

Odor Receptor Proteins Recloned: Molecular Realities of Olfactory Discrimination in Fish

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We live in a crowded world of odors; how do we discriminate only one, the scent of garlic for example? Discrimination requires coordination of processes within the nervous system at both peripheral and central levels. Odor molecules are recognized when they bind to specific (and selective) receptor proteins in sensory neurons. Our olfactory epithelium contains around 2×10^6 such neurons which converge onto about 2000 second-order neurons in our olfactory bulb. The synaptic sites of this convergence are called glomeruli for their anatomical appearance; about 1000 sensory neurons converge on each glomerulus. Processing of olfactory information begins in the bulb; initial decisions are sent on to other brain centers via output neurons for further processing. This is the general scheme for a wide range of animals including all vertebrates and arthropods⁽¹⁾. For example, the moth *Manduca sexta* has fewer olfactory sensory cells than we have, about 2×10^5 , but the anatomy and the convergence factor onto glomeruli are essentially the same.

How many different receptor types are available in an olfactory epithelium to cope with the available odors? How is the olfactory system organized so that information is passed on in a meaningful way? These questions are elegantly addressed in two recent papers which report the cloning of approximately twenty different genes for olfactory receptor proteins from the channel catfish⁽²⁾, and describe the distribution of these proteins among the receptor neurons in the olfactory epithelium⁽³⁾. These reports conclude that there are about 100 different genes encoding catfish olfactory receptor proteins, that each olfactory neuron expresses one or at most only a few receptor genes, and that the expression of an individual receptor gene is distributed evenly and apparently randomly among the entire population of olfactory neurons. The authors discuss the developmental implications of such an organization, and contrast receptor organization of the catfish with that of the rat, a terrestrial smeller with a receptor system previously characterized by this group⁽⁴⁾.

To understand the rationale for the approach to cloning the receptor genes, it must be remembered that odor-activation of membrane-bound receptors is transduced into a change in the ionic conductance of the neuronal membrane. This basic fact drove research efforts through the 1980s to identify olfactory transduction and receptor mechanisms in a variety of organisms. Earlier observations of high levels of adenylate cyclase activity in olfactory epithelia⁽⁵⁾ led to the demonstra-

tion of odorant-dependent production of cAMP in isolated olfactory cilia^(6,7). Subsequent studies identified olfactory specific G-proteins that could provide coupling between receptors and adenylate cyclase enzymes, and ion channels that were directly activated by cAMP, thus establishing an olfactory transduction pathway⁽⁸⁾. Not all odorants induced cAMP, and it was shown that IP₃ functioned as a second messenger for many of these other odorants⁽⁹⁾. Like cAMP, IP₃ is thought to act directly on its own ion channels in the outer cilia membrane of olfactory neurons. These two pathways appear widespread among olfactory systems of a wide range of animals, including mammals, fish, amphibians and even arthropods^(8,9,10). Central to both pathways is that G-proteins mediate the receptor induced second messenger production, i.e. olfactory receptor proteins are 'G-protein coupled receptors'.

The first successful strategy for isolating olfactory receptor proteins exploited this feature of G-protein coupling. G-protein coupled receptors, also known as seven-transmembrane domain receptors, comprise a widely represented family which includes rhodopsin and a multitude of neurotransmitter receptors. Linda Buck and Richard Axel⁽⁴⁾ designed oligonucleotides based on conserved domains of previously sequenced G-protein coupled receptors and used these oligonucleotides as primers in polymerase chain reactions (PCR) to identify and clone a large family of putative olfactory receptor proteins from the rat. Based on genomic analysis the full complement of rat olfactory receptors was estimated at about 1000⁽⁴⁾.

Confirmation that these proteins are odor receptor proteins requires that odorants can be shown to activate them; this was recently demonstrated for several of the rat proteins⁽¹¹⁾. However, functional studies of the rat receptors have been difficult for several reasons, all relating to the nature of the odorants. Terrestrial animals smell airborne odorants which are volatile and hydrophobic; such odorants are difficult to apply experimentally in a quantifiable manner in an aqueous medium. More fundamentally, the behavioral or biological significance of most terrestrial (volatile) odorants is unclear and it has proven very difficult to find any specific odorants that activate one of the rat receptors. The fish, as an aquatic animal, represents a solution to this ligand problem. Aquatic animals smell water soluble molecules like amino acids and purines. These odorants are more limited in number than airborne odorants and many have identified behavioral roles. This more highly defined nature of the aquatic odorants utilized by fish was a major reason for initiating the receptor search in the catfish.

To identify, clone and assess the diversity of the catfish olfactory receptor proteins⁽²⁾, PCR primers were constructed based on conserved domains of the rat olfactory receptor proteins. PCR products were sequenced, and a product having the highest identity with the rat olfactory receptors was used to screen a catfish olfactory cDNA library, identifying three distinct clones. New PCR primers, based on these catfish derived clones, generated PCR products that led to the identification of three additional and distinct cDNAs. RNase protection experiments confirmed that all six clones were uniquely expressed in olfactory epithelium, and tissue *in situ*

analysis indicated that expression occurred only in mature olfactory receptor neurons (mRNA of one receptor, however, was detected in very low levels in testis). The abundance of receptor mRNA is relatively high in the neurons, estimated at around 200 copies per cell. Genomic analysis indicates that the receptor genes are free of introns, which is typical of the G-protein coupled receptor family. While no functional analysis was performed, the facts that the clones represent members of the G-protein coupled receptor family, share certain unique sequence motifs with the rat olfactory receptor family, and represent mRNAs uniquely expressed in olfactory epithelium are highly suggestive that these clones do represent olfactory receptor proteins of the catfish.

Olfactory discrimination depends on a relatively large number of different receptor proteins; the authors estimate that the catfish has about 100 different olfactory receptor genes. Genomic Southern blot analysis of each clone indicated that hybridization cross-reactivity between the six clones is minimal and that all but one recognizes multiple DNA fragments. These observations suggested that each of the original six clones represents a distinct receptor subclass. For example, screening of a genomic library with one clone (#32) identified 5 additional unique genes, all nearly identical but differing slightly within transmembrane domains III and IV. The authors suggest these small sequence differences reflect discriminating ligand-binding differences between the respective receptors; the ligand binding site of the seven-transmembrane domain receptors is thought to lie in the plane of the membrane, within a basket formed by the transmembrane domains⁽¹²⁾, and thus variation within these domains may indicate ligand binding specificity. Tissue *in situ* analysis indicated that each clone is expressed in 0.5-2.0% of the total neuronal population, and that none of the original 6 clones are coexpressed in individual neurons. These observations are the basis for the estimate that there are 100 catfish receptor genes. The authors note the approximate difference in receptor gene diversity between the rat and catfish, 1000 vs. 100, and suggest that this may reflect the difference in odor complexity in terrestrial vs. aquatic environments; there are vastly more airborne odorants than water soluble odorants, which may have led to increased receptor diversity among terrestrial animals.

Discrimination between odorants, however, requires more than mere recognition by receptors; sensory neurons must communicate this recognition to the brain. The current view is that there is a conserved relationship between the receptor gene that a sensory neuron expresses and the physical location in the bulb of the glomerulus to which that neuron projects. This argues that glomeruli are spatially and functionally defined and is supported by studies demonstrating spatially-conserved odorant-dependent activity patterns within the bulb^(13,14). The olfactory system is thus thought to be topographically organized, but the function of this topography and the developmental mechanisms which establish and maintain it are unknown. Understanding the regulation of receptor expression should help us to understand how sensory cells acquire their identity and how the topography is established.

To discern the topographical organization of sensory neu-

ron phenotypes in the catfish, the distribution patterns of specific receptor genes were examined through extensive tissue *in situ* analysis⁽³⁾. The catfish olfactory epithelium is located in a structure called the rosette, which is a series of lamellae joined together by a central raphe. The neuroepithelium, which contains the olfactory receptor neurons, is organized as a patch in the center of each lamella surrounded by non-neuronal epithelium. These neuroepithelial patches are located on both the proximal and distal surfaces of each lamella. The *in situ* studies demonstrated that receptors represented by each clone are evenly distributed throughout the neuroepithelia of all lamellae. Furthermore, each receptor appears to be randomly distributed within the neuroepithelial patches; precise patterns of receptor distribution are not conserved between proximal and distal surfaces of a single lamella or between comparable surfaces of neighboring lamellae. The developmental conundrum is how such apparently randomly distributed neurons become specified to express the same receptor gene and converge on the same spatial target in the olfactory bulb.

The authors suggest two mechanisms for this epithelial organization. First, while it is clear that the location of neuroepithelial patches may be spatially determined, the apparent randomness of the receptor distribution within a patch minimizes such spatial determination in favor of some stochastic process determining which receptor gene should be expressed in which neuron. Alternatively, it is possible that unspecified neurons might randomly converge onto the bulb and form glomerular synapses, with some retrograde signal then conferring identity onto the primary neurons to induce receptor gene expression appropriate to that glomerulus. It is worth noting that studies to date support the first mechanism; in both arthropods and vertebrates, glomerular formation does not occur until after the ingrowing primary axons have arrived at their appropriate sites^(15,16). In the moth *Manduca sexta*, ingrowing primary axons first form protoglomeruli with glia cells, and only after this do second order neurons enter these protoglomeruli to form synapses and the mature glomerulus⁽¹⁵⁾. Generally, ingrowing sensory axons appear to know their targets prior to their arrival, and neuronal identity of sensory neurons is conferred in the absence of synaptic formation. However, other systems (e.g. visual and auditory) appear to have a strictly defined topography that can be explained by conserved spatial positioning. On the basis of function, it makes sense that olfactory receptors should distribute themselves in an apparently random manner to match the true random distribution of the odor stimulus molecules, and that evolution should ensure a precise mechanism for establishing this diffuse receptor distribution pattern. If cells can acquire identity through positional information, it seems plausible that such a mechanism can be adapted to respond to some stochastic process perhaps involving nearest neighbor inhibition. The problem of receptor gene expression is compounded by the chromosomal organization of the olfactory receptor genes; various reports suggest that groups of genes are clustered and exist in either orientation within a cluster. In the catfish, for example, three of the subfamily-32 genes lie within a 30 kb DNA fragment⁽²⁾. Intriguingly, the authors raise the question of genomic rearrangement of regulatory

domains being involved in determining which of the 100 fish or 1000 rat genes will be expressed in a given neuron.

A major difference between the catfish and rat olfactory epithelia is that the rat neuroepithelium is organized in distinct patches, each containing a specific subset of olfactory receptor genes. Within a patch, receptor gene expression appears to be randomly distributed. These observations are mentioned in the second catfish paper⁽³⁾ as unpublished and personal communication, and were also presented at the most recent Association for Chemoreception Sciences meeting (4/93). The authors contrast these multiple patches of the rat with the single patch of the fish, and suggest that for the rat this patch compartmentalization 'into discrete units of lesser complexity reduces the problems inherent in regulating the expression of specific receptors among ~1000 genes, and may facilitate the guidance of a complex array of axonal projections from the epithelium to the bulb'⁽³⁾. It may also be that the fish's single patch represents a basic unit, and that multiple patches are the result of evolutionarily selected duplication of these basic units to cope with the more diverse airborne odorants.

Topography is a curious phenomenon of sensory systems. While it 'makes sense' that our visual and auditory systems physically map the external world into our brain, it makes less sense that topography should exist at all for olfactory signals which have no inherent spatial order with respect to individual odor molecules. The apparent randomness of receptor distribution is probably the consequence of evolutionary selection optimizing a tissue to deal with a truly random distribution of the signal. However, topography in sensory systems is also likely to exist for developmental reasons, to ensure that signal detection is wired into the brain in an appropriate manner so that it can be reliably processed. The olfactory system may represent the outcome of a complex developmental problem, combining apparent disorder with order, allowing for the ordered processing of disordered information. These papers^(2,3) represent insightful and important work that raises critical questions surrounding the mechanisms of neuronal development of sensory systems

and that opens significant new avenues of research in the field of olfaction.

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