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Expression of Candidate Pheromone Receptor Genes in Vomeronasal Neurons

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Abstract

In mammals, olfactory sensory perception is mediated by two anatomically and functionally distinct organs: the main olfactory epithelium (MOE) and the vomeronasal organ (VON). Pheromones activate the VNO and elicit a characteristic array of innate reproductive and social behaviors, along with dramatic neuroendocrine responses. Recent approaches have provided new insights into the molecular biology of sensory transduction in the VNO. Differential screening of cDNA libraries constructed from single sensory neurons from the rat VNO has led to the isolation of a family of genes which are likely to encode mammalian pheromone receptors. The isolation of these receptors from the VNO might permit the analysis of the molecular events which translate the bindings of pheromones into innate stereotypic behaviors and help to elucidate the logic of pheromone perception in mammals.

Introduction

The perception of odors in humans is often viewed as an aesthetic sense, a sense capable of evoking emotion and memory leading to measured thoughts and behaviors. Smell, however, is also a primal sense. In most species, odors can elicit innate and stereotyped behaviors that are likely to result from the nonconscious perception of odors. These different pathways of olfactory sensory processing are thought to be mediated by two anatomically and functionally distinct olfactory sensory organs, the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). Pheromones activate the VNO and elicit a characteristic array of innate reproductive and social behaviors along with dramatic neuroendocrine responses.

Until recently, neither the pheromone receptors nor the signal transduction pathways activated by pheromone in vomeronasal neurons have been identified. In the MOE, the repertoire of odorant receptor genes consists of ~1000 genes, each encoding a distinct seven transmembrane domain protein (Buck and Axel, 1991; Parmentier *et al.*, 1992; Ben Arie *et al.*, 1994). Analysis of the expression patterns of this family of odorant receptor genes (Ngai *et al.*, 1993; Ressler *et al.*, 1993, 1994; Vassar *et al.*, 1993, 1994), coupled with earlier electrophysiologic and tracing experiments (Stewart *et al.*, 1979; Lancet *et al.*, 1982; Kauer *et al.*, 1987; Imamura *et al.*, 1992; Mori *et al.*, 1992; Katoh *et al.*, 1993), have provided a logic for olfactory discrimination. Individual sensory neurons in the MOE are likely to express only one of the 1000 receptor genes (Ngai *et al.*, 1993; Chess *et al.*, 1994; C. Dulac and R. Axel, unpublished studies). Neurons expressing a given receptor, although randomly distributed in domains of the epithelium, project their axons to a small number of topographically fixed loci (or glomeruli) in the main olfactory bulb (Vassar *et al.*, 1994; Ressler *et al.*, 1994). These data support a model of olfactory coding in which discrimination of odor quality would result from the detection of specific spatial patterns of activity in the olfactory bulb.

The isolation of the genes encoding the pheromone receptors from VNO neurons might similarly provide insight into the chemical nature of the pheromones themselves, the logic of olfactory coding in the VNO and the way in which perception of this class of odors leads to innate behaviors. Our efforts to identify the genes encoding the mammalian pheromone receptors by virtue of potential homology with the family of odorant receptor genes expressed in the MOE have been unsuccessful. We therefore developed a cloning strategy in which cDNA libraries were constructed from individual rat VNO neurons (Dulac and Axel, 1995). Difference cloning permitted us to identify ~30 genes that define a novel family of presumed seven transmembrane domain receptors that are evolutionarily independent of the odorant receptors of the MOE. Expression of the individual members of this gene family is restricted to a distinct set of VNO neurons such that different neurons express different

receptor genes. These genes are likely to encode mammalian pheromone receptors.

Isolation of the pheromone receptor genes

Our initial efforts to identify the genes encoding the pheromone receptors were based upon the assumption that the MOE and the VNO might share a common evolutionary origin such that DNA sequence homology may exist between the two receptor families. However, low stringency hybridization of MOE receptor probes to rat vomeronasal cDNA libraries, as well as polymerase chain reactions (PCR) using conserved motifs from both the family of odorant receptor genes and from the superfamily of known seven transmembrane domain receptors, were consistently unsuccessful. Moreover, the components of the olfactory signal transduction cascade in the MOE [(the olfactory-specific G-protein, G_{olf} (Jones and Reed, 1989), the olfactory-specific adenylate cyclase (Bakalyar and Reed, 1990) and the cyclic nucleotide responsive ion channel (Dhallan *et al.*, 1990)] were not detectable in VNO neurons by *in situ* hybridization or by screening cDNA libraries (data now shown). These observations suggested that the pheromone receptors and the signal transduction pathways which they activate might have evolved independently in the VNO and the MOE.

We therefore developed a cloning procedure that made no assumptions concerning the structural class of the receptor molecules. Rather, we only assumed that the expression of the pheromone receptors would be restricted to the VNO, and that individual neurons within the VNO were likely to express different receptor genes. In the MOE, ~1% of the mRNA in a given sensory cell encodes a given receptor (Vassar *et al.*, 1994). However, the 1000 different receptor genes are each expressed in different neurons such that the frequency of a specific receptor RNA will be diluted to 0.001% of the mRNA message population. The generation of libraries from individual neurons provided an experimental solution to the problem of detecting a specific mRNA in a heterogeneous population of neurons (Dulac and Axel, 1995). RT-PCR was therefore used to generate double-stranded cDNA, as well as cDNA libraries from individual vomeronasal sensory neurons. We expected that the frequency of a specific receptor cDNA in libraries from single neurons would be ~1%. Differential screening of such libraries from single neurons should therefore permit the isolation of pheromone receptor genes.

This experimental approach led to the isolation of two independent clones encoding an identical cDNA sequence present within the cDNA library of VNO neuron 1 at a frequency of 0.5%, but not present in cDNA from VNO neuron 2 or from main olfactory sensory neurons. This cDNA was used as a probe to isolate full-length clones from a cDNA library with larger inserts constructed with RNA prepared from several dissected VNOs. A full-length clone, VN1, encodes a seven transmembrane domain receptor (see below).

The pattern of expression of this cDNA was determined by performing RNA *in situ* hybridization with sections through the rat VNO (Figure 1). In cross section, a thick multicellular sensory epithelium lines half of the lumen of the VNO. *In situ* hybridization demonstrates that mature VNO neurons uniformly express the olfactory marker protein (OMP) (Figure 1A). In contrast, the cDNA specific for VNO neuron 1 localized to a subpopulation of VNO neurons (Figure 1C). No hybridization was observed in the MOE, or in any other neural or non-neural cells (Figure 1D). Thus, difference cloning from libraries prepared from single neurons has allowed the isolation of a novel seven transmembrane domain receptor expressed in VNO sensory neurons.

The sequence of several members of the receptor gene family

We observed that VN1 is expressed in ~4% of the vomeronasal sensory neurons. This suggested the existence of a gene family with individual member genes expressed in different subsets of neurons. We therefore used both PCR and high- and low stringency hybridization to VNO cDNA libraries to identify possible members of a receptor gene family expressed in other VNO neurons. Hydropathy analysis suggests that each of the seven sequences contain seven hydrophobic stretches that represent potential transmembrane domains (Figure 2). Sequence analysis suggests that these putative receptors are likely to adopt a structure similar to that of the previously characterized superfamily of seven transmembrane receptors. However, the VNO receptors do not share any of the conserved sequence motifs exhibited by members of the previously identified superfamily (Probst *et al.*, 1992; Baldwin, 1993). One region of homology, however, is observed with the family of mammalian prostaglandin receptors throughout the second and third transmembrane domains. Twenty-five percent identity is observed between VN2 and the rat E3 prostaglandin receptor over these two domains, but no significant sequence homology is observed in other regions of the molecule. Prostaglandins are potent pheromones eliciting mating in fish (Stacey and Sorensen, 1986; Sorensen *et al.*, 1998), but their role as mammalian phero- mones is unknown. However, this level of homology over a small region of the protein does not permit us to argue that the receptors may recognize prostaglandins.

Overall, the seven VNO cDNA sequences share 47–87% sequence identity. As observed previously for the odorant receptors from the MOE (Buck and Axel 1991), this family of VNO receptors exhibits significant divergence within the transmembrane domains, the presumed site of ligand binding (Strader *et al.*, 1994). This pattern of divergence

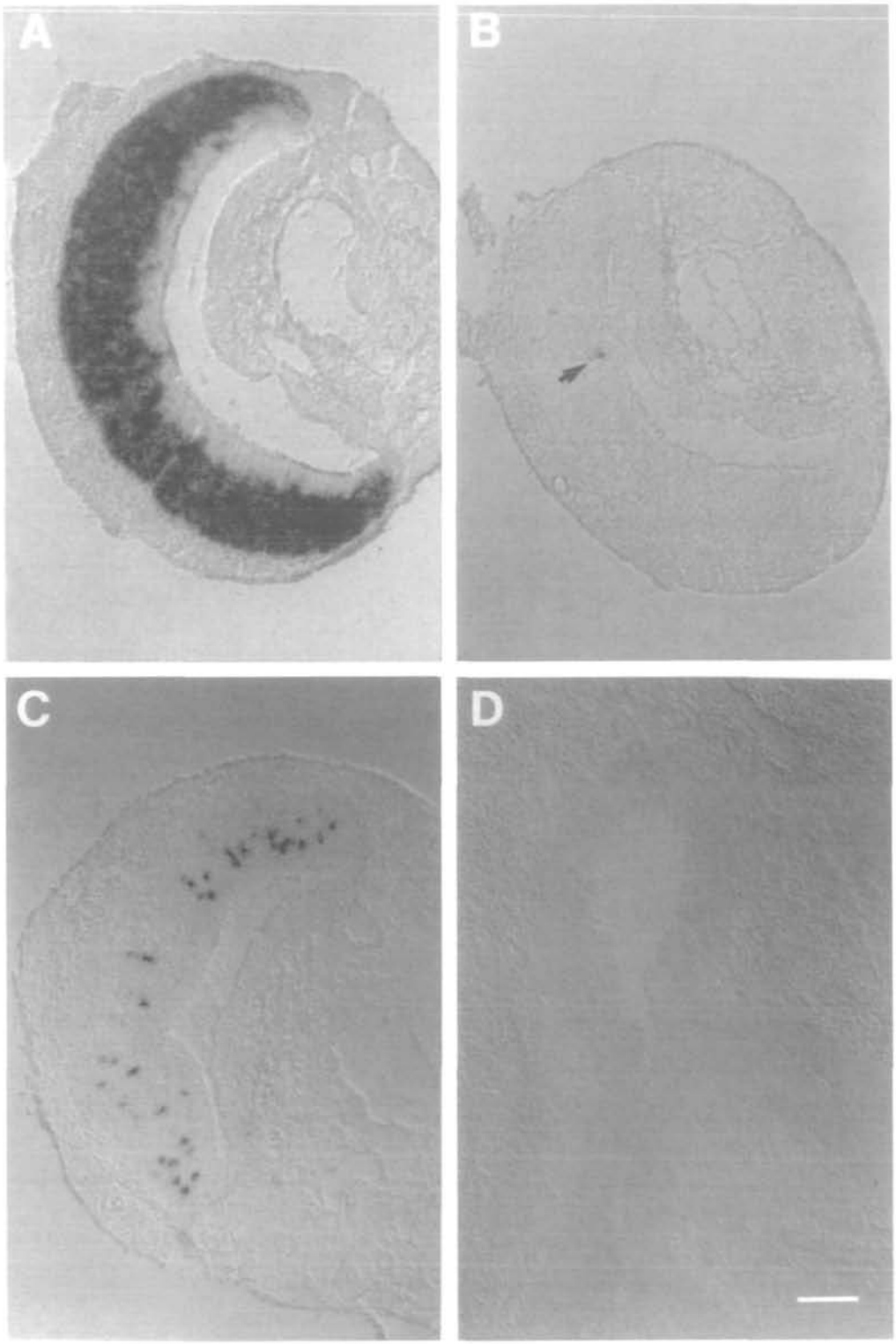

Figure 1 Expression of VN1 receptor RNA is restricted to a subset of VNO neurons. Coronal sections of the VNO dissected from adult male rats were annealed with digoxigenin-labeled antisense RNA probes for the OMP **(A)**, the M12 receptor (a receptor expressed in abundance in the MOE) **(B)** and the VNO-specific receptor VN1 **(C)**. **(D)** *In situ* hybridization of VN1 to a coronal section of turbinates from the newborn MOE. The arrow in (B) indicates a single positive VNO neuron expressing the MOE receptor, M12. In (A), N denotes the neuroepithelium; L, the lumen of VNO; and V, the vomeronasal vein. In (D), the arrow points to the MOE; NC denotes the nasal cavity. Scale bar = 120 μm.

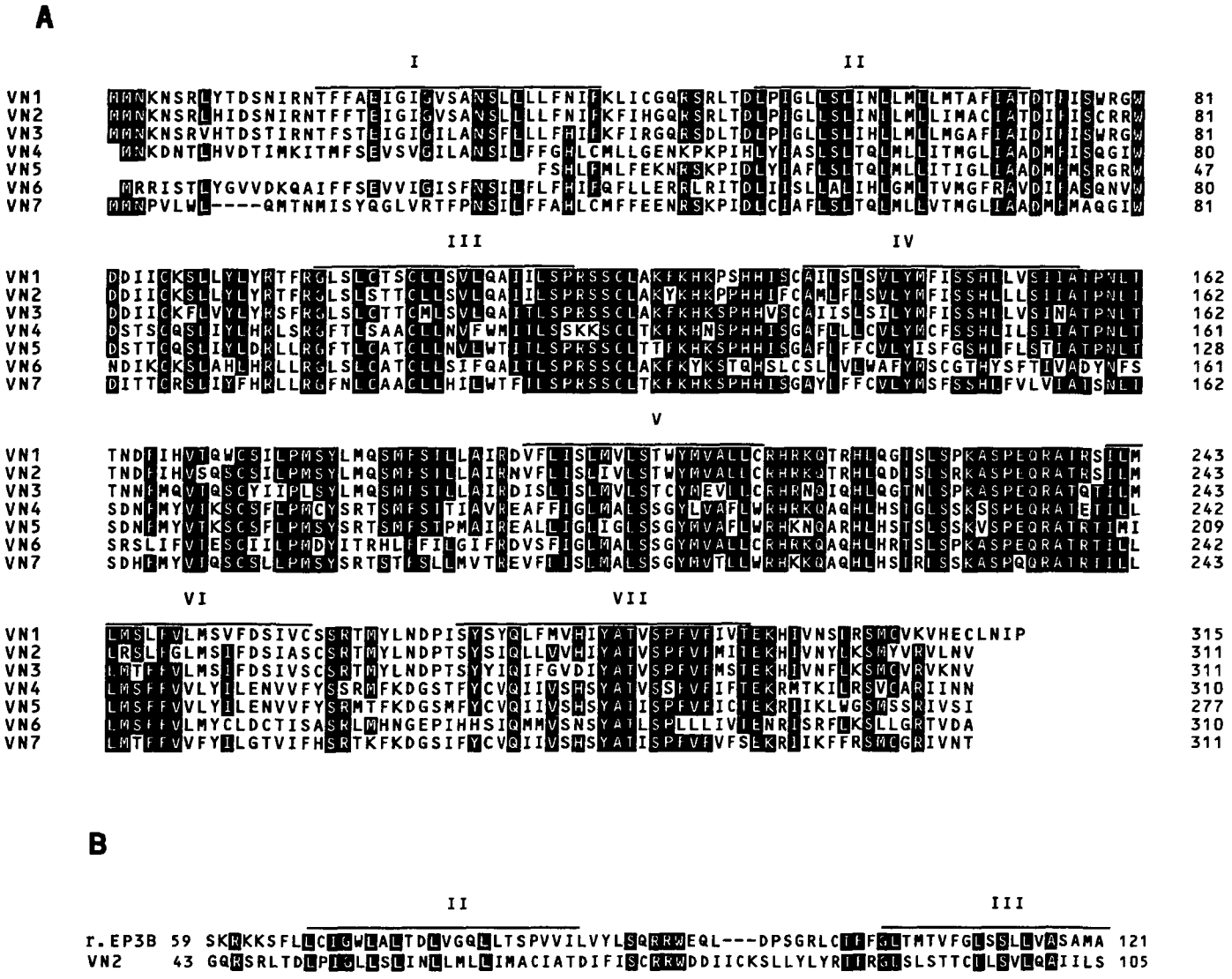

Figure 2 Deduced amino acid sequences of the pheromone receptor cDNAs. **(A)** The deduced amino acid sequences of seven putative pheromone receptor cDNAs are aligned. Predicted positions of the seven transmembrane domains are indicated (I–VII). Amino acid residues common to at least five of the seven sequences are shown as white lettering on black background. **(B)** An alignment between the sequences of the second and third transmembrane domains of the rat prostaglandin receptor E3 (rEP3B) and the VNO receptor VN2 showing 28% identity over this region of the receptor sequence.

suggests that the different members may permit the binding of different structural classes of ligands.

The pattern of receptor expression in the VNO

RNA *in situ* hybridization experiments were performed with digoxigenin-labeled RNA antisense probes from each of the six subfamilies under high stringency conditions, such that it was likely that a given probe will only detect members within its own subfamily. The results with each of the six probes were qualitatively indistinguishable. In each case, we observed a punctate distribution of cells expressing a given receptor RNA (Figure 3). No differences in the patterns of *in situ* hybridization were observed between males and females. Each probe detected ~1–4% of the VNO sensory neurons. These data contrast with hybridization patterns observed with the probe for the olfactory marker protein OMP, which demonstrated uniform labeling of the VNO epithelium. Control sections hybridized with sense receptor probes revealed no specific signal. Expression of this gene family was observed only in VNO neurons; no labelling was observed in the sensory neurons of the MOE. Finally, no expression of the VNO receptors was observed upon *in situ* hybridization to sections through brain, kidney, testes and liver (data not shown). Moreover, analysis of several sections through the entire VNO suggested that neurons expressing a given receptor are not topologically localized but rather are randomly distributed along the anterior–posterior axis.

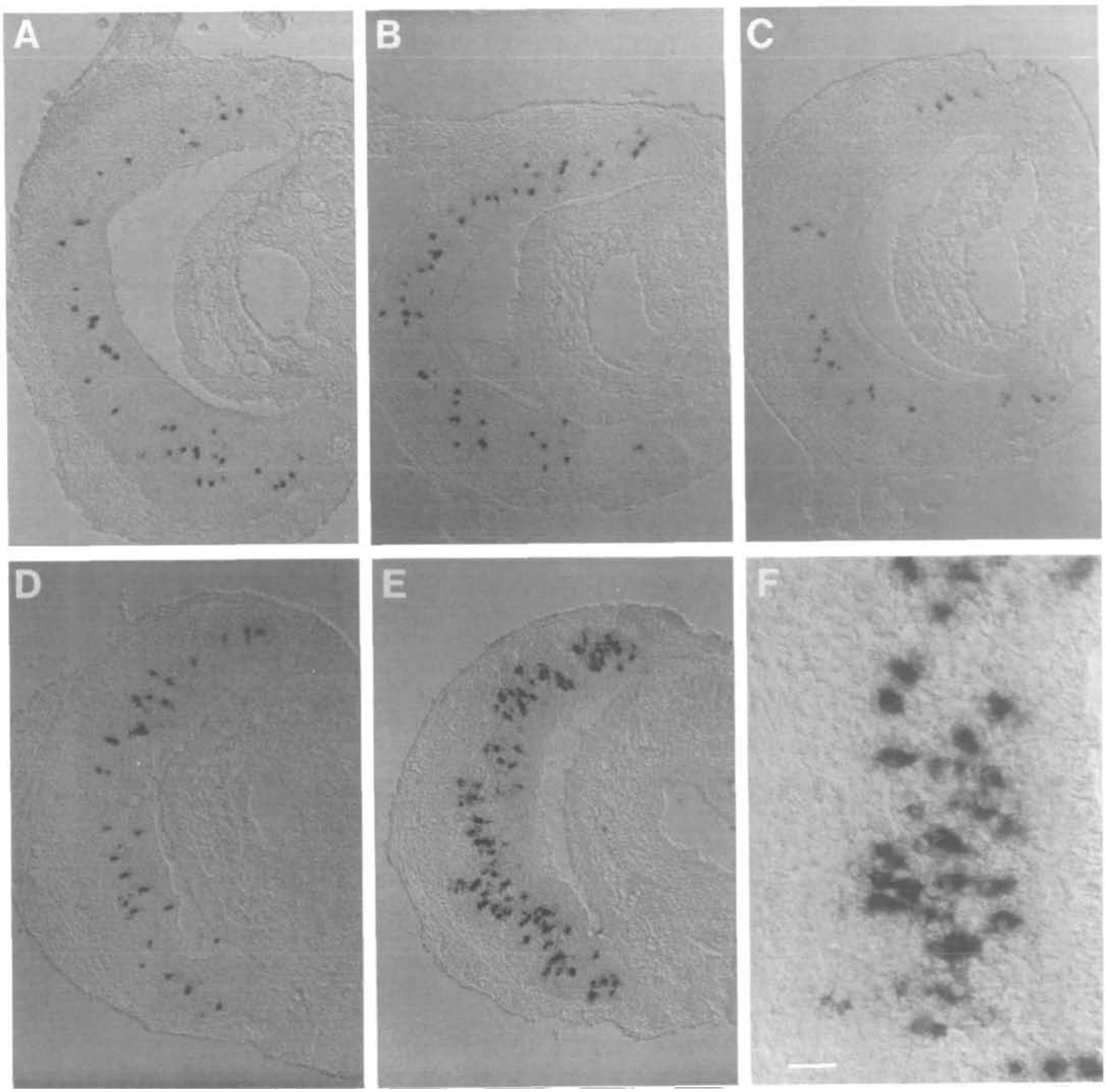

Figure 3 Localization of the individual receptors to distinct subpopulations of cells within the VNO. *In situ* hybridization to coronal sections of a dissected VNO using digoxigenin-labeled probes from either the individual receptors or a mix of the six receptors. Digoxigenin-labeled antisense RNA probes from receptor VN1 **(A)**, receptor VN3 **(C)**, receptor VN4 **(D)** or a mix of six probes specific for each receptor subfamily **(E)** were annealed to a coronal section of the VNO dissected from male rats. **(B)** The annealing of receptor VNl probe to a section through the VNO from a female rat. **(F)** A high power magnification of (E). VNO cDNA clones 1–7 label 2.7, 3.8, 1.1, 1.2, 1.1, 1.5 and 3% of the cells in the neuroepithelium respectively. The mix of seven probes label 15% of the cells. Scale bar = 120 μm.

In the MOE, a given neuron is likely to express only one receptor from the family of 1000 receptor genes. Moreover, neurons expressing a given receptor project their axons to one or a small number of topographically defined glomeruli within the olfactory bulb. The regulated expression of odorant receptors assuring that only one receptor is expressed in individual olfactory neurons is an important element in the coding of olfactory information in the main olfactory

system. Quantitative analysis of the *in situ* hybridizations of the VNO receptor probes indicate that neurons within the VNO similarly express only a single receptor gene.

The observation that 1–4% of the VNO neurons express a given receptor subfamily suggests that each cell expresses only a subset of receptor genes. If we demonstrate that each of the different receptor probes hybridizes with distinct non-overlapping subpopulations of neurons, this would provide evidence that neurons differ with respect to the receptors they express. Sections were annealed with probes specific for each of the six receptor subfamilies, either individually or with a mixture of six probes (Figure 3). If each receptor is expressed in a distinct non-overlapping subpopulation of neurons, then the sum of the cells identified with the six probes should equal the number of cells identified with the mixed probe. In accord with this suggestion, we observe that the percentage of olfactory neurons detected with the mixed probe (15%) is significantly greater than the percentage detected with any of the individual probes alone, and approximates the sum of the percentage of positive neurons detected with the six individual probes (12%). These values are present in the legend to Figure 3. These results suggest that the six receptor subfamilies are expressed in distinct non-overlapping populations of olfactory neurons and provide support for a model in which a single sensory neuron expresses a single receptor gene.

How large is the gene family?

Since the range of molecules detected by the VNO is thought to be far smaller than the number of odors detected by the MOE, we anticipated that the repertoire of pheromone receptors would be far smaller as well. Gene cloning and Southern blotting with genomic DNA provide an estimate of the size of the pheromone receptor repertoire. A screen of genomic libraries with a mix of probes detect ~35 positive clones per genome. This value is in accord with the results of genomic blot hybridization at low stringency, which identifies ~30 discrete genes with the available probes. This minimum estimate of 30–35 genes clearly provides a lower limit of the size of the VNO receptor repertoire since it is likely that the seven genes we have cloned do not allow us to detect all the members of the pheromone receptor gene family.

In situ hybridization experiments with individual probes provide an independent estimate of the number of receptor genes expressed in the VNO. Each of the seven putative pheromone receptor genes labels ~1–4% of the vomeronasal sensory neurons, whereas a mix of the genes representing the six subfamilies detects ~15% of the VNO neurons. These data suggest that a given neuron expresses only one pheromone receptor gene. Since the six subfamily probes detect ~20 genes in the chromosome at high stringency, and label 15% of the VNO neurons, we estimate that the repertoire of pheromone receptors may consist of ~100 distinct genes.

Pheromone receptors in humans

Until recently, the VNO in humans was thought to be an atretic organ of vestigial function. Recent reports, however, identify a structurally intact VNO in virtually all biopsy specimens examined (Garcia-Velasco and Mondragon 1991; Moran *et al.*, 1991; Stensaas, *et al.* 1991). Activation of neurons has been observed in the human VNO in response to purified components from skin extracts (Monti-Bloch *et al.*, 1994), but the physiological or behavioral consequences of VNO activation remain elusive. Moreover, it has been difficult to identify human pheromones that elicit innate behavioral arrays since behavior in humans is far more likely to be tempered by learning and experience.

In preliminary experiments we have identified homologs of the rodent VNO receptors in human genomic DNA. Low stringency screens of a human genomic library with a mix of rat VNO receptor cDNAs identify human homologs at a frequency of ~15 per haploid genome. Partial sequence of two clones reveals 41 and 48% identity with the closest rat homologs. However, both genomic clones reveal stop codons within the coding region, indicating that these two human sequences are pseudogenes. Characterization of additional genomic or cDNA clones from the human VNO will be required to determine whether humans indeed express functional VNO receptors. The identification of putative pheromone receptors may provide insight into the chemical nature of the pheromones, the mechanisms by which the perception of pheromones leads to innate behaviors and the possible role of this sensory system in humans.

Discussion

We have identified a novel family of seven transmembrane domain proteins that is likely to encode the mammalian pheromone receptors. Differential screening of cDNA libraries constructed from single sensory neurons initially led to the isolation of a family of putative receptor genes. Each member of the gene family is expressed in a small subpopulation of neurons such that the seven putative receptor genes we have cloned identify 15% of the cells in the VNO. The expression of this gene family is restricted to neurons within the VNO and is not observed in sensory neurons of the MOE nor in other, non-neuronal cells. This array of properties is consistent with those predicted for the mammalian pheromone receptors. Proof that these sequences indeed encode pheromone receptors will require the demonstration that these receptor proteins bind pheromones and are able to transduce pheromone binding into alterations in membrane potential.

Four large gene families have now been identified whose members are expressed in subsets of chemosensory neurons in the nematode *C. elegans* (Troemel *et al.*, 1995; Sengupta *et*

al., 1996), and in the MOE (Buck and Axel, 1991) and VNO of vertebrates. What functions could we ascribe to these gene families if they do not encode odorant receptors? In the MOE, neurons expressing a given olfactory receptor project their axons to one or a small number of glomeruli within the olfactory bulb (Vassar *et al.*, 1994; Ressler *et al.*, 1994). It is formally possible, therefore, that these receptors recognize guidance cues that are spatially distributed within the olfactory bulb. An even more parsimonious model would argue that these receptors may recognize odorants in one pole of the cell, the dendrite, and recognize guidance molecules at the axon termini.

The relationship between the two olfactory organs

The sequences of the odorant receptors of the MOE and the pheromone receptors of the VNO share no apparent homology, indicating that the two olfactory sensory systems of mammals have evolved independently. This suggestion is in accord with the observation that the signal transduction machinery of the MOE cannot be detected in the neurons of the VNO. What is the evolutionary origin of the VNO? Pheromone-responsive neurons and neurons responsive to the more general class of odorants are likely to have been present throughout vertebrate evolution. With the emergence of terrestrial forms, segregation of the two types of neurons may have occurred, generating a distinct VNO that facilitates the access and binding of the two classes of odorous ligand. Thus, terrestrial vertebrates from amphibians to mammals, including humans, retain two distinct olfactory systems, the VNO and the MOE (Potiquet, 1891; Bertmar, 1981; Garcia-Velasco and Mondragon, 1991; Moran *et al.*, 1991; Stensaas *et al.*, 1991; Eisthen, 1992).

These two functional classes of sensory neurons are also apparent in invertebrate olfactory systems. These observations immediately pose the question as to whether homologs of the two different families of vertebrate olfactory receptors are present within the genome of invertebrates. Attempts to identify genes related to the large family of MOE receptors *Drosophila* (H. Amrein, L. Vosshall and R. Axel, unpublished studies; J. Carlson, personal communication) have thus far been unsuccessful. Several large families of seven transmembrane receptor genes expressed in subsets of *C. elegans* chemosensory and olfactory neurons have recently been identified (Troemel *et al.*, 1995; Sengupta *et al.*, 1996). However, these sequences share no homology with the mammalian receptor sequences from either the VNO or MOE. It is possible that the identification of additional families of receptors will reveal a common evolutionary ancestor to the vertebrate and invertebrate olfactory systems. Alternatively, the differences in the chemical nature of the odorants and differences in the physiological consequences of odor recognition might suggest independent origins for the invertebrate and vertebrate olfactory system.

The logic of olfactory coding in the MOE and VNO

Analysis of the patterns of expression of receptor genes in the main olfactory system has provided significant insight into mechanisms for the diversity and specificity of odor recognition in mammals. Similarly, the isolation of the pheromone receptors from the VNO is likely to help to elucidate the logic of olfactory perception in the vomeronasal system. The initial step in olfactory discrimination by the MOE requires the interaction of odorous ligands with one of the multiple seven transmembrane domain receptors expressed in the MOE. Discrimination among odorants requires that the brain determine which of numerous receptors has been activated. Since individual olfactory sensory neurons in the MOE are likely to express only a single receptor gene, the problem of distinguishing which receptors have been activated is reduced to a problem of distinguishing which neurons have been activated.

Recent experiments demonstrate that neurons expressing a given receptor, and therefore responsive to a given odorant, project their axons to one or a small number of discrete loci or glomeruli within the olfactory bulb (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996). The positions of specific glomeruli are topographically fixed, and are conserved in the brains of all animals within a species. These data provide physical evidence that the olfactory bulb provides a two-dimensional map that identifies which of the numerous receptors have been activated within the sensory epithelium. Such a model is in accord with previous experiments demonstrating that different odors elicit spatially defined patterns of glomerular activity in the olfactory bulb (Stewart *et al.*, 1979; Kauer *et al.*, 1987; Lancet *et al.*, 1982; Imamura *et al.*, 1992; Mori *et al.*, 1992; Katoh *et al.*, 1993). Thus, the quality of an olfactory stimulus would therefore be encoded by the specific combination of glomeruli activated by a given odorant.

At one level, the vomeronasal system shares anatomic and physiologic features with the main olfactory system, suggesting that similar experiments with pheromone receptors might also provide insight into how the recognition of odors by the VNO leads to the elaboration of innate behaviors. Primary olfactory sensory neurons within the VNO project a single unbranched axon which then synapses with dendrites of mitral cells in the accessory olfactory bulb, the first relay station for vomeronasal signaling in the brain. At a molecular level, we have identified a family of putative pheromone receptor genes that encode seven transmembrane domain proteins. Individual VNO neurons are likely to express only a single receptor gene. Cells expressing a specific receptor are randomly dispersed within the apical zone of the sensory epithelium. Thus, the pattern of pheromone receptor expression shares striking similarities with the expression of odorant receptors in the MOE.

At first glance, the anatomy and molecular organization of the VNO and MOE as well as that of the main and accessory olfactory bulb appear quite similar. There are, however, important differences. In the MOE, the mitral cells, the major output neurons of the olfactory bulb, project a primary dendrite to a single glomerulus, suggesting a one-to-one correspondence between mitral cell and sensory axon, such that a given mitral cell can respond to the activation of only a single class of sensory neurons. The task of discerning which sensory neurons have been activated must therefore be accomplished by integration at higher cortical centers. Mitral cells of the accessory bulb, however, exhibit a more complex primary dendritic array, allowing synapse formation with more than one glomerulus and therefore more than one class of sensory neurons (Macrides *et al.*, 1985; Takami and Graziadei, 1991). These observations suggest that, in the vomeronasal system, integration permitting the detection of a specific combination of different receptors activated by pheromones may occur in the accessory olfactory bulb.

The VNO and the main olfactory system reveal striking differences in the secondary projections to the cortex and in the responses elicited by the two sensory systems. VNO neurons project directly to the amygdala and hypothalamus, leading to innate and stereotypic behavioral responses (Winans and Scalia, 1970; Broadwell, 1975; Scalia and Winans, 1975; Krettek and Price, 1977, 1978; Kevetter and Winans, 1981). In contrast, the projections from the main olfactory organ activate higher cortical centers, resulting in a measured emotional or cognitive response. The projections from the vomeronasal system to the hypothalamus also control the release of luteinizing hormone release hormone and prolactin release hormone, increasing luteinizing hormone and prolactin levels both centrally and peripherally (reviewed in Keverne, 1983; Meredith and Fernandez-Fewell, 1994). In this manner, stimulation of the vomeronasal system can coordinate the activation of central neural pathways with dramatic neuroendocrine changes to elicit a characteristic array of innate reproductive and social behaviors.

The coding of olfactory information is likely to be far simpler in the vomeronasal system than in the main olfactory pathway. The receptor repertoire in the VNO is an order of magnitude smaller than in the MOE. Moreover, integration in the vomeronasal pathway is apparent in the accessory bulb, and the secondary projections synapse on a small number of loci in the amygdala. This is in sharp contrast to the complexity of the higher cortical pathways required for processing olfactory information from the MOE. Thus, the vomeronasal system may permit the analysis of the molecular events which translate the bindings of pheromones into innate stereotypic behaviors.

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