Neuron, Vol. 19, 371–379, August, 1997, Copyright ©1997 by Cell Press

A New Multigene Family of Putative Pheromone Receptors

Nicholas J. P. Ryba*‡ and Roberto Tirindelli†
*Taste and Smell Unit
Oral Infection and Immunity Branch
National Institute of Dental Research
National Institutes of Health
Bethesda, Maryland 20892-1188
†Istituto di Fisiologia Umana
Universita di Parma
43100 Parma
Italy

Summary

The vomeronasal organ (VNO) mediates detection of pheromones related to social and reproductive behavior in most terrestrial vertebrates. We have identified a new multigene family of G protein–linked receptors (V2Rs) that are specifically expressed in the VNO. V2Rs have no significant homology to other putative pheromone receptors (V1Rs) or to olfactory receptors but are related to the Ca^{2+}-sensing receptor and metabotropic glutamate receptors. V2Rs are expressed at high levels in small subpopulations of VNO neurons. V2Rs are primarily expressed in a different layer of VNO neurons from V1Rs, thus both gene families are likely to encode mammalian pheromone receptors.

Introduction

Most terrestrial vertebrates possess two anatomically distinct olfactory systems that serve rather different functions. The main olfactory epithelium is responsible for the conventional sense of smell, which in animals may be critical for survival (Brunet et al., 1996) and in humans can evoke complex thoughts and emotions. The vomeronasal organ (VNO) mediates detection of chemical signals that result in innate, subconscious, stereotyped responses (Halpern, 1987; Wysocki, 1989; Meredith and Fernandez-Fewell, 1994). These chemical signals are defined as pheromones and provide a range of social and sexual information. The results of activation of the VNO are dramatic (Wysocki and Lepri, 1991). For example, removal of the VNO in naïve male rodents results in a marked reduction in mating frequency (Clancy et al., 1984; Meredith, 1986). Male-specific aggression is also at least partly a function of the VNO (Clancy et al., 1984). In female mice, VNO activation accelerates sexual development and estrus in the presence of males and delays estrus in groups of females (Johns et al., 1978; Reynolds and Keverne, 1979; Lomas and Keverne, 1982). The chemicals that act as pheromones are largely uncharacterized although there are indications that components of rat and mouse urine can induce several pheromone responses (Johns et al., 1978; Wysocki et al., 1980; Mucignat-Caretta et al., 1995).

‡To whom correspondence should be addressed.

In the olfactory epithelium, many odorants stimulate the production of cyclic AMP through a G protein–coupled signal transduction pathway (Jones and Reed, 1989; Bakalyar and Reed, 1990; Dhallan et al., 1990). About 1000 genes encode distinct proteins with seven transmembrane helices that are likely to function as olfactory receptors (Buck and Axel, 1991). Analysis of the expression patterns of these receptors suggest that individual neurons express a single receptor allele from the repertoire of 1000 different genes (Chess et al., 1994). Neurons expressing particular receptors are randomly distributed within a few broad zones of the olfactory epithelium (Ressler et al., 1993; Vassar et al., 1993). However, the axons from neurons expressing one specific receptor project to a few discrete and stereotyped loci (glomeruli) in the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). This supports a model in which discrimination of odors would be achieved by detection of spatial patterns of activation of the olfactory bulb (Shepherd, 1994; Axel, 1995; Buck, 1996).

The vomeronasal epithelium and olfactory epithelium both originate from the olfactory placode, have similar pseudostratified structures, exhibit neurogenesis throughout life, and respond to olfactory stimulation. However, recent studies (Dulac and Axel, 1995; Halpern et al., 1995; Berghard and Buck, 1996; Berghard et al., 1996; Jia and Halpern, 1996; Wu et al., 1996) suggest that distinct signal transduction pathways are involved in chemosensory reception in the olfactory epithelium and the VNO. One family of 30–100 putative pheromone receptors (V1Rs) has been cloned (Dulac and Axel, 1995). Their expression appears concentrated in the apical half of the receptor cell layer of the VNO. Neurons in this region of the VNO express $G\alpha_{i2}$ (Halpern et al., 1995; Berghard and Buck, 1996; Jia and Halpern, 1996). In contrast, neurons located in the basal half of the receptor cell layer express $G\alpha_o$. We have now identified a new family of G protein–linked receptors that are specifically expressed in the VNO, primarily in the $G\alpha_o$-expressing neurons. V2Rs have no significant homology to V1Rs (Dulac and Axel, 1995) or to olfactory receptors (Buck and Axel, 1991) but are related to the Ca^{2+}-sensing receptor (CaSR) (Brown et al., 1993) and metabotropic glutamate receptors (mGluRs) (Nakanishi, 1992). We have sequenced about 30 distinct cDNAs for V2Rs (including two full-length receptors) and demonstrate that they are expressed at high levels in small subpopulations of VNO neurons. These characteristics are consistent with V2Rs functioning as pheromone receptors.

Results

Cloning and Sequences of Several V2Rs

A degenerate probe to the transmembrane region of olfactory receptors was used to search for receptors expressed in the VNO. An initial cDNA was isolated from rat VNO ($V2R_{1\text{-}1}$). The sequence of $V2R_{1\text{-}1}$ was homologous to the G protein–coupled CaSR (Figure 1A). Rescreening the same VNO cDNA library (1×10^5 plaques)

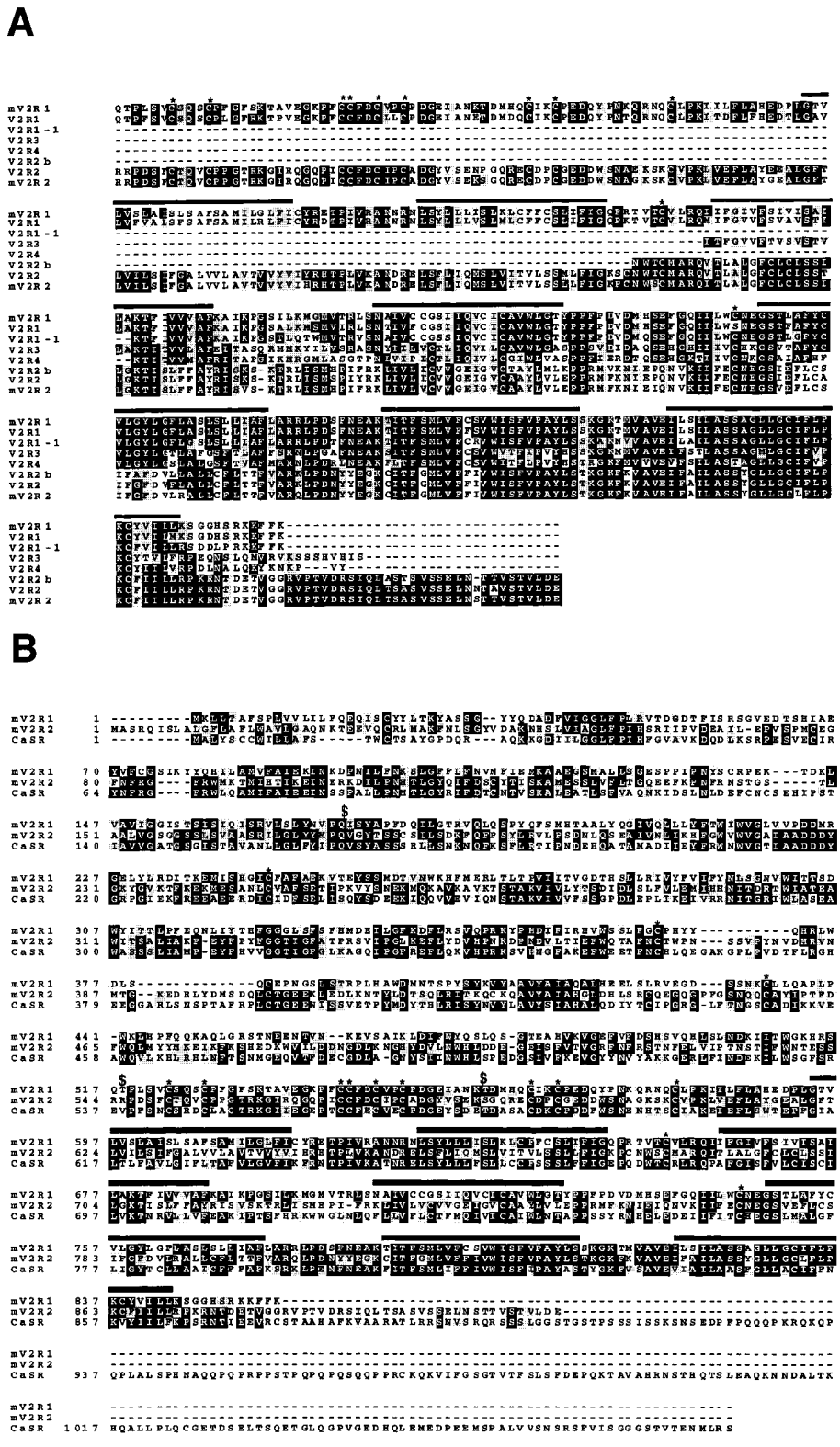

Figure 1. Deduced Protein Sequence of the V2Rs

(A) Comparison of the cysteine-rich and transmembrane domains of the initial cDNA clone ($V2R_{1\text{-}1}$) and other members of four subfamilies of V2Rs including full-length mouse homologs ($mV2R_1$ and $mV2R_2$). Identical and conserved residues are indicated by shading. The seven putative transmembrane helices are indicated by black bars above the sequence; extracellular cysteine residues that are also present in mGluRs are marked (*).

(B) Comparison of the CaSR with $mV2R_1$ and $mV2R_2$; transmembrane helices and conserved cysteines are indicated as in (A), and exon–intron boundaries that we have characterized in $mV2R_1$ are marked ($).

Table 1. Protein and DNA Sequence Identity between Putative Pheromone Receptors and the CaSR

	Identity of Protein and cDNA sequence (%)									
	$mV2R_1$	$V2R_1$	$V2R_{1\text{-}1}$	$mV2R_2$	$V2R_2$	$V2R_{2b}$	$V2R_3$	$V2R_4$	CaSR	
$mV2R_1$		91	91	49	52	51	60	59	48	Similarity of DNA (Coding sequence)
$V2R_1$	94		90	52	53	54	60	60	52	
$V2R_{1\text{-}1}$	94	92		53	55	54	58	58	52	
$mV2R_2$	55 (51[a], 66[b])	65	64		91	92	51	54	52	
$V2R_2$	63	66	63	96		94	49	52	51	
$V2R_{2b}$	65	66	63	96	96		51	52	49	
$V2R_3$	72	73	70	64	64	64		70	48	
$V2R_4$	70	69	67	63	63	63	80		52	
CaSR	56	67	66	63	66	67	61	61		
	Similarity of predicted peptides									

The predicted peptides for the V2Rs shown in Figure 1 were compared with each other (lower left half). The cDNAs encoding these predicted peptides were also compared (upper right half). For the comparison of the mV2Rs (a), the N-terminal extracellular domain and (b) the transmembrane domain were also compared individually.

at moderate stringency with $V2R_{1\text{-}1}$ led to the isolation of 34 cDNA clones, encoding 25 different receptors related to but distinct from $V2R_{1\text{-}1}$. Sequence analysis indicated that these could be grouped into a smaller number of subfamilies of closely related receptors. Members of a subfamily share ~90% sequence identity at both the DNA and protein level (Table 1). However, the different subfamilies that we have identified so far have as little as 50% DNA sequence identity with each other, and are equally divergent from each other as they are from the CaSR (Table 1).

Even though $V2R_{1\text{-}1}$ was isolated by homology screening using an olfactory receptor–based probe, V2Rs have no significant sequence identity to known olfactory receptors or the V1Rs. Full-length clones of mouse homologs of $V2R_1$ and $V2R_2$ were isolated using genomic sequence analysis to generate primers for 5′-RACE and 3′-RACE. Expression of the mouse V2Rs was confirmed by reverse transcription–polymerase chain reaction (RT–PCR) using primers outside the coding sequences. The full-length PCR products were subcloned and sequenced (Figure 1B). Unlike G protein–coupled receptors related to rhodopsin, the CaSR has a signal peptide followed by a long extracellular N terminus that precede the seven *trans*-membrane helices and a long intracellular C-terminal domain (Brown et al., 1993). V2Rs have signal peptides and sequence similarity to the CaSR throughout the extracellular and membrane-spanning domains but, like several of the mGluRs (Nakanishi, 1992), have short C termini. Extracellular cysteine residues conserved between the CaSR and the mGluRs are also found in the V2Rs (Figure 1). Interestingly, in the N-terminal extracellular region, these V2Rs appear to be more divergent from each other than their transmembrane domain (Figure 1, Table 1).

V2Rs Are Specifically Expressed in the VNO

To determine the tissue distribution of V2Rs, we performed Northern analysis using members of the four V2R subfamilies. V2Rs hybridized to mRNA from the VNO but not to mRNA from the olfactory epithelium or any other tissue examined (Figure 2). In situ hybridizations to tissues other than the VNO including the olfactory epithelium, olfactory bulb, regions of the brain, the parathyroid, and several major organs were also negative (data not shown). Low level expression of some related receptors in other tissues cannot be excluded entirely as computer-aided search of the expressed-sequence tag database identified two isolates of a single cDNA clone (accession numbers AA170592 and AA170693) from mouse spleen that appears to be a V2R. However, both in situ hybridization and RT–PCR indicated that V2Rs are not generally expressed in the spleen (data not shown).

Individual V2Rs Are Expressed in Subpopulations of VNO Neurons

In rats and mice, the VNO is a closed tubular structure running in an anterior–posterior direction at the base of the nasal septum. In cross section, the concave neurosensory epithelium lines the ventromedial side of the organ (see Figure 3C). In situ hybridization experiments were performed with digoxigenin-labeled antisense cRNA probes for 11 different V2Rs, including members of each of the four subfamilies. We also used a V1R and G-protein subunits as controls. High stringency conditions were used to ensure that probes detected only very closely related transcripts. The cDNAs that we used to generate probes for the $V2R_2$, $V2R_3$, and $V2R_4$ subfamily members contained extensive 3′-nontranslated sequence that was divergent even between members of one subfamily, further adding to the specificity of hybridization. However, at least in some cases, it is likely that probes recognized more than one member of a subfamily.

The sensory neurons in the VNO all contain the G-protein subunit $G\gamma_8$ (Figure 3C). In situ hybridization of $G\alpha_{i2}$ and $G\alpha_o$ probes (Figures 3A and 3B) define distinct subpopulations of the sensory receptor neurons as has been reported previously (Halpern et al., 1995; Berghard and Buck, 1996; Jia and Halpern, 1996). The $G\alpha_{i2}$ and $G\alpha_o$-containing neurons are located in the apical and basal halves of the receptor cell layer, respectively. The neurons expressing these G proteins are distributed in broad and essentially continuous layers. In contrast, a V1R probe hybridized with a scattered population of a few individual neurons that overlap the layer of $G\alpha_{i2}$ expression (Figure 3D); this distribution is typical of V1Rs (Dulac and Axel, 1995). Under the same conditions, a $V2R_{1\text{-}1}$ probe also hybridized with a small subpopulation of the VNO sensory neurons (Figures 3E and 3F). The

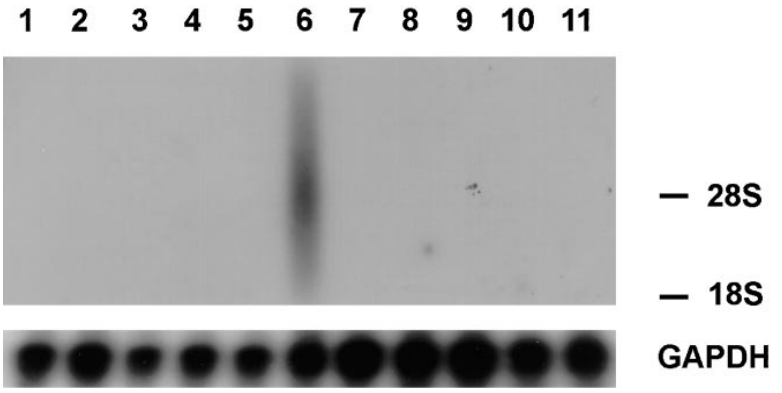

Figure 2. Northern Blot Analysis of Rat A^+-RNA from:

1) cortex; 2) subcortical region; 3) cerebellum and pons; 4) olfactory bulb; 5) olfactory epithelium; 6) VNO; 7) testes; 8) liver; 9) lungs; 10) heart; and 11) kidney. A mix of $V2R_{1-4}$ probes were hybridized and washed at low stringency; rescreening of the blot with a GAPDH probe indicated equal amounts of RNA were loaded in each lane.

cell bodies of the sensory neurons that contained $V2R_{1-1}$ were primarily basal to those expressing $V1R_4$, suggesting that like the G-protein subunits, the two receptor families define layers in the VNO. Sense probes to the receptors did not hybridize (data not shown). No differences in the pattern of hybridization were observed for any V2R between male and female animals (see Figures 3E and 3F).

A predominantly basal localization of the cell bodies of neurons expressing a number of different V2Rs was observed (Figure 4). However, interestingly, by examining several V2Rs, we found additional laminar organization in their expression pattern (Figure 4). For instance, $V2R_4$ expression was tightly restricted to a narrow laminar at the very base of the epithelium (Figure 4A). Another $V2R_4$ subfamily member probe derived exclusively from the 3′-nontranslated region, and with no cross hybridization to $V2R_4$ under high stringency conditions, showed a very similar pattern of hybridization (Figure 4B). To confirm that similar V2Rs were expressed in similar distributions of VNO neurons, we performed in situ hybridization under reduced stringency conditions with the $V2R_4$ probe (Figure 4C). Hybridization was to an almost continuous narrow layer at the base of the epithelium. Both strongly staining cells, expressing $V2R_4$, and more weakly staining cells, expressing $V2R_4$ homologs, were detected. Other V2R subfamilies, for example $V2R_3$ (Figure 4D), were also expressed within the basal half of the sensory cell layer, but the distribution of neurons expressing these receptors was less constrained. Finally, the $V2R_1$ subfamily was expressed in neurons in a broader layer that extended into more apical regions of the VNO (Figures 3E, 3F, and 4E). Similar morphological distributions of V2R expression were observed in >10 adult rats (both male and female) and along the entire length of the VNO.

Each of the individual V2R probes hybridized to between 0.5% and 3% of the receptor cells at high stringency (see legend to Figure 4). A mixture of four V2R cRNA probes, one from each of the four subfamilies,

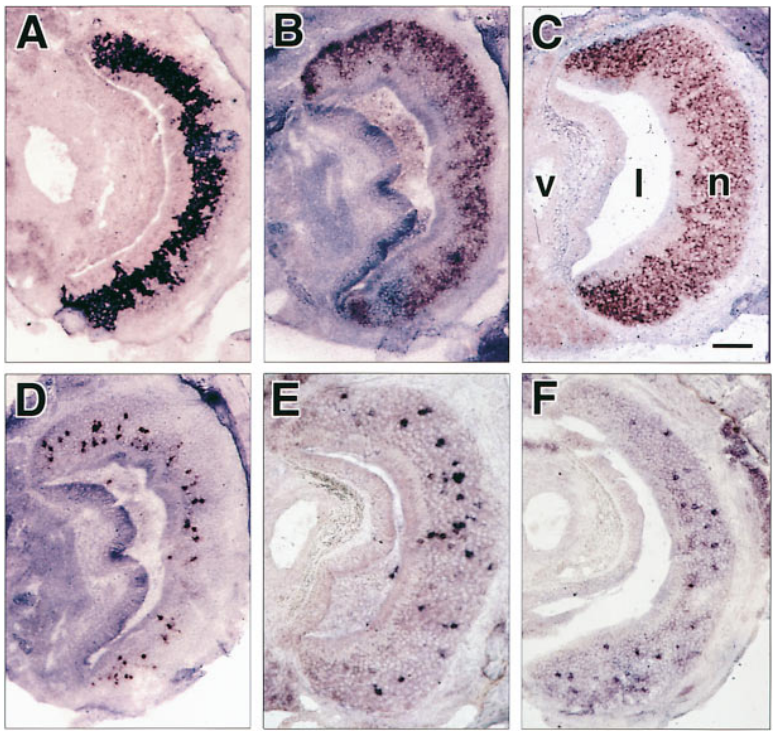

Figure 3. In Situ Hybridization to Coronal Sections of Adult Rat VNO

The antisense cRNA probe was:
(A) $G\alpha_{i2}$.
(B) $G\alpha_o$.
(C) $G\gamma_8$.
(D) $V1R_4$.
(E and F) $V2R_{1-1}$.
(A–E) Male rat VNO.
(F) Female. Hybridization was carried out at high stringency; hybridizing cells stain dark brown. The vomeronasal vein (v), the lumen (l), and the neurosensory epithelium (n) are identified in (C); scale bar = 100 μm.

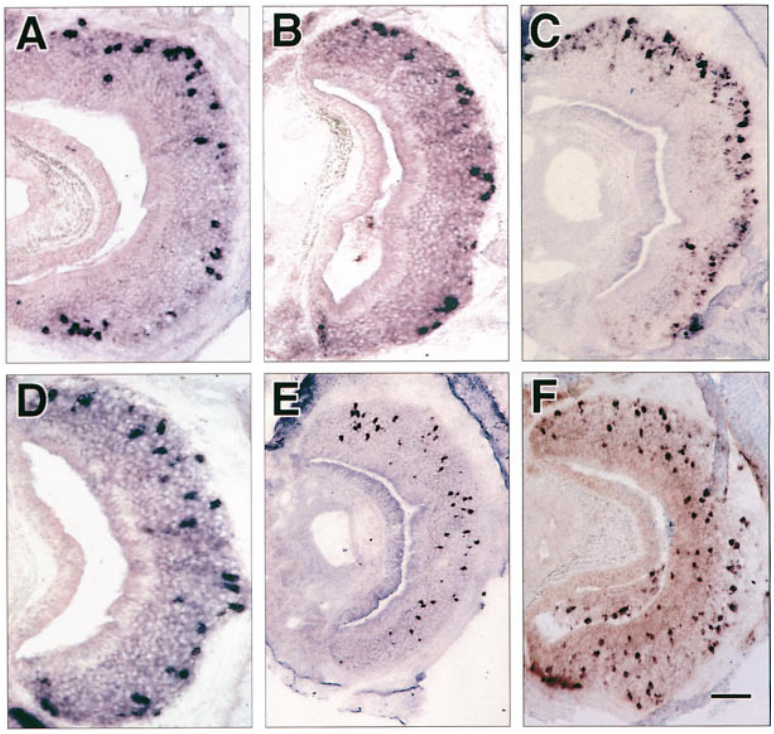

Figure 4. In Situ Hybridization of Different Members of the V2R Family

(A and C–F) Male VNO.
(B) Female VNO.
Probes were:
(A) $V2R_4$.
(B) A different $V2R_4$ subfamily member probe derived exclusively from nontranslated sequence that showed minimal hybridization to $V2R_4$.
(C) The $V2R_4$ probe hybridized at reduced stringency.
(D) $V2R_3$.
(E) $V2R_1$.
(F) A mixture of four antisense probes, one to each subfamily ($V2R_{1-4}$); scale bar = 100 μm. The frequencies of hybridizing cells were: $V2R_1$, 2.2%; $V2R_2$, 0.8%; $V2R_3$, 1.3%; $V2R_4$, 1.1%; and mix, 6.3%.

hybridized with a larger number of neurons than was detected by any single probe (Figure 4F). The number of neurons detected by combined probes to four V2R subfamilies was reproducibly the approximate sum of the neurons detected by the individual probes, as is expected if the different receptor subfamilies are expressed in nonoverlapping populations of neurons.

The apical distribution of neurons expressing V1Rs and the more basal distribution of V2Rs in the VNO are suggestive of their coupling to distinct G proteins (Figure 3). However, the hybridization pattern of $V2R_{1\text{-}1}$, $V2R_1$, and mixed V2Rs (Figures 3E, 3F, 4E, and 4F) indicated that some neurons expressing this class of receptors might be located within the apical $G\alpha_{i2}$ layer of the VNO. To examine this in more detail, digoxigenin-labeled V2R probes were mixed with a fluorescein-labeled $G\alpha_{i2}$ probe. After sequential detection of hybridized probes using different substrates for alkaline phosphatase, we observed dark staining of V2R-expressing neurons together with pink staining of the whole $G\alpha_{i2}$-positive region (Figure 5). Several V2Rs including $V2R_3$ and $V2R_4$ were only expressed in cells outside the $G\alpha_{i2}$-positive region (Figures 5A, 5B, 5D, and 5E). However, some $V2R_1$-expressing neurons were surrounded by cells expressing $G\alpha_{i2}$ while others were outside the $G\alpha_{i2}$-positive region (Figures 5C and 5F). This distribution strongly suggests that at least some neurons expressing V2Rs also express high levels of $G\alpha_{i2}$; however, conclusive proof will require other experimental approaches.

The Pattern of V2R-Subtype Expression Is Similar in Rats and Mice

Sequence analysis indicates that very similar subfamilies of V2Rs are expressed in rats and mice (Figure 1). The pattern of V2R expression is also similar in these two species: in mice as well as rats, V2Rs are generally restricted to the basal half of the sensory cell layer of the VNO (Figure 6). Moreover, the pattern of expression of mouse V2R subfamilies also paralleled the pattern observed for rat. The expression of $V2R_1$-subfamily members includes cells that are more apical than those expressing $V2R_3$ and $V2R_4$ (Figure 6). Thus, it appears that several signaling molecules are expressed in stereotyped layers in the rodent VNO. These include G-protein subunits (Halpern et al., 1995; Berghard and Buck, 1996; Jia and Halpern, 1996), V1Rs (Dulac and Axel, 1995), and a number of V2R subfamilies.

Discussion

We have identified a novel multigene family of seven-transmembrane domain receptors that is likely to mediate pheromone responses in rats and mice. These receptors have diverse sequences and are related to the G protein–linked extracellular CaSR (Brown et al., 1993) and mGluRs (Nakanishi, 1992). Subfamilies of receptors are expressed at high levels in distinct subpopulations of VNO sensory neurons but are not expressed in the olfactory epithelium or any other neural or non-neural tissues that we have examined. These are properties that are expected for pheromone receptors, but as with the olfactory receptors (Buck and Axel, 1991) and the first family of putative pheromone receptors (Dulac and Axel, 1995), conclusive proof of their function will require demonstration that specific pheromones bind to individual receptors and that this results in a neural response.

The *V2R* family of receptors is the third large gene family that is likely to encode chemoreceptors in mammals. Like the V1Rs (Dulac and Axel, 1995) and the olfactory receptors (Buck and Axel, 1991), the V2Rs are predicted to contain a seven-helix transmembrane domain; other than that, these three families of receptors

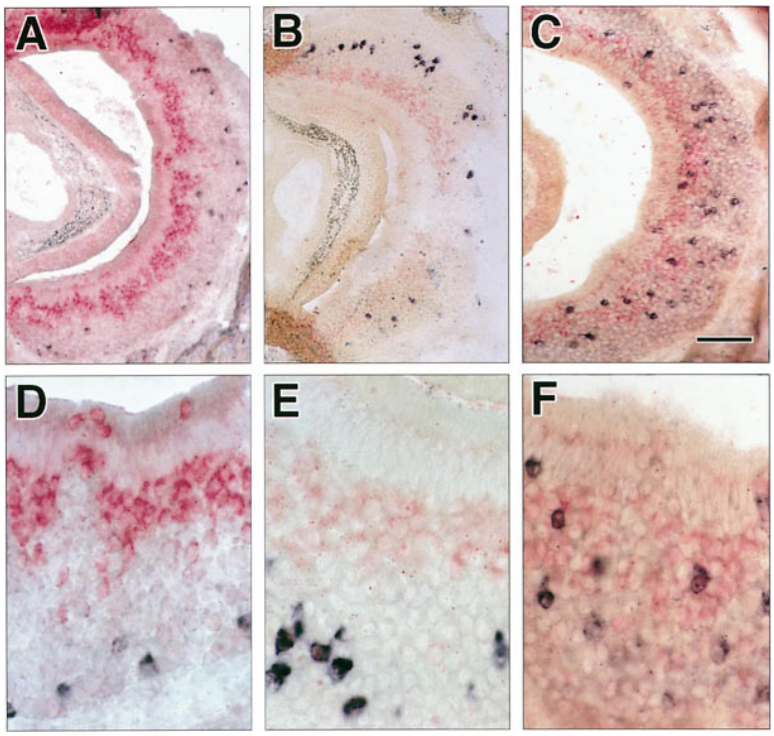

Figure 5. Mixtures of a Digoxigenin-Labeled V2R Probe and a Fluorescein-Labeled $G\alpha_{i2}$ Probe Were Hybridized to Sections of Male VNO at High Stringency

V2R-positive neurons stained dark, while the $G\alpha_{i2}$-positive cells stained pink–red. Probes were:
(A and D) $V2R_4$.
(B and E) $V2R_3$.
(C and F) $V2R_1$; scale bars = 100 μm (A–C) and 40 mm (D–F).

share no significant homology. Similarly, the V2Rs have no significant homology to the large family of receptors expressed in subsets of olfactory neurons in the nematode, Caenorhabditis elegans (Troemel et al., 1995). The structural diversity of chemoreceptors is therefore surprisingly large, particularly as all four classes, though predicted to be G-protein linked, fall into completely different classes of receptors that are essentially unrelated in sequence. Moreover, it is surprising that whereas the sense of smell appears to be mediated almost exclusively by a single very large family of olfactory receptors, two smaller families of receptors are expressed in the VNO and presumably mediate pheromone responses.

There is good evidence that ligand binding to the CaSR (Brown et al., 1993) and mGluRs (Nakanishi, 1992) is a function of the N-terminal extracellular domain. Therefore, it is likely that this region of the V2Rs also participates in ligand binding. This may explain the high degree of protein sequence conservation within parts of the transmembrane region of these receptors (for example, helices VI and VII, Figure 1B) relative to olfactory receptors or the V1Rs, where this region of the protein is the highly variable (Buck and Axel, 1991; Ngai et al., 1993; Dulac and Axel, 1995).

Size of the *V2R* Gene Family

The question of exactly how many V2Rs are expressed in the VNO remains to be determined. However, we have several lines of evidence that indicate considerable diversity. Sequence analysis of the 34 clones obtained by screening of a rat VNO cDNA library demonstrated 25 distinct sequences. Although none of these was a full-length clone, raising the possibility that some might be expressed pseudogenes, none contained internal stop codons. We have subsequently isolated full-length clones of two divergent subclasses of *V2R* (Figure 1).

In situ hybridization results are also consistent with expression of multiple different V2Rs in the VNO. Each individual V2R hybridized to only 0.5%–3% of the sensory neurons. Even though high stringency conditions were used with probes that were extensively or exclusively made up of the 3′-nontranslated region, it is likely that some probes detected several closely related subfamily members. Thus, the number of neurons expressing any particular receptor may be even lower. If VNO neurons in the basal half of the sensory cell layer all express a single *V2R* gene, many different genes would need to be expressed.

Unlike the olfactory receptors and the V1Rs, the coding sequences for V2Rs contain introns (Figure 1B). Therefore, to examine genomic diversity of V2Rs, we screened well-characterized rat and mouse genomic libraries with single exon probes. We have cloned several genes from these genomic libraries including $G\gamma 8$ (Ryba and Tirindelli, 1995), olfactory marker protein (Rogers et al., 1987), odorant binding protein (Pevsner et al., 1988), olfactory receptors (Buck and Axel, 1991), and Olf-1 (Wang and Reed, 1993). Positive clones detected by single exon probes to single copy genes are present in both the rat and mouse library at frequencies between 1:150,000 and 1:200,000, while multigene family probes detect proportionally higher numbers of positive clones. Single exon probes for the cysteine-rich region (Figure 1B) of two different V2R-extracellular domains identify 40–60 times the number of clones detected for single copy genes. So far, all seven genomic clones that have been isolated and partially sequenced are distinct *V2R* genes and are from the same subfamily as the probe that was used. The transmembrane region of V2Rs detects even larger numbers of positive clones (up to 200

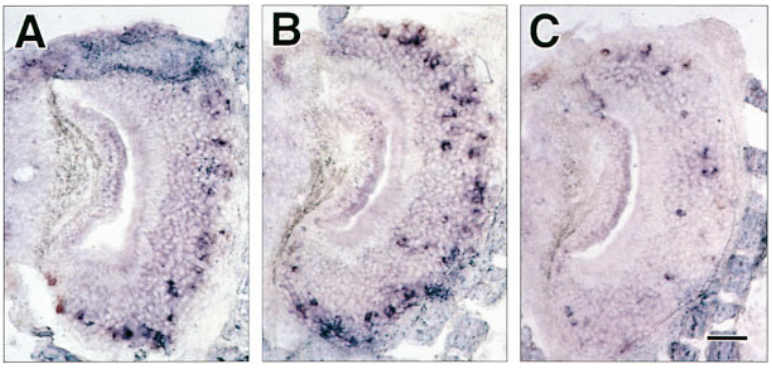

Figure 6. In Situ Hybridization of Different V2R Subfamily Members to Sections of Female Mouse VNO

Probes were:
(A) $V2R_4$.
(B) $V2R_3$.
(C) $V2R_1$. Scale bar = 70 μm.

times that detected by single copy exons). These clones have been less extensively characterized, but the two that have been sequenced encode V2Rs. Caution should be applied in making a direct estimate of the number of V2Rs that are expressed based on the genomic diversity that we have observed. Many of the genomic clones may be pseudogenes as is the case for other duplicated gene families. For example, several olfactory receptor–related genomic sequences have been reported to be pseudogenes (Glusman et al., 1996). Therefore, we cannot make an absolute estimate of the number of V2Rs. However, all of our data are consistent with between 30 and 100 V2Rs being expressed in the rodent VNO and indicate that the *V2R* family may be similar in size to the *V1R* family (Dulac and Axel, 1995).

Laminar Distribution of Receptors and G Proteins in the VNO

The patterns of cells expressing V1Rs suggested that there may be a laminar organization in the VNO (Dulac and Axel, 1995). Studies of the distribution of $G\alpha_{i2}$ and $G\alpha_o$ confirm this (Halpern et al., 1995; Berghard and Buck, 1996; Jia and Halpern, 1996) and suggest that expression of V1Rs may be restricted to the region of the epithelium where $G\alpha_{i2}$ is highly expressed. Again, the primarily basal distribution of cells expressing V2Rs reinforces this laminar organization of the VNO. In contrast, in the olfactory epithelium, little laminar organization has been observed in the sensory cell layer, and the laminar organization that exists reflects developmental state (Verhagen et al., 1989; Walton et al., 1993; Ryba and Tirindelli, 1995). However, since at least some V2R-expressing neurons are located within the layer of neurons that also express high levels of $G\alpha_{i2}$, it may be too simple to conclude that V1Rs activate $G\alpha_{i2}$ and V2Rs activate $G\alpha_o$. It is also important to stress that the laminar organization observed in the VNO is unlikely to represent differences in developmental state. Generation of new VNO neurons in mice occurs at the boundary between the sensory and nonsensory epithelium rather than in the basal layer as is the case for the main olfactory epithelium (Barber and Raisman, 1978).

The expression patterns of the V2Rs that we have studied appear similar but distinct: that of $V2R_1$ overlaps both the $G\alpha_{i2}$ and $G\alpha_o$ layers of the epithelium while cells expressing $V2R_3$ and $V2R_4$ do not. Moreover, $V2R_3$ was expressed in a broader and less basal population of neurons than $V2R_4$. In other respects, the cells that expressed $V2R_4$ appeared to be similar to VNO neurons throughout the $G\alpha_o$ layer. Morphologically, they appeared normal, and they appeared to be located within the neural cell layer expressing $G\alpha_o$, $G\gamma_8$ (Figure 3), and olfactory marker protein (data not shown). The layers of V2R expression are not as well defined as the zones of receptor expression in the olfactory epithelium (Ressler et al., 1993; Vassar et al., 1993): the layers appear to overlap with each other, and there often appears to be an outlying cell or two. However, similar distributions were consistently observed in many sections from >10 different animals. Indeed, the expression patterns of homologous receptors appear to be conserved between rats and mice. VNO neurons are generated throughout life by division of precursor cells located at the boundary of the sensory and nonsensory epithelia (Barber and Raisman, 1974, 1978) and migrate into stratified layers on the concave, sensory side of the VNO. The stereotyped distributions of receptors and G-protein α subunits imply that there must be some linkage between gene expression and the processes that guide migration of the neural cell body. The laminar organization of these components suggests that there may be radial gradients of guidance cues. It will be interesting to determine the nature of these cues and to examine the link between them and gene expression in the VNO.

Neurons in the main olfactory epithelium that express particular olfactory receptors project to discrete and fixed locations in the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). This suggests a mechanism whereby distinct odors may produce distinct patterns of neural activity in the olfactory bulb. Recently, odor-specific patterns have been measured in zebrafish and bees (Friedrich and Korsching, 1997; Joerges et al., 1997). Even though insects do not appear to express olfactory receptors related to those found in vertebrates, it appears that neurons with similar specificity converge to allow odor recognition. It is tempting to speculate that similar focusing of vomeronasal neurons expressing particular receptors may occur. Moreover, VNO neurons that express $G\alpha_{i2}$ project to anterior regions, and those expressing $G\alpha_o$ project to posterior regions of the accessory olfactory bulb (Shinohara et al., 1992; Halpern et al., 1995; Jia and Halpern, 1996). Therefore, it may be that similar mechanisms control axon guidance to appropriate sites in the accessory olfactory bulb and cell body migration in the VNO. Our

demonstration of a novel family of putative pheromone receptors combined with the cloning of V1Rs (Dulac and Axel, 1995) sets the stage for investigation of how neuronal localization and targeting are coordinated with gene expression in the VNO.

Experimental Procedures

Cloning of V2Rs

A rat vomeronasal cDNA library in λZAPII was screened at low density (5×10^3 plaques) and low stringency (hybridization: 6 × SSC at 60°C; washes at 0.2 × SSC at 25°C) with a PCR-derived probe designed to be a degenerate mix of sequences encoding olfactory receptors. The probe was prepared by PCR amplification with primers to conserved regions at the end of helix III and within helix VI: ATGGCITAYGAYCGCTATG and GTGRGWGSCRCAIGTWG TWA by amplification of cDNA from olfactory epithelium. Four weakly hybridizing plaques were isolated, and the cDNAs were sequenced. One of these had similarity to the CaSR and was used as a probe for isolation of other V2Rs from the same library. The PCR probe was highly degenerate and essentially uncharacterized. However, the reason for its weak hybridization to $V2R_{1\text{-}1}$ is not immediately apparent as known olfactory receptors share little identity with V2Rs. The 5′ and 3′-ends of $mV2R_1$ and $mV2R_2$ were identified by Marathon-RACE (Clontech) using primers derived from mouse genomic sequence. Full-length mV2Rs were isolated by RT–PCR using specific primers; the sequences of these clones are shown in Figure 1B.

Genomic Screening, Northern Analysis, and In Situ Hybridization

Single exon probes for genomic screening were prepared by restriction digestion of rat cDNA clones or by PCR. The genomic libraries in λ vectors (Stratagene) were screened at moderate density (~12,000 plaques/14 cm plate). Hybridization was in 6 × SSC at 65°C; washes were in 0.1 × SSC at 50°C. For Northern analysis, A^+-RNA was isolated from a variety of tissues and brain areas; 2 μg aliquots were fractionated on 0.9% agarose gels containing formaldehyde and transferred to nylon. Hybridization with cDNA probes was in 0.5 M phosphate (pH 7) at 65°C; washes were as above. Coronal sections of fresh-frozen VNO were attached to silanized slides (Digene); sections were prepared for hybridization by standard methods (Schaeren-Wiemers and Gerlin-Moser, 1993). In situ hybridization was carried out with digoxigenin and fluorescein-labeled cRNA probes synthesized and detected with alkaline-phosphatase-conjugated antibody following protocols from Boehringer Mannheim and Amersham. In double-labeling experiments, alkaline phosphatase was inactivated by incubation at pH 2 prior to incubation with the second antibody. Generally, high stringency in situ hybridization was carried out (at 72°C in 5 × SSC, 50% formamide); for some experiments, reduced stringency was used with hybridization at 62°C. Washing was at 72°C in 0.2 × SSC in both cases.

Acknowledgments

We thank T. Bolzoni for supplying tissue, M. Hoon and R. Siraganian for stimulating discussion and critical review of the manuscript, and R. Dreyfuss for photography.

Received June 26, 1997; revised July 16, 1997.

References

Axel, R. (1995). The molecular logic of smell. Sci. Am. *273*, 219–230.

Bakalyar, H.A., and Reed, R.R. (1990). Identification of a specialized adenylyl cyclase that may mediate odorant detection. Science *250*, 1403–1406.

Barber, P.C., and Raisman, G. (1974). Cell division in the vomeronasal organ of the adult mouse. Brain Res. *81*, 21–30.

Barber, P.C., and Raisman, G. (1978). Replacement of receptor neurones after section of the vomeronasal nerves in the adult mouse. Brain. Res. *147*, 297–313.

Berghard, A., and Buck, L. (1996). Sensory transduction in vomeronasal neurons: evidence for $G\alpha_o$, $G\alpha_i$, and adenylyl cyclase II as major components of a pheromone signaling cascade. J. Neurosci. *16*, 909–918.

Berghard, A., Buck, L.B., and Liman, E.R. (1996). Evidence for distinct signaling mechanisms in two mammalian olfactory sense organs. Proc. Natl. Acad. Sci. USA *93*, 2365–2369.

Brown, E.M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M.A., Lytton, J., and Hebert, S.C. (1993). Cloning and characterization of an extracellular Ca^{2+}-sensing receptor from bovine parathyroid. Nature *366*, 575–580.

Brunet, L.J., Gold, G.H., and Ngai, J. (1996). General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. Neuron *17*, 681–693.

Buck, L.B. (1996). Information coding in the vertebrate olfactory system. Annu. Rev. Neurosci. *19*, 517–544.

Buck, L.B., and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell *65*, 175–187.

Chess, A., Simon, I., Cedar, H., and Axel, R. (1994). Allelic inactivation regulates olfactory receptor gene expression. Cell *78*, 823–834.

Clancy, A.N., Coquelin, A., Macrides, F., Gorski, R.A., and Nobel, E.P. (1984). Sexual behavior and aggression in male mice: involvement of the vomeronasal system. J. Neurosci. *4*, 2222–2229.

Dhallan, R.S., Yau, K.W., Schrader, K.A., and Reed, R.R. (1990). Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. Nature *347*, 184–187.

Dulac, C., and Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals. Cell *83*, 195–206.

Friedrich, R.W., and Korsching, S.I. (1997). Combinatorial and chemotropic odorant coding in the zebrafish olfactory bulb visualized by optical imaging. Neuron *18*, 737–752.

Glusman, G., Clifton, S., Roe, B., and Lancet, D. (1996). Sequence analysis in the olfactory receptor gene cluster on human chromosome 17: recombinatorial events affecting receptor diversity. Genomics *37*, 147–160.

Halpern, M. (1987). The organization and function of the vomeronasal system. Annu. Rev. Neurosci. *10*, 325–362.

Halpern, M., Shapiro, L.S., and Jia, C. (1995). Differential localization of G proteins in the opossum vomeronasal system. Brain Res. *677*, 157–161.

Jia, C., and Halpern, M. (1996). Subclasses of vomeronasal neurons: differential expression of G proteins ($G_{i\alpha 2}$ and $G_{o\alpha}$) and segregated projections to the accessory olfactory bulb. Brain Res. *719*, 117–128.

Joerges, J., Küttner, A., Galizia, C.G., and Menzel, R. (1997). Representations of odours and odour mixtures visualized in the honeybee brain. Nature *387*, 285–288.

Johns, M.A., Feder, H.H., Komisaruk, B.R., and Mayer, A.D. (1978). Urine-induced reflex ovulation in anovulatory rats may be a vomeronasal effect. Nature *272*, 446–448.

Jones, D.T., and Reed, R.R. (1989). Golf: an olfactory neuron specific-G protein involved in odorant signal transduction. Science *244*, 790–795.

Lomas, D.E., and Keverne, E.B. (1982). Role of the vomeronasal organ and prolactin in the acceleration of puberty in female mice. J. Reprod. Fertil. *66*, 101–107.

Meredith, M. (1986). Vomeronasal organ removal before sexual experience impairs male hamster mating behavior. Physiol. Behav. *36*, 737–743.

Meredith, M., and Fernandez-Fewell, G. (1994). Vomeronasal system, LHRH, and sex behaviour. Psychoneuroendocrinology *19*, 657–672.

Mombaerts, P., Wang, F., Dulac. C., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996). Visualizing an olfactory sensory map. Cell *87*, 675–686.

Mucignat-Caretta, C., Caretta, A., and Cavaggioni, A. (1995). Acceleration of puberty onset in female mice by male urinary proteins. J. Physiol. (Lond.) *486*, 517–522.

Nakanishi, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. Science *258*, 597–603.

Ngai, J., Chess, A., Dowling, M.M., Necles, N., Macagno, E.R., and Axel, R. (1993). The family of genes encoding odorant receptors in the channel catfish. Cell *72*, 667–680.

Pevsner, J., Reed, R.R., Feinstein, P.G., and Snyder, S.H. (1988). Molecular cloning of odorant-binding protein: member of a ligand carrier family. Science *241*, 336–339.

Ressler, K.J., Sullivan, S.L., and Buck, L.B. (1993). A zonal organization of odorant receptor gene expression in the olfactory epithelium. Cell *73*, 597–609.

Ressler, K.J., Sullivan, S.L., and Buck, L.B (1994). Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. Cell *79*, 1245–1255.

Reynolds, J., and Keverne, E.B. (1979). The accessory olfactory system and its role in the pheromonally mediated suppression of oestrus in grouped mice. J. Reprod. Fertil. *57*, 31–35.

Rogers, R.E., Dasgupta, P., Gubler, U., Grillo, M., Khew-Goodall, Y., and Margolis, F.L. (1987). Molecular cloning and sequencing of a cDNA for olfactory marker protein. Proc. Natl. Acad. Sci. USA *84*, 1704–1708.

Ryba, N.J.P., and Tirindelli, R. (1995). A novel GTP-binding protein γ-subunit, Gγ8, is expressed during neurogenesis in the olfactory and vomeronasal neuroepithelia. J. Biol. Chem. *270*, 6757–6767.

Schaeren-Wiemers, N., and Gerlin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and culture cells: in situ hybridization using digoxigenin-labeled cRNA probes. Histochemistry *100*, 431–440.

Shepherd, G.M. (1994). Discrimination of molecular signals by the olfactory receptor neuron. Neuron *13*, 771–790.

Shinohara, H., Asano, T., and Kato, K. (1992). Differential localization of G-proteins Gi and Go in the accessory olfactory bulb of the rat. J. Neurosci *12*, 1275–1279.

Troemel, E.R., Chou, J.H., Dwyer, N.D., Colbert, H.A., and Bargmann, C.I. (1995). Divergent seven transmembrane receptors are candidate chemosensory receptors in C. elegans. Cell *83*, 201–218.

Vassar, R., Ngai, J., and Axel, R. (1993). Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. Cell *74*, 309–318.

Vassar, R., Chao, S.K., Sitcheran, R., Nunez, J.M., Vosshall, L.B., and Axel, R. (1994). Topographic organization of sensory projections to the olfactory bulb. Cell *79*, 981–991.

Verhagen, J., Oestreicher, A.B., Gispen, W.H., and Margolis, F.L. (1989). The expression of the growth associated protein B50/GAP43 in the olfactory system of neonatal and adult rats. J. Neurosci. *9*, 683–691.

Walton, K.M., Martell, K.J., Kwak, S.P., Dixon, J.E., and Largent, B.L. (1993). A novel receptor-type protein tyrosine phosphatase is expressed during neurogenesis in the olfactory neuroepithelium. Neuron *11*, 387–400.

Wang, M.M., and Reed, R.R. (1993). Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. Nature *364*, 121–126.

Wu, Y., Tirindelli, R., and Ryba, N.J.P. (1996). Evidence for different chemosensory signal transduction pathways in olfactory and vomeronasal neurons. Biochem. Biophys. Res. Commun. *220*, 900–904.

Wysocki, C.J. (1989). Vomeronasal chemoreception: its role in reproductive fitness and physiology. In Neural Control of Reproductive Function (New York: Alan R. Liss), pp. 545–566.

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

Wysocki, C.J., Wellington, J.L., and Beauchamp, G.K. (1980). Access of urinary nonvolatiles to the mammalian vomeronasal organ. Science *207*, 781–783.