same sex or opposite sex, results in an increase in production of IP_3. This stimulation is mimicked by GTPγS and blocked by GDPβS. Furthermore we found that 2-heptanone present in both male and female urine was capable of stimulating increased production of IP_3 in the female VNO but not the male VNO. Finally, 2,5-dimethylpyrazine present only in female urine was also only capable of stimulating increased production of IP_3 in the female VNO.

Key words: IP_3, mice, pheromones, signal transduction, urinary compounds, VNO

Introduction

The mammalian olfactory system recognizes a wide range of molecules that represent important information about an animal's environment. The main olfactory system is used to locate food and detect predators or prey, whereas a second system, called the vomeronasal system or accessory olfactory system recognizes species-specific chemical signals (pheromones) which are used to coordinate social and reproductive behaviors. The accessory olfactory system consists of the vomeronasal organ (VNO), the vomeronasal glands and the accessory olfactory bulb (AOB). The VNO is distinctly separated from the nasal cavity in most amphibia, reptiles and nonprimate mammals, but is absent in birds and adult catarrhine monkeys and apes (Stoddard, 1980). The VNO has the appearance of a paired, tubular structure divided by the nasal septum, each side having a crescent-shaped lumen lined with receptor neurons on the medial concave side and filled with fluid from the vomeronasal glands (Døving and Trotier, 1998). Lateral to the lumen are large blood vessels and sinuses that are innervated by the autonomic nervous system inducing vasodilations and vaso-constrictions that produce a pump-like action for stimulus access to the lumen (Meredith, 1994).

The vomeronasal neurons can be divided into two main groups: (1) the most apical zone of the neuroepithelium contains vomeronasal neurons that project to the anterior aspect of the accessory olfactory bulb and express the G-protein $G\alpha_{i2}$ and members of either V1R or V3R families of putative pheromone receptors; and (2) the basal zone contains vomeronasal neurons that project to the posterior aspect of the accessory olfactory bulb and express the G-protein $G\alpha_0$ and members of the V2R family of receptors (Halpern *et al.*, 1995; Berghard and Buck, 1996; Jia and Halpern, 1996; Wekesa and Anholt, 1999; Dulac, 2000; Pantages and Dulac, 2000). V1R receptors are the same general type G-protein-coupled receptor as the olfactory receptor and there are estimated to be ~150 different types of receptors (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; Rodriguez *et al.*, 2002). V2Rs on the other hand are similar to metabotropic glutamate receptors in that they have a long extracellular

N-terminal region believed to be involved in ligand binding. There are estimated to be 100 V2Rs in rodents, arrayed into several sub-families (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Vomeronasal transduction in vertebrates is distinct and driven by phospholipase C (PLC)-induced production of IP_3 and the subsequent increase in intracellular calcium concentration (Luo *et al.*, 1994; Inamura *et al.*, 1997; Wekesa and Anholt, 1997; Holy *et al.*, 2000; Inamura and Kashiwayanagi, 2000; Leinders-Zufall *et al.*, 2000; Cinelli *et al.*, 2002; Wekesa *et al.*, 2003). Although there is some consensus on the role of PLC in the transduction process, there is still debate on the type of G-protein involved and the role of IP_3 (Lucas *et al.*, 2003). Studies in mice using bacterial toxins such as pertussis that lead to ADP-ribosylation of G-protein alpha subunits of G_0 and G_{i2} have been unable to block the production of IP_3 in the VNO, thus suggesting a role for Gq/11 in signal transduction (Wekesa *et al.*, 2003)

Previous experiments have shown that behavioral responses to the same pheromone differ depending on the sex and endocrine status of the respondent. For example, in male mice, exposure to female pheromones facilitates luteinizing hormone secretion (Johnston and Bronson, 1982; Coquelin *et al.*, 1984) and stimulates ultrasonic vocalizations (Nyby *et al.*, 1979), whereas exposure to male pheromones elicits inter-male aggressive behavior (Guillot and Chapouthier, 1996). In contrast, exposure of females to female pheromones delays puberty and suppresses estrus (Van der Lee and Boot, 1959), while male pheromones accelerate puberty (Vandenbergh, 1969; Lombardi and Vandenbergh, 1977), cause estrous cycle synchronization (Whitten, 1959) and induce pregnancy block to recent mating (Bruce, 1959). In rodents, the major source of pheromones seems to be the urine; however very few volatile (Novotny *et al.*, 1985) and non-volatile (Vandenbergh *et al.*, 1975) urinary substances producing a definite endocrine or behavioral response have been identified. The best-characterized non-volatile urinary components in male mouse urine are the 'major urinary proteins' (Clissold *et al.*, 1984). It has been suggested that the major urinary proteins are involved in puberty acceleration (Vandenbergh *et al.*, 1975; Clark *et al.*, 1985; Mucignat-Caretta *et al.*, 1995), estrus synchronization and puberty delay (Jemiolo *et al.*, 1989; Novotny *et al.*, 1999). The major urinary proteins of mice belong to the super family of lipocalins, a structurally homologous but diverse family of extracellular proteins, characterized by their ability to bind small, principally hydrophobic molecules (Flower, 1996). Urine also contains smaller molecules that have been shown to have pheromonal effects. Two such molecules are 2,5-dimethylpyrazine and 2-heptanone which reportedly are believed to have diverse chemosignaling functions. 2-Heptanone found in both male and female urine has been shown to extend the estrous cycle in female mice whereas 2,5-dimethylpyrazine, found only in female urine, acts to delay puberty in females (Novotny *et al.*, 1985; Jemiolo *et al.*, 1989; Novotny, 2003).

Using a procedure that we have developed for the preparation of a VNO membrane fraction enriched in dendritic microvillar membranes, we report that two urinary compounds, 2-heptanone and 2,5-dimethylpyrazine, stimulate the production of IP_3 when applied to microvillar VNO membranes from pre-pubertal females but not from pre-pubertal males.

Materials and methods

Animals

CD-1 mice *Mus musculus* were initially purchased from Charles River Laboratories (Wilmington, MA). They were maintained in a breeding colony in the Department of Biological Sciences at Alabama State University, Montgomery, AL. Animals were housed in facilities inspected and approved by the Institutional Animal Care and Use Committee and cared for according to the NIH Guidelines for care and use of laboratory animals. Mice were kept in single sex pairs in Nalgene cages 26 × 21 × 14 cm, at 24–28°C room temperature and a 12/12 h light/dark cycle. Food and water were given *ad libitum.* Mice used for VNO membrane preparations were prepubertal (20–21 days old) whereas mice used for urine collection were adults (60–90 days old).

Membrane preparation

VNOs were dissected from their crevices in the nasal cavity, removed from the cartilaginous capsule and frozen on dry ice. The tissues were minced with a razor blade then crushed with a Teflon pestle and subjected to sonication for 2–5 min in ice-cold phosphate-buffered saline (PBS) in a Bransonic bath sonicator. The resulting suspension was layered on a 45% (w/w) sucrose cushion and centrifuged at 4°C for 30 min at 30 000 r.p.m. in a Beckman SW55Ti rotor. The membrane fraction was collected and centrifuged as before for 15 min to pellet the membranes. The membranes were resuspended in 100 µl of ice-cold PBS. Protein concentration was then determined according to the method of Lowry *et al.* (1951).

The procedure used for the preparation of microvillar membranes is modeled after well-established methods for harvesting olfactory cilia from olfactory neuroepithelium (Anholt *et al.*, 1986; Anholt, 1995). The sonication of membranes results not only in the detachment of olfactory microvilli, but also in the detachment of microvilli from sustentacular cells and plasma membrane fragments from other components of the neuroepithelium. Electron microscopic examination of these preparations revealed vesicles, axonemal structures devoid of a plasma membrane and axonemal structures associated with membrane fragments (Anholt *et al.*, 1986; Anholt, 1995). The membrane preparation we refer to as 'microvillar membranes' is, therefore,

likely to contain contaminants derived from other components of the VNO, including microvillar membranes from supporting cells and it is difficult to estimate the purity of the preparation precisely. However, our preparation appears to be sufficiently enriched in chemosensory membranes for the purpose of our studies.

Second messenger assay

Adult gender-specific urine was collected over a 5 day period, pooled, spun for 5 min at 5000 *g*, decanted and frozen as aliquots at –80°C until assayed. Female urine was collected from adult sexually active females at the end of their estrous cycle, whereas male urine was collected from sexually active adult males. IP_3 kits were purchased from Perkin Elmer Life Sciences Inc. (Boston, MA). For IP_3 assays, reactions were incubated for 1 min at 37°C in 25 mM Tris-acetate buffer pH 7.2, 5 mM Mg-acetate, 1 mM DTT, 0.5 mM ATP, 0.1 mM $CaCl_2$, 10 μM GTP and 20 μg VNO membrane protein. Reactions were terminated by the addition of 1 M trichloroacetic acid. The chemicals, 2-heptanone and 2,5-dimethylpyrazine were purchased from Aldrich Chemical Co. (Milwaukee, WI). The non-hydrolysable forms of G-protein guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) were purchased from Boehringer Mannheim (Indianapolis, IN). Differences between experimental and control animals were analyzed by analysis of variance (ANOVA).

Results

GTP-dependent increases in IP_3 levels induced by male and female urine in VNO membranes from female mice

To study transduction pathways activated by pheromonal stimuli, we developed preparations enriched in microvillar membranes from VNOs of prepubertal females. Incubation of microvillar VNO membranes from prepubertal females with adult male and female urine results in a significant increase in the production of IP_3 ($P < 0.05$; Figure 1). We were further able to show that the response that we measured was G-protein dependent by blocking the response with GDPβS and stimulating with GTPγS.

Increase in IP_3 levels induced by 2-heptanone and 2,5-dimethylpyrazine in VNO membranes from prepubertal female mice

Incubation of microvillar membranes from VNOs of prepubertal female mice with 2-heptanone and 2,5-dimethylpyrazine resulted in a robust increase of IP_3 as compared to the control ($P < 0.05$; Figure 2). This effect was blocked by incubation with GDPβS. Although the response was significantly higher than control, it was not as high as stimulation with male or female urine. These results complement behavioral studies that have shown that these compounds have possible chemosignaling function in female mice. It has been shown that 2-heptanone extends estrus in female mice

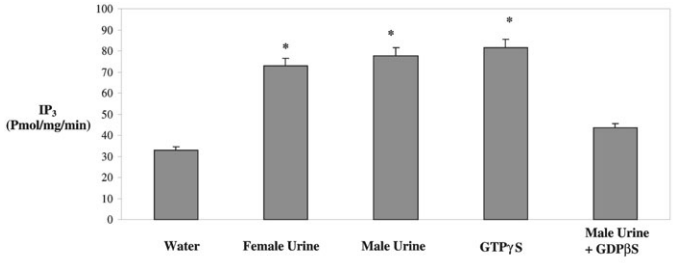

Figure 1 GTP dependence of the production of IP_3 by male and female urine in female VNO membranes. Reactions were performed without stimulus, in the presence of 10% adult female urine, 10% adult male urine, 10 μM GTPγS, or 10 % male urine together with 100 μM GDPβS. Significant stimulation compared to basal activity is observed in the presence of male urine, female urine and GTPγS (*$P < 0.05$) and not in the presence of male urine with GDPβS. The data points and error bars represent the averages and standard errors of at least six independent experiments, each consisting of duplicate measurements.

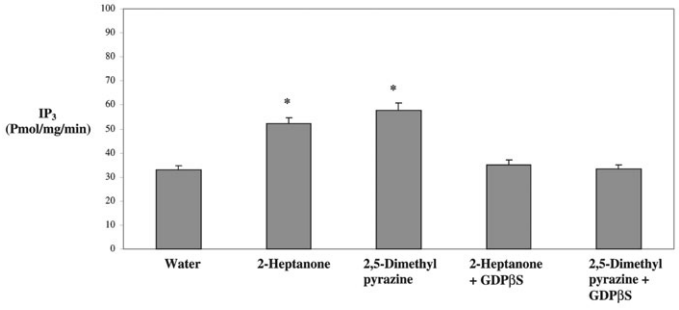

Figure 2 Female VNO response to urinary chemicals. Reactions were performed without stimulus, in the presence of 10 μM 2-heptanone, 10 μM 2,5-dimethylpyrazine, 10 μM 2-heptanone together with 100 μM GDPβS and 10 μM 2,5-dimethypyrazine together with 100 μM GDPβS. Significant stimulation compared to basal activity is observed in the presence of 2-heptanone and 2,5-dimethylpyrazine (*$P < 0.05$). The data points and error bars represent the averages and standard errors of at least six independent experiments, each consisting of duplicate measurements.

(Jemiolo *et al.*, 1989), whereas 2,5-dimethylpyrazine delays puberty in female mice (Novotny *et al.*, 1985).

GTP-dependent increases in IP_3 levels induced by male and female urine in VNO membranes from prepubertal male mice

Incubation of microvillar VNO membranes from prepubertal males with adult male or female urine results in a significant increase in the production of IP_3 ($P < 0.05$; Figure 3). This response is mimicked by GTPγS and blocked by GDPβS.

Incubation of microvillar membranes from prepubertal male VNO with 2-heptanone and 2,5-dimethylpyrazine does not result in an increase in IP_3 levels. Stimulation of prepubertal male VNO membranes by 2,5-dimethylpyrazine and 2-heptanone did not produce a significant increase in IP_3 (Figure 4). These results suggest that these two urinary

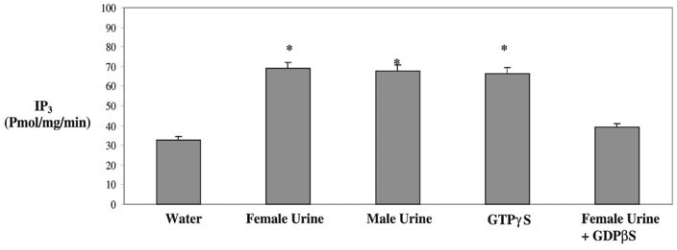

Figure 3 GTP dependence of the production of IP_3 by male and female urine in male VNO membranes. Reactions were performed without stimulus, in the presence of 10% adult female urine, 10% male urine, 10 μM GTPγS, or 10 % female urine together with 100 μM GDPβS. Significant stimulation compared to basal activity is observed in the presence of male urine, female urine and GTPγS (*$P < 0.05$) and not in the presence of male urine with GDPβS. The data points and error bars represent the averages and standard errors of at least six independent experiments, each consisting of duplicate measurements.

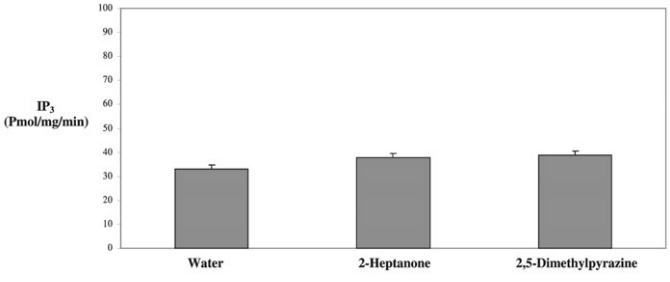

Figure 4 Male VNO response to urinary chemicals. Reactions were performed without stimulus, in the presence of 10μM 2-heptanone or 10 μM 2,5-dimethylpyrazine. No significant stimulation compared to basal activity is observed in the presence of 2-heptanone or 2,5-dimethylpyrazine. The data points and error bars represent the averages and standard errors of at least six independent experiments, each consisting of duplicate measurements.

chemicals are specific for female chemosignals and not male. It is possible that these two compounds may mediate their effects through another second messenger such as cAMP. This is highly unlikely since most of the current data indicates that IP_3 is the dominant second messenger within the vomeronasal system.

Discussion

There are several studies that have shown sexual dimorphism in the vertebrate vomeronasal organ in terms of size of the VNO or number of neurons in the VNO. There is evidence that more neurons are present in the VNO of males than female rats (Segovia and Guillamon, 1982). Zufall *et al.* (2002) tested the effects of 2-heptanone and 2,5-dimethylpyrazine on female mice vomeronasal neurons as measured by an electrovomeronasogram (EVG). They found that mouse vomeronasal neurons are extremely sensitive and highly selective in their response to specialized pheromonal cues and appear to recognize only one or very few pheromonal components (Zufall *et al.*, 2002). Holy *et al.* (2000) using a multielectrode array to record from sheets of VNO neuroepithelium found that there are neurons in male and female VNOs that respond uniquely to pheromones of one sex. There is further evidence that neurons within defined subdivisions of the VNO respond differentially to pheromones and moreover the responses differ in the VNO of females and males (Halem *et al.*, 2001).

Most of the previous biochemical experiments have focused on interactions between males and females thus ignoring chemical communication between members of the same sex. It is well known that female mice send chemical signals to other females that may lead to estrus suppression, puberty delay, or estrus synchronization (Keverne, 1983; Vandenbergh, 1994). Males on the other hand send signals to other males during the formation of hierarchies in social systems (Keverne, 1983; Vandenbergh, 1994). Also most of the previous experiments have focused mainly on female response to male chemical cues. Here we show that males can respond to female cues with an increase in IP_3, thus suggesting that pheromonal cues from females to males that lead to male specific behaviors such as increase in ultrasonic vocalization or reduction in aggression may be mediated by IP_3. The signal transduction pathway of such communication between members of the same sex has also not been explored up to now. Our data indicates that pheromones in male mice urine are capable of stimulating an increase in IP_3 in the male VNO just as female urine is capable of stimulating an increase in IP_3 in female VNOs. This suggests that communication between members of the same sex or of the opposite sex is mediated by increases in IP_3 as the second messenger.

Our study also shows that presentation of 2-heptanone and 2,5-dimethylpyrazine is able to stimulate the production of IP_3 in the female VNO but not the male VNO. When compared with the control, i.e. treatment with water, 2,5-dimethylpyrazine and 2-heptanone were effective in stimulating the production of IP_3 in the female VNO ($P < 0.05$) but not the male VNO. This is an interesting observation that matches behavioral data which show that these two compounds have specific pheromonal effects in females and not males. Even though whole male or female urine is capable of stimulating an increase in IP_3 in males and females, our result suggests that females may respond to specific chemicals within the urine. This is unlike other systems such as in insects where the same chemical compound solicits different pheromonal effects depending on the sex of the recipient. We also noticed in our study that the production of IP_3 in response to the individual urinary components was significantly higher than the controls but much lower than the response from whole male and female urine. This observation can be explained by the notion that urine contains a cocktail of pheromones and that the high stimulus by urine is due to activation of a vast majority of pheromone receptors coupled to the IP_3 pathway. Our

biochemical results complement behavioral studies that have shown that 2-heptanone which is present in both male and female urine extends the estrus cycle whereas 2,5-dimethylpyrazine present in only female urine delays puberty in females (Novotny, 2003).

The differences observed in behavioral responses of males and females to a specific pheromone may be due to differences in central processing. A pheromonal effect has to be conducted through the glomeruli of the AOB to the amygdala and hypothalamus and an appropriate response initiated. Perhaps, it is this sexual dimorphism that allows differences in the behavior responses to a particular pheromone. Experiments using immediate-early gene (IEG) protein expression as markers of neuronal activity have shown that chemical signals in soiled bedding stimulate a sexually dimorphic pattern of c-fos protein immunoreactivity in central sites along the VNO projection pathway (Bakker *et al.*, 1996; Kelliher *et al.*, 1998; Halem *et al.*, 1999, 2001). This explanation is highly unlikely in our case since these two compounds were incapable of stimulating the male VNO.

In most sensory systems, sex differences in perception begin at the sensory receptor. Here we have shown that the vomeronasal organ responds to urinary stimuli in a sex dependent manner. This is important in coordinating social and reproductive behaviors. Sex differences in the VNO response to pheromones could reflect differences in the distribution of VNO receptor subtypes (Herrada and Dulac, 1997). On the other hand, the differences may be due to sex dimorphisms in the size of the VNO and number of neurons (Segovia and Guillamon, 1982) or to sex differences in steroid-sensitive centrifugal inputs mediating the VNO response to pheromones (Halem *et al.*, 2001).

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References

Anholt, **R.R.H.** (1995) *Preparation of olfactory cilia*. In Spielman, A.L. and Brand, J.G. (eds), Experimental Cell Biology of Taste and Olfaction, Current Techniques and Protocols. CRC Press, New York, pp. 163–168.

Anholt, **R.R.H., Aebi, U.** and **Snyder, S.H.** (1986) *A partially purified preparation of isolated chemosensory cilia from olfactory epithelium of the bullfrog,* Rana catesbina. J. Neurosci., 6, 1962–1969.

Bakker, **J., Baum, M.J.** and **Slob, A.K.** (1996) *Neonatal inhibition of brain estrogen synthesis alters adult neural fos response to mating and pheromonal stimulation in the male rat*. Neuroscience, 74, 251–260.

Berghard, **A.** and **Buck, L. B.** (1996) *Sensory transduction in vomeronasal neurons: evidence for G_0, G_{i2} and adenylyl cyclase II as major components of a pheromone signaling cascade*. J. Neurosci., 16, 909–918.

Bruce, **H.M.** (1959) *An exteroceptive block to pregnancy in the mouse*. Nature, 84, 105.

Cinelli, **A.R., Wang, D., Chen, P., Liu, W.** and **Halpern, M.** (2002) *Calcium transients in the garter snake vomeronasal organ*. J. Neurophysiol., 87, 1449–1472.

Clark, **A.J., Charal, P., Bingham, R.W., Barrett, D.** and **Bishop, J.O.** (1985) *Sequence structures of a mouse major urinary protein gene and pseudogene compared*. EMBO J., 4, 3159–3165.

Clissold, **P.M., Hainey, S.** and **Bishop, J.O.** (1984) *Messenger RNAs coding for mouse major urinary proteins are differentially induced by testosterone*. Biochem. Genet., 22, 379–387.

Coquelin, **A., Clancy, A.N., Macrides, F., Noble, E.P.** and **Gorski, R.A.** (1984) *Pheromonally induced release of luteinizing hormone in male mice: involvement of the vomeronasal system*. J. Neurosci., 4, 2230–2236.

Døving, **K.B.** and **Trotier, D.** (1998) *Structure and function of the vomeronasal organ*. J. Exp. Biol., 201, 2913–2925.

Dulac, **C.** (2000) *Sensory coding of pheromone signals in mammals*. Curr. Opin. Neurobiol., 10, 511–518.

Flower, **D.R.** (1996) *The lipocalin protein family*. Biochem. J., 318, 1–14.

Guillot, **P.V.** and **Chapouthier, G.** (1996) *GABAergic neurotransmission in the olfactory bulb and intermale aggression in mice: modulation by steroids*. Behav. Genet., 26, 497–504.

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

Halpern, **M., Shapiro, L.S.** and **Jia, C.** (1995) *Differential localization of G proteins in the opossum vomeronasal system*. Brain Res., 677, 157–61.

Herrada, **G.** and **Dulac, C.** (1997) *A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution*.Cell, 90, 763–770.

Holy, **T.E., Dulac, D.** and **Mesiter, M.** (2000) *Responses of vomeronasal neurons to natural stimuli*. Science, 289, 1569–1572.

Inamura, **K.** and **Kashiwayanagi, M.** (2000) *Inward current responses to urinary substances in rat vomeronasal sensory neurons*. Eur. J. Neurosci., 12, 3529–3536.

Inamura, **K., Kashiwayanagi, M.** and **Kurihara, K.** (1997) *Inositol-1,4,5-trisphosphate induces responses in receptor neurons in rat vomeronasal sensory slices*. Chem. Senses, 22, 93–103.

Jemiolo, **B., Andreolini, F., Xie, T.M., Wiesler, D.** and **Novotny, M.V.** (1989) *Puberty-affecting synthetic analogs of urinary chemosignals in the house mouse,* Mus domesticus. Physiol. Behav., 46, 293–298.

Jia, **C.** and **Halpern, M.** (1996) *Subclasses of vomeronasal receptor neurons; differential expression of G proteins ($G_{i2}\alpha$ and $G_0\alpha$) and segregated projections to the accessory olfactory bulb*. Brain Res., 719, 117–128.

Johnston, **R.E.** and **Bronson, F.H.** (1982) *Endocrine control of female mouse odors that elicit LH surges and attraction in males*. Biol. Reprod., 27, 1174–1180.

Kelliher, **K.R., Chang, Y.M., Wersinger, S.R.** and **Baum, M.J.** (1998) *Sex difference and testosterone modulation of pheromone-induced neuronal Fos in the ferret's main olfactory bulb and hypothalamus*. Biol. Reprod., 59, 1454–1463.

Keverne, **E.** (1983) *Pheromonal influences on the endocrine regulation of reproduction*. Trends Neurosci., 6, 381–384.

Leinders-Zufall, **T., Lane, A.P., Punche, 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.

Lombardi, **J.R.** and **Vandenbergh, J.G.** (1977) *Pheromonally induced sexual maturation in females: regulation by the social environment of the male*. Science, 196, 545–546.

Lowry, **O.H., Rosebrough, N.J., Farr, A.L.** and **Randall, R.J.** (1951) *Protein measurement by Folin phenol reagent*. J. Biol. Chem., 193, 265–275.

Lucas, **P., Ukhanov, K., Leinders-Zufall, T.** and **Zufall, F.** (2003) *A diacylglycerol-gated channel in vomeroansal neuron dendrites is impaired in TRPC2 mutant mice: mechanism of pheromone transduction*. Neuron, 40, 551–561.

Luo, **Y., Lu, S., Chen, P., Wang, D.** and **Halpern, M.** (1994) *Identification of chemoattractant receptors and G-proteins in the vomeronasal system of garter snakes*. J. Biol. Chem., 269, 16867–16877.

Matsunami, **H.** and **Buck, L.** (1997) *A multigene family encoding a diverse array of putative pheromone receptors in mammals*. Cell, 90, 775–784.

Meredith, **M.** (1994) *Chronic recording of vomeronasal pump activation in awake behaving hamster*. Physiol. Behav., 56, 345–354.

Mucignat-Caretta, **C., Caretta, A.** and **Cavaggioni, A.** (1995) *Acceleration of puberty onset in female mice by male urinary proteins*. J. Physiol., 482, 517–522.

Novotny, **M.V.** (2003) *Pheromones, binding proteins and receptor responses in rodents*. Biochem. Soc. Trans., 31, 117–122.

Novotny, **M., Harvey, S., Jemiolo, B.** and **Alberts, J.** (1985) *Synthetic pheromones that promote intermale aggression in mice*. Proc. Natl Acad. Sci. USA, 82, 2059–2061.

Novotny, **M., Ma, W., Zidek, L.** and **Daev, E.** (1999) *Recent biochemical insights into puberty acceleration, estrus induction and puberty delay in the house mouse*. In Johnston, R.E., Muller-Schwarze, D. and Sorenson, P.W. (eds), Advances in Chemical Signals in Vertebrates. Kluwer, Academic/Plenum, New York, pp. 99–116.

Nyby, **J., Wysocki, C.J., Whitney, G., Sinzinni, G.** and **Schneider, J.** (1979) *Elicitation of male mouse ultrasonic vocalizations*. J. Comp. Physiol. Psychol., 93, 957–975.

Pantages, **E.** and **Dulac, C.** (2000) *A novel family of candidate pheromone receptors in mammals*. Neuron, 28, 835–845.

Rodriguez, **I., Del Punta, K., Rothman, S., Ishii, T.** and **Mombaerts, P.** (2002). *Multiple new and isolated families within the mouse superfamily of V1R vomeronasal receptors*. Nat. Neurosci., 5, 134–140.

Ryba, **N.J.P.** and **Tirindelli, R.** (1997) *A new multigene family of putative pheromone receptors*. Neuron, 19, 371–379.

Segovia S. and **Guillamon, A.** (1982) *Effects of sex steroids on the development of the vomeronasal organ in the rat*. Dev. Brain. Res., 5, 209–212.

Stoddard, **M.D.** (1980) The Ecology of Vertebrate Olfaction. Chapman & Hall, London.

Vandenbergh, **J.G.** (1969) *Male odor accelerates female sexual maturation in mice*. Endocrinology, 81, 345–349.

Vandenbergh, **J.G.** (1994) *Pheromones and mammalian reproduction*. In Knobil, E. and Neill, J.D. (eds), Physiology of Reproduction, Vol. 2. Raven Press, New York, pp. 343–362.

Vandenbergh, **J.G., Whitsett, J.M.** and **Lombardi, J.R.** (1975) *Partial isolation of a pheromone accelerating puberty in female mice*. J. Reprod. Fertil., 43, 515–523.

Van der Lee, **S.** and **Boot, L.M.** (1959) *Spontaneous pseudopregnancy in mice*. Acta Physiol. Pharmacol. Nederl., 4, 442–444.

Wekesa, **K.S.** and **Anholt, R.R.H.** (1997) *Pheromone regulated production of inositol-(1,4,5)-trisphosphate in the mammalian vomeronasal organ.* Endocrinology, 138, 3497–3504.

Wekesa, **K.S.** and **Anholt, R.R.H.** (1999) *Differential expression of G proteins in the mouse olfactory system*. Brain Res., 837, 117–126.

Wekesa, **K.S., Miller, S.** and **Napier, A.** (2003) *Involvement of $G_{q/11}$ in signal transduction in the mammalian vomeronasal organ*. J. Exp. Biol., 206, 827–832.

Whitten, **W.K.** (1959) *Occurrence of anoestrus in mice caged in groups*. J. Endocrinol., 18, 102–107.

Zufall, **F., Kelliher, K.R.** and **Leinders-Zufall, T.** (2002) *Pheromone detection by mammalian vomeronasal neurons*. Microsc. Res. Tech., 58, 251–260.

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