Distinct Evolutionary Patterns between Chemoreceptors of 2 Vertebrate Olfactory Systems and the Differential Tuning Hypothesis

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Most tetrapod vertebrates have 2 olfactory systems, the main olfactory system (MOS) and the vomeronasal system (VNS). According to the dual olfactory hypothesis, the MOS detects environmental odorants, whereas the VNS recognizes intraspecific pheromonal cues. However, this strict functional distinction has been blurred by recent reports that both systems can perceive both types of signals. Studies of a limited number of receptors suggest that MOS receptors are broadly tuned generalists, whereas VNS receptors are narrowly tuned specialists. However, whether this distinction applies to all MOS and VNS receptors remains unknown. The differential tuning hypothesis predicts that generalist MOS receptors detect an overlapping set of ligands and thus are more likely to be conserved over evolutionary time than specialist VNS receptors, which would evolve in a more lineage-specific manner. Here we test this prediction for all olfactory chemoreceptors by examining the evolutionary patterns of MOS-expressed odorant receptors (ORs) and trace amine–associated receptors (TAARs) and VNS-expressed vomeronasal type 1 receptors (V1Rs) and vomeronasal type 2 receptors (V2Rs) in 7 tetrapods (mouse, rat, dog, opossum, platypus, chicken, and frog). The phylogenies of V1Rs and V2Rs show abundant lineage-specific gene gains/losses and virtually no one-to-one orthologs between species. Opposite patterns are found for ORs and TAARs. Analysis of functional data and ligand-binding sites of ORs confirms that paralogous chemoreceptors are more likely than orthologs to have different ligands and that functional divergence between paralogous chemoreceptors is established relatively quickly following gene duplication. Together, these results strongly suggest that the functional profile of the VNS chemoreceptor repertoire evolves much faster than that of the MOS chemoreceptor repertoire and that the differential tuning hypothesis applies to the majority, if not all, of MOS and VNS receptors.

Introduction

Most tetrapod vertebrates have 2 olfactory systems with distinct morphologies and signal transduction pathways: the main olfactory system (MOS) and the vomeronasal system (VNS) (Grus and Zhang 2006). According to the dual olfactory hypothesis (Scalia and Winans 1975), the 2 systems were thought to have 2 distinct functions: the MOS detects environmental odorants, whereas the VNS detects intraspecific pheromonal cues. However, exceptions to this distinction began appearing in the literature shortly after the dual olfactory hypothesis was published, and the overlapping functions of the 2 systems have been the subject of many recent reviews (Restrepo et al. 2004; Baxi et al. 2006; Spehr M, Spehr J, et al. 2006; Kelliher 2007). It was found that MOS sensory neurons can be activated by some pheromones in rabbits and pigs (Hudson and Distel 1983; Dorries et al. 1995) and that the disruption of the MOS signaling pathway affects mouse mating, parenting, and aggressive behaviors, which are widely thought to be mediated by pheromones (Belluscio et al. 1998; Mandiyan et al. 2005). On the other hand, the VNS has been shown to be important for foraging in some snakes, salamanders, and opossums (Schwenk 1993; Placyk and Graves 2002; Halpern et al. 2005). Furthermore, some odorants and pheromones activate neurons in both systems (Sam et al. 2001; Xu et al. 2005; Spehr M, Spehr J, et al. 2006; Chamero et al. 2007). These data clearly demonstrate that neither of the 2 olfactory systems is used exclusively for perceiving one class of chemical cues. Rather, it seems that the 2 systems are acting in concert to doubly process some chemosignals, albeit through distinct signal transduction pathways (Restrepo et al. 2004; Spehr M, Spehr J, et al. 2006).

Despite the fact that the MOS and the VNS have overlapping functions and overlapping ligands, some empirical evidence suggests that VNS receptors may be narrowly tuned to specific ligands, whereas MOS receptors may be broadly tuned to a complex combination of ligands (Leinders-Zufall et al. 2000; Katada et al. 2005). Because each MOS or VNS sensory neuron expresses only one allele of one chemoreceptor gene, the above differential tuning hypothesis applies equally to olfactory neurons and olfactory chemoreceptor proteins. To avoid confusion, we use “receptor” to refer to both neuron and protein and use “chemoreceptor” to refer to protein. It is known that both nonvolatile MHC peptides and the volatile urinary 2-heptanone activate sensory neurons in the MOS and VNS. In the study of VNS activation by MHC peptides, it was found that important anchoring residues of the peptides were required for signal transduction, that activation was independent of peptide concentration, and that peptides mutated at any site other than the anchoring residues still activated the VNS neurons (Leinders-Zufall et al. 2004). In contrast, changing these key residues had less effect on MOS activation as some peptides with altered sequences still activated the MOS receptors if concentration was increased (Spehr, Kelliher, et al. 2006). Similarly, whereas the VNS response to 2-heptanone was independent of its concentration (Leinders-Zufall et al. 2000), MOS neurons responding to 2-heptanone did so in a concentration-dependent manner (Spehr M, Spehr J, et al. 2006).

However, it is unknown whether the above observations from a limited number of MOS and VNS responses can be generalized to most or all MOS and VNS receptors. Here we attempt an evolutionary genomic test of the hypothesis of differential tuning between MOS and VNS receptors, by examining the evolutionary patterns of chemoreceptors expressed in the MOS and VNS. Our test is...
based on the idea that broadly tuned generalist MOS chemoreceptors are detecting an overlapping set of ligands and should thus be more likely to be conserved over evolutionary time than narrowly tuned specialist VNS chemoreceptors, which would evolve in a more lineage-specific manner. In other words, when the ligands encountered by 2 animal species differ, their MOS chemoreceptors need not be different because the same MOS chemoreceptors can detect many different ligands. In contrast, their VNS chemoreceptors are likely to be different because the ligand recognition by VNS chemoreceptors is more specific. Our test of the hypothesis of faster evolution of the functional profile of the VNS receptor repertoire than that of the MOS receptor repertoire is feasible because the 2 olfactory systems each express 2 superfamilies of chemoreceptors. Sensory neurons in the MOS express either odorant receptors (ORs) or trace amine-associated receptors (TAARs) (Mombaerts 2004; Liberles and Buck 2006), whereas those in the VNS express vomeronasal type 1 receptors (V1Rs) or vomeronasal type 2 receptors (V2Rs) (Mombaerts 2004). This expressional distinction is so clear that when the VNS is lost in birds, all V1R and V2R genes were inactivated (Shi and Zhang 2007), whereas OR and TAAR genes are still preserved in the genome (Niimura and Nei 2005; Grus et al. 2007). The 4 types of receptors are all 7-transmembrane G-protein–coupled receptors (GPCRs), but they do not have any significant sequence similarity. Although the 4 gene superfamilies have been subject to intense evolutionary analysis (Young et al. 2002, 2005; Grus and Zhang 2004; Grus et al. 2005, 2007; Aloni et al. 2006; Hoppe et al. 2006; Hashiguchi and Nishida 2007; Niimura and Nei 2007; Shi and Zhang 2007; Young and Trask 2007), none of the previous studies have either quantitatively compared the evolutionary patterns of the 4 superfamilies or compared them in the context of determining their potential functional differences. We show a clear-cut distinction in the evolutionary pattern between MOS and VNS chemoreceptors. Coupled with an analysis of functional data and ligand-binding sites of chemoreceptors, our results provide strong support to the differential tuning hypothesis at the level of entire MOS and VNS chemoreceptor repertoires.

Materials and Methods

Olfactory Chemoreceptors from 6 Tetrapods

We compared the V1R, V2R, OR, and TAAR gene repertoires from 6 tetrapods that have publicly available high-quality genome sequences. These species include frog (*Xenopus tropicalis*), chicken (*Gallus gallus*), platypus (*Ornithorhynchus anatinus*), opossum (*Monodelphis domestica*), dog (*Canis familiaris*), and mouse (*Mus musculus*). We analyzed all previously reported putatively functional olfactory chemoreceptors of these species. The numbers of V1Rs compiled were 187 in mouse (Shi et al. 2005), 8 in dog (Grus et al. 2005; Young et al. 2005), 98 in opossum (Shi and Zhang 2007), 270 in platypus (Grus et al. 2007), and 21 in frog (Shi and Zhang 2007). Chicken does not have functional V1Rs. The numbers of V2Rs compiled were 70 in mouse (Yang et al. 2005), 79 in opossum (Shi and Zhang 2007), 249 in frog (Shi and Zhang 2007), and 15 in platypus (Grus et al. 2007). Chicken and dog do not have functional V2Rs. The numbers of ORs compiled were 1,084 in mouse (Young et al. 2002; Zhang and Firestein 2002), 658 in dog (Aloni et al. 2006), 871 in opossum (Aloni et al. 2006), 261 in platypus (Grus et al. 2007), 77 in chicken (Niimura and Nei 2005), and 405 in frog (Niimura and Nei 2005). The numbers of TAARs compiled were 15 in mouse (Lindemann et al. 2005), 2 in dog (Grus et al. 2007), 21 in opossum (Grus et al. 2007), 4 in platypus (Grus et al. 2007), 3 in chicken (Grus et al. 2007), and 3 in frog (Grus et al. 2007).

Rat Chemoreceptors

To make the mouse–rat comparison, we obtained 1,195 ORs (Rat Genome Sequencing Consortium 2004), 17 TAARs (Lindemann et al. 2005), 106 V1Rs (Shi and Zhang 2007), and 59 V2Rs (Shi and Zhang 2007) from the rat (*Rattus norvegicus*).

Phylogenetic Reconstruction

For each of the 4 olfactory chemosensory receptor superfamilies, protein sequences were aligned by ClustalX (Thompson et al. 1997) with manual adjustment. Phylogenetic trees were reconstructed using the Neighbor-Joining method (Saitou and Nei 1987) for OR, TAAR, V1R, and V2R superfamilies, respectively, using the 6 vertebrate species. One thousand bootstrap replications (Felsenstein 1985) were used except in the OR tree. The comprehensive OR tree had only 400 bootstrap replications, whereas the subtrees constructed for each OR family for better resolution had 1,000 bootstrap replications. Trees for the 4 receptor superfamilies were also reconstructed for the mouse and rat. MEGA (Kumar et al. 2004) was used for these evolutionary analyses.

Proportion of Genes Belonging to Lineage-Specific Clades

In the phylogeny for a chemoreceptor superfamily, a lineage-specific clade of *n* genes must have resulted from at least *n* – 1 gene gains/losses since the species diverged from its closest relative in our set of 6 vertebrates. The total proportion of genes belonging to lineage-specific clades is the sum of the *n* – 1 genes for all lineage-specific clades divided by the total number of genes for that chemoreceptor superfamily in that species. A lineage-specific clade is a monophyletic clade of chemoreceptors from a single species. We also used a more stringent definition of lineage-specific clades by the additional requirement that the monophyletic clade should have a bootstrap support of at least 70%. To determine the proportion of MOS chemoreceptors belonging to lineage-specific clades, we summed the *n* – 1 genes for a species for both ORs and TAARs and divided it by the total number of ORs and TAARs in that species. A similar approach was used to determine the proportion of VNS chemoreceptors belonging to lineage-specific clades. In the mouse–rat gene tree, a mouse gene and a rat gene are considered to be one-to-one orthologs if
they form a monophyletic clade that does not include any other genes. Because orthologous gene pairs are rare for VNS chemoreceptors, to avoid missing any orthologs, we did not require bootstrap support in defining orthologs in any superfamily to ensure a fair comparison.

Functional Comparison of Dog ORs

To investigate the relationship between OR sequence divergence and functional divergence, we analyzed a functional data set of dog ORs (Benbernou et al. 2007). These ORs were originally classified into subfamilies of OR family 6 (Benbernou et al. 2007). However, simple analysis showed that they do not all share the pairwise amino acid sequence identity >40%, which is required for ORs to be classified in the same family (Glusman et al. 2000). To determine their correct classification, we used these ORs to Blast against the dog ORs in the HORDE database (http://bioportal.weizmann.ac.il/HORDE/). Based on our analysis, these 38 dog ORs belong to 2 class II OR families, OR6 and OR11. We were also able to further classify these ORs into subfamilies based on the criterion that subfamily members share >60% identity in protein sequence (Glusman et al. 2000). Benbernou et al. (2007) tested the ORs into subfamilies based on the criterion that subfamily

Analysis of the OR Ligand-Binding Pocket

For each of the 557 pairs of one-to-one mouse–rat orthologous ORs, we randomly picked 10 amino acid sites. For each random set of 10 sites, we determined id, the number of orthologous pairs that had an identical sequence at these 10 sites. We repeated this process 1,000 times for both the entire coding sequence and for only the transmembrane domains to get 2 distributions for id. The simulated values were compared with the actual id for the 10 sites in the experimentally determined ligand-binding pocket (Katada et al. 2005). We also conducted a similar analysis for 266 paralogous mouse ORs that belong to the 128 mouse-specific clades in the mouse–rat tree, involving 192 pairwise OR comparisons.

Results

Distinct Phylogenetic Patterns between MOS and VNS Chemoreceptors

We reconstructed the phylogenies of all putatively functional V1Rs, V2Rs, ORs, and TAARs, respectively, from frog, chicken, platypus, opossum, dog, and mouse (fig. 1). The species were carefully chosen to represent major tetrapod lineages and to avoid overrepresentation of placental mammals, for which many genera have been sequenced. The difference between the phylogenies of VNS and MOS chemoreceptors is striking. V1Rs and V2Rs form almost exclusively lineage-specific clades in their trees (fig. 1A and B), the exception being the V2R2 clade. By contrast, MOS chemoreceptors show a common pattern of multiluxa clades, although there are also some small lineage-specific clades (fig. 1C–F).

To quantify the difference in phylogenetic pattern between VNS and MOS chemoreceptors, we calculated the proportion of genes in each chemoreceptor superfamily that arose from lineage-specific gene gains and losses. Using this metric and considering relatively well-supported lineage-specific clades (>70% bootstrap), we found a significantly higher proportion of lineage-specific chemoreceptors in the VNS than in the MOS for frog (χ² = 11.89, P < 0.001), platypus (χ² = 127.99, P < 10⁻²⁹), opossum (χ² = 120.76, P < 10⁻⁴⁷), and mouse (χ² = 201.28, P < 10⁻⁴⁴) (fig. 2A). The pattern is less prominent for the frog than for the mammals, probably because the frog lineage is so long (fig. 2A) that it has less power to detect lineage-specific events. Similar results were obtained when all lineage-specific clades were considered regardless of the bootstrap support (supplementary fig. S1, Supplementary Material online).

In the above analyses, we used 6 distantly related species to represent major tetrapod lineages. To examine if the distinct evolutionary patterns of VNS and MOS chemoreceptors observed at this large evolutionary distance also occur between closely related taxa, we compared the mouse and rat, 2 species that diverged approximately 18 MYA (Murphy et al. 2004). We constructed new trees using mouse and rat chemoreceptors (supplementary fig. S2, Supplementary Material online) and calculated the same metric for a comparison of the 4 chemoreceptor superfamilies in the 2 species. Again, we found a higher proportion of genes from lineage-specific gains and losses in the VNS chemoreceptors than in the MOS chemoreceptors for both mouse (χ² = 401.82, P < 10⁻⁸⁸) and rat (χ² = 52.48, P < 10⁻¹²) (fig. 2B). We also determined the number (m) of potentially orthologous gene pairs between the 2 species in each chemoreceptor superfamily by counting the number of monophyletic clades consisting of 1 mouse gene and 1 rat gene (Supplementary data sets 1–4, Supplementary Material online). We then calculated the fraction (f) of genes in each chemoreceptor superfamily that have one-to-one mouse–rat orthologs, using f = m/min(x, y), where x and y are the numbers of mouse and rat genes in the superfamily, respectively. The f values for V1Rs (18/106 = 0.17) and V2Rs (4/59 = 0.07) are significantly lower than those for ORs (557/1084 = 0.51) and TAARs (7/15 = 0.47) (P < 0.015 in each of the 4 comparisons, Fisher’s exact test).

Although about half of mouse ORs and TAARs have potential orthologs in rat, these genes with orthologs are not distributed evenly among different ORs and TAARs. Tetrapod ORs have been classified into 2 classes: fish-like Class I ORs and terrestrial Class II ORs (Freitag et al. 1995; Mombaerts 2004). The f value is 76/123 = 0.62 for Class I ORs, significantly greater than that (481/961 = 0.50) for Class II ORs (P < 0.01, Fisher’s exact test). Mammalian TAARs have also been classified into 3 families: TAAR1-4, TAAR5, and TAAR6-9 (Lindemann
FIG. 1.—Unrooted phylogenetic trees of all putatively functional frog, chicken, platypus, opossum, dog, and mouse (A) V1Rs, (B) V2Rs, (C) ORs, (D) Class I ORs, (E) Class II ORs from families 5 and 8 as defined by the HORDE database, and (F) TAARs. Because there are too many Class II ORs to show clearly, families 5 and 8 are randomly chosen to illustrate the phylogenetic pattern of Class II ORs. Other families show a similar pattern. The trees were reconstructed using the Neighbor-Joining method with Poisson-corrected protein distances. The scale bars show 0.1 amino acid substitutions per site. The phylogenetic patterns show that VNS chemoreceptors tend to form lineage-specific clades, which are rarely found among MOS chemoreceptors.
et al. 2005). All mouse and rat TAARs in families TAAR1-4 and TAAR5 are part of potentially orthologous gene pairs. In contrast, only 4 of the 22 mouse and rat TAARs in family TAAR6-9 are part of potentially orthologous gene pairs. Interestingly, family TAAR6-9 also has had independent expansion in the opossum lineage (Grus et al. 2007; Hashiguchi and Nishida 2007).

Functional Divergence after Chemoreceptor Gene Duplication

It is generally believed that paralogous proteins are much more likely than one-to-one orthologs to have divergent functions (Zhang 2003). This hypothesis can be verified for the chemoreceptors. Functional evidence based on site-directed mutagenesis studies and computational predictions suggests that the ligand-binding domain of ORs lies in a hydrophobic binding pocket created in the transmembrane domains (Baldwin 1994; Man et al. 2004; Zhang et al. 2004; Katada et al. 2005). Specifically, 10 residues in transmembranes 3, 5, and 6 in mouse OR 73 (in the OR5D subfamily) were identified to constitute its ligand-binding pocket (Katada et al. 2005). Site-directed mutagenesis at these residues altered the ligand-binding profile of mouse OR 73 to eugenol and related ligands, suggesting that these residues play a role in OR recognition of ligands. We found that, between mouse–rat orthologous ORs, the 10 ligand-binding sites are more conserved than randomly chosen 10 sites from the entire protein ($P = 0.003$) or from the transmembrane domains ($P = 0.012$) (fig. 3). For the ligand-binding sites, 78.1% (435/557) of orthologous mouse–rat OR pairs have identical sequences, whereas this fraction is on average 55.3% for 10 randomly chosen sites from the entire protein or 60.3% for 10 randomly chosen sites from the transmembrane domains. In contrast, when we examined ORs that belong to the mouse-specific clades in the mouse–rat OR tree (supplementary fig. S2C, Supplementary Material online), the 10 ligand-binding sites are more variable than randomly chosen sites from the entire protein ($P = 0.098$) or the transmembrane domains ($P = 0.128$). Thus, consistent with the expectation, this comparison suggests that one-to-one orthologous ORs tend to recognize the same ligands, whereas paralogous ORs tend to recognize different ligands.

The above results, however, do not tell how quickly a newly duplicate OR establishes its function. Here we compare the relationship between OR sequence divergence (proxy for time) and functional divergence using a functional data set of 38 paralogous ORs from the dog (Benbernou et al. 2007). Each of these 38 ORs was previously examined for response to 7 aliphatic aldehydes of C6–C12 at 3 different concentrations (Benbernou et al. 2007). For dog ORs belonging to the same subfamily, OR functional divergence was positively correlated with protein sequence divergence ($R^2 = 0.155; P < 0.01$; fig. 4). In other words, more divergent ORs responded more differently to ligands. However, for ORs belonging to different subfamilies or different families, no correlation was observed between sequence divergence and functional
divergence ($R^2 = 0.000132; P = 0.77$; fig. 4). These observations suggest that functional changes may occur only in newly duplicated OR genes and that once the function is established in an OR, it is no longer altered. Although the probability is low, we cannot exclude the possibility that in some lineages, there are functional reversions after divergence.

Discussion

The 2 vertebrate olfactory systems, the VNS and the MOS, overlap in some of their activating ligands. But our analysis showed that they differ greatly in the evolutionary patterns of their chemoreceptors. Whereas the phylogenies of VNS chemoreceptors exhibit almost exclusively lineage-specific clades, those of MOS chemoreceptors show both multispecies clades and lineage-specific clades. One potential caveat is that each chemoreceptor gene superfamily studied here form one to multiple gene clusters in chromosomes and different chromosomal regions may have different intrinsic rates of gene duplication. However, it seems unlikely that by chance the 2 VNS chemoreceptor superfamilies are both in high duplication regions, whereas the 2 MOS chemoreceptor gene superfamilies are both in low duplication regions. This possibility becomes even lower when we consider that V1R, V2R, and OR genes are actually scattered in multiple chromosomes (Young et al. 2002; Zhang and Firestein 2002; Grus and Zhang 2004; Zhang et al. 2004; Yang et al. 2005). Thus, the contrast between the evolutionary patterns of MOS and VNS receptors reflects a difference at the selection level rather than the mutation level.
cept for the V2R2 clade, which has a different evolutionary
receptors among the initial 6 tetrapod species examined, ex-
a high rate that there are no one-to-one orthologous chemo-
tively faster changes of the VNS receptor repertoire than the
occurred after the evolutionary separations of the species
studied here, 2) loss of ancestral genes in specific lineages,
from one spe-
specific clades. We defined a lineage-specific clade as
VNS, including both a separate VNO. It has already been
to occur. The striking difference in the evolutionary pattern
lies in the first case, although all 3 are likely
organisms that respond to the same ligand
Although all 4 chemoreceptor families we investigated are
present in teleost fish (Alioto and Ngai 2005, 2006;
Niimura and Nei 2005; Hashiguchi and Nishida 2007;
Pfister et al. 2007; Shi and Zhang 2007), we focused our
comparative analysis on tetrapods for the following rea-
s. In teleost fish, both the VNS and MOS are expressed in
developmental stages of the olfactory epithelium because tel-
fish do not have a separate VNO. It has already been
shown that there were shifts in olfactory chemoreceptor
prevalence following the vertebrate transition from aquatic
habitats to terrestrial habitats with Class I ORs and V2Rs
dominate the aquatic vertebrates and Class II ORs
and V1Rs dominating in terrestrial vertebrates (Shi and
Zhang 2007). Functional changes of the chemoreceptors
likely accompanied this evolutionary shift. For example,
based on the comparative sequence analysis, teleost
V2Rs are thought to be amino acid receptors, whereas
mammalian V2Rs do not contain the conserved residues
necessary for amino acid binding (Alioto and Ngai 2006). Additionally, in contrast to patterns observed in
mammalian V1Rs, teleost V1Rs are highly conserved
among distantly related taxa (Saraiva and Korsching
2007), suggesting a different role for these teleost chemo-
receptors. Furthermore, the difference in environment be-
tween aquatic and terrestrial vertebrates has altered the
nature of the ligands, such that classes of common teleost
odorants, such as bile acids, amino acids, steroids, and pros-
taglandins, are not all common classes of tetrapod odorants.

The basis of our analysis is in recognizing lineage-
specific clades. We defined a lineage-specific clade as
a monophyletic clade of multiple receptors, all from one spe-
cies. Such clades were formed by 1) gene duplication that
occurred after the evolutionary separations of the species
studied here, 2) loss of ancestral genes in specific lineages,
or 3) a combination of the above 2 processes. The majority
of our focus lies in the first case, although all 3 are likely
to occur. The striking difference in the evolutionary pattern
between VNS and MOS chemoreceptors indicates substan-
tially faster changes of the VNS receptor repertoire than the
MOS receptor repertoire during evolution. The VNS chem-
receptor superfamilies acquired and lost genes with such
a high rate that there are no one-to-one orthologous chemo-
receptors among the initial 6 tetrapod species examined, ex-
cept for the V2R2 clade, which has a different evolutionary

Fig. 4.—Pairwise amino acid sequence divergence and OR response
profile distance are significantly correlated between closely related dog
OR paralogs (pluses) but not between more distantly related OR paralogs
(circles). OR response data taken from Benbernou et al. (2007).

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cept for the V2R2 clade, which has a different evolutionary

origin (Yang et al. 2005; Shi and Zhang 2007), different
expression pattern and transport mechanism (Martini
et al. 2001; Silvotti et al. 2005), and possibly different func-
tion (Young and Trask 2007) from other V2Rs. Even be-
tween the closely related mouse and rat, less than 11% of
VNS chemoreceptors have one-to-one orthologs, compared
with over 48% of MOS chemoreceptors. These results are in
stark contrast to other groups of GPCRs, which all have at
least 84% one-to-one orthologs between mouse and rat
(Gloriam et al. 2007). Our analysis of OR functions and li-
gand-binding sites showed that paralogous chemoreceptors
are much more likely than one-to-one orthologs to have di-
vergent functions and that the functional divergence tends to
be established shortly after gene duplication. Taken to-
gether, our results suggest that the functional profile of the
VNS receptor repertoire evolves much faster than that of
the MOS receptor repertoire, which is consistent with
the prediction of the differential tuning hypothesis that
broadly tuned generalist MOS receptors detect an overlap-
ning set of ligands and thus are more likely to be conserved
over evolutionary time than narrowly tuned specialist VNS
receptors, which would evolve in a more lineage-specific
manner.

There are additional lines of evidence for the differential
Tuning hypothesis. In the study that identified the 10
ligand-binding pocket residues of mouse OR 73, Katada
et al. (2005) aimed to understand the molecular mechanism
allowing for the identification of tens of thousands of po-
tential ligands by ~1,000 ORs. The complex combinatorial
mechanism of OR ligand binding by which each OR can
bind multiple ligands and each ligand can be bound by mul-
tiple ORs (Malnic et al. 1999) is much different from the
highly specific receptor–ligand pairing for other GPCRs
(Katada et al. 2005). These authors showed that the major-
ity of ligand-binding residues for ORs are nonpolar, and the
majority of interactions between ORs and ligands are weak
hydrophobic or van der Waals interactions (Katada et al.
2005). In contrast, they note that the β2-adrenergic receptor
has polar and charged residues in its ligand-binding pocket,
allowing for stronger ionic interactions between ligand and
receptor and higher ligand affinities (Wieland et al. 1996;
Katada et al. 2005). Although the ligand-binding pocket has
not been identified for either class of VNS chemoreceptor,
we found that transmembranes 3, 5, and 6 have a signifi-
cantly higher number of polar residues (ORs mean
34.2%, V1Rs mean 37.3%; Fisher’s exact test,
\( P < 10^{-28} \) ) and charged residues (ORs means 6.6%,
V1Rs mean 7.2%; Fisher’s exact test, \( P < 10^{-4} \) ) in
V1Rs (from mouse, dog, opossum, platypus, and frog) than
in ORs (from the same species), suggesting that there is po-
tential for stronger and more specific ligand–receptor in-
teractions in V1Rs than in ORs, consistent with the differential
tuning hypothesis.

A number of functional studies of ORs are consistent
with the hypothesis of differential tuning in VNS and MOS
chemoreceptors. For instance, the same ligands can activate
distantly related ORs (Malnic et al. 1999; Sanz et al. 2005;
Benbernou et al. 2007; Stary et al. 2007). In contrast, only
a few VNS receptors are activated by 2-heptanone and
ESP1 (Leinders-Zufall et al. 2000; Kimoto et al. 2005). Ad-
ditionally, all VNS neurons that respond to the same ligand

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have the same response profile, not responding to any of the other V1R ligands (Leinders-Zufall et al. 2000). In contrast, ORs that respond to octanal had a wide range of response profiles to related odorants (Araneda et al. 2004; Benbernou et al. 2007). Furthermore, concordant birth-and-death evolutionary patterns between the V2R superfamily and the 2 V2R ligand families strongly suggest high ligand specificity of VNS chemoreceptors (Chamero et al. 2007; Shi and Zhang 2007). We conclude that multiple lines of evidence, particularly the distinct evolutionary patterns of MOS and VNS chemoreceptors, strongly support differential tuning between MOS and VNS receptors at the level of entire receptor repertoires.

**Supplementary Material**

Supplementary data sets 1–4 and figures S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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