Review

Evolving olfactory systems on the fly

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The detection of odour stimuli in the environment is universally important for primal behaviours such as feeding, mating, kin interactions and escape responses. Given the ubiquity of many airborne chemical signals and the similar organisation of animal olfactory circuits, a fundamental question in our understanding of the sense of smell is how species-specific behavioural responses to odorants can evolve. Recent comparative genomic, developmental and physiological studies are shedding light on this problem by providing insights into the genetic mechanisms that underlie anatomical and functional evolution of the olfactory system. Here we synthesise these data, with a particular focus on insect olfaction, to address how new olfactory receptors and circuits might arise and diverge, offering glimpses into how odour-evoked behaviours could adapt to an ever-changing chemosensory world.

Structure, function and variation in the olfactory system

Brain evolution has generated a diversity of animal responses to identical environmental features. This is seen most clearly in the olfactory system through highly species-specific behavioural responses to odours. For example, although we experience disgust at farmyard smells of animal waste, these excretory products form a language of private chemical communication within the pigsty or sheep-pen disclosing sexual and social status [1]. Olfactory response variability is also pronounced in the insect world: the malaria mosquito, *Anopheles gambiae*, is highly attracted to carbon dioxide (CO_2) in human breath, identifying a potential source for its next blood-meal [2], whereas the fruit fly, *Drosophila melanogaster*, is repelled by this conspecific alarm signal or indicator of unripe fruit [3,4]. Even relatively closely-related species can exhibit profoundly distinct odour-evoked responses. *D. sechellia* and *D. simulans* are fruit fly cousins whose last common ancestor is estimated to have existed only 250 000 years ago [5]. *D. sechellia*, endemic to the Seychelles archipelago, is an olfactory specialist, attracted to the odours of the *Morinda citrifolia* 'noni' fruit, a food source and oviposition site. By contrast, the olfactory generalist *D. simulans* is repelled by noni fruit odours and is instead attracted to a wide range of other vegetative substrates [6,7]. The evolutionary advantages of species-specific odour-evoked behaviours in these and many other examples are clear: olfactory niche formation alleviates direct competition for nutritional resources and prevents unproductive interspecies mating attempts. But what molecular and developmental mechanisms underlie adaptive olfactory specialisation?

Insights into olfactory evolution can be gained from recent progress in two areas. First, we now have a fairly detailed understanding of the organisation and function of the peripheral olfactory system [8,9] (Figure 1). In both insects and vertebrates, odour molecules are detected by odorant receptors (ORs) localised to the dendrites of olfactory sensory neurones (OSNs) in the antenna (insects), or the olfactory epithelium (vertebrates) [10]. The vast majority of OSNs express only a single OR [11] and the axons of like OSNs converge onto one or a few stereotyped glomeruli within the antennal lobe in insects, or the olfactory bulb in vertebrates [12,13]. Here they synapse with specific second-order neurones (insect projection neurones, or vertebrate mitral and tufted cells) which transmit olfactory information to higher brain centres (the insect mushroom body and lateral protocerebrum, or vertebrate olfactory cortex). Physiological studies have shown that many odours activate specific combinations of ORs that have distinct sensitivities and dynamic properties [14]. Peripheral circuits thus transform odour identity and

Glossary

Antennal basiconic 3 (ab3): one of the ten basiconic classes of olfactory sensory hairs (sensilla) in *D. melanogaster*. This sensillum houses two neurones, ab3A and ab3B, which express OR22a/OR22b and OR85b receptors, respectively.
***Dscam1* (*Down syndrome cell-adhesion molecule 1*)**: a *D. melanogaster* gene potentially generating >100 000 alternative splice variant transcripts that encode a diversity of cell-surface immunoglobulin-domain proteins involved in neuronal recognition.
EC50 (half-maximal effective concentration): the concentration of a chemical (e.g. odour or drug) that induces a response halfway between the baseline and maximum after a specified exposure time.
GR21a, GR63a (gustatory receptors 21a, 63a): two members of the insect "gustatory" receptor repertoire (related to insect ORs) that are co-expressed in antennal neurones and together comprise the peripheral sensory receptor for CO_2.
Imaginal disc: tissue primordium in holometabolous insect larvae that gives rise to external appendages in the adult (e.g. antennae, legs, wings).
Labelled line: a sensory pathway relying on a dedicated peripheral receptor, circuitry and response centre in the brain.
Locus control region: a gene regulatory sequence characterised by its ability to enhance the expression of a cluster of linked genes in a tissue-specific manner at a distance by opening chromatin structure.
MicroRNA: a ~22 nucleotide RNA sequence involved in post-transcriptional gene regulation by binding complementary sequences in the 3′ untranslated region of multiple target mRNAs.
Perireceptor olfactory proteins: proteins acting upstream of ORs in odour detection. These are normally secreted into the fluid that bathes OSN dendrites and include odorant-binding proteins and odorant-degrading enzymes.
Positive selection: a mechanism of natural selection describing selection for an allele (or a site within an allele) that increases fitness. Also known as directional selection.
Sensory organ precursor (SOP): progenitor cell for insect external sensory organs.

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0168-9525/$ – see front matter doi:10.1016/j.tig.2010.04.004 Trends in Genetics 26 (2010) 307–316

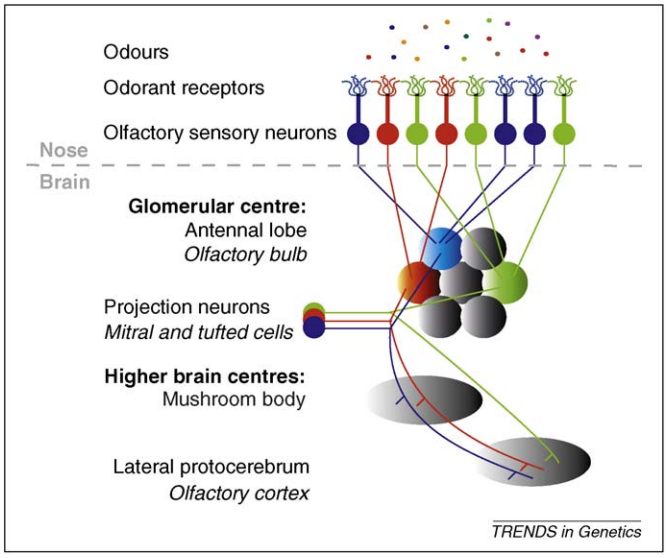

Figure 1. Anatomy of olfactory circuitry. In both insects and vertebrates, axons of olfactory sensory neurones expressing the same odorant receptor converge onto specific glomeruli in the primary olfactory centre: the antennal lobe (insects) or the olfactory bulb (vertebrates). Here, they synapse with second-order neurones called projection neurones (insects), or mitral and tufted cells (vertebrates). Olfactory information is then transmitted to the mushroom body and lateral protocerebrum (insects), or the olfactory cortex (vertebrates). Local interneurones that form broad synaptic contacts in many glomeruli are not shown.

intensity into a unique spatiotemporal glomerular 'code' [15]. These activity patterns are further shaped by morphologically diverse classes of local interneurones that broadly innervate the primary olfactory centre [16].

Second, comparative genomic and functional studies on olfactory systems in phylogenetically close species are beginning to identify differences that might shed light on species-specific olfactory behaviours. The most comprehensive studies are those on *D. melanogaster* and *A. gambiae* (Figure 2). These insects possess two types of olfactory organs – antennae and maxillary palps – but both differ dramatically in morphology (Figure 2a). Remarkable divergence is also seen at the receptor level and, with one exception (the atypical OR83b co-receptor [17]), there are no obvious orthologous mosquito and fruit fly ORs (Figure 2b). Electrophysiological analyses of the odour–response profiles of these receptor repertoires have revealed overlapping but distinct tuning properties (Figure 2c), and these correlate with stimuli that are likely to be important for these ecologically divergent insects [14,18]. Finally, the fruit fly and mosquito antennal lobes exhibit differences in glomerular number and morphology (Figure 2d) [19,20], neuroanatomical distinctions that are probably reflected in more centrally located circuits as well.

In this review we consider the issue of olfactory system evolution, encompassing the genetic mechanisms underlying the emergence of receptors detecting novel odorants as well as circuits driving new behaviours to already perceptible olfactory stimuli. We focus on the olfactory system of the powerful genetic model, *D. melanogaster*, but draw examples from other invertebrate and vertebrate models where the insights they provide are relevant to our discussion.

In the beginning: evolution of a new OR gene

Olfactory circuits capable of detecting previously imperceptible environmental odours most probably originate with the birth of new OR genes. Genomic analyses of OR repertoires both within and between species suggest mechanisms for the generation of new OR loci. For example, many receptor genes exist in tandem arrays [21,22], a hallmark of gene duplication by non-allelic homologous recombination (Figure 3a). This could be the principal mechanism for receptor repertoire expansion as there is an element of positive feedback to this process: duplicated genes increase the likelihood of additional allelic mispairings and further gene duplication events. However, OR genes are not fixed within tandem arrays and closely related genes are often found at distant sites within the same chromosome or on other chromosomes [21,22]. The mechanisms underlying intra- or interchromosomal OR gene translocation are less well understood, but might be a by-product of large-scale chromosomal rearrangements. Physical segregation of a new receptor gene from a parental tandem array might protect it from further intra-array recombination events (that can lead to gene deletion as well as duplication) and thereby facilitate the acquisition of new functional or expression properties.

OR gene loss might be as prevalent as gain, through their deletion by non-allelic homologous recombination, or through pseudogenisation by nonsense or frameshift mutations (Figure 3b). Indeed, the olfactory specialisation of *D. sechellia* has been correlated with an increased rate of OR gene loss relative to other drosophilids [23,24], suggesting that this fly has discarded sensory inputs no longer relevant to its restricted food-seeking behaviours. Similarly, significant losses of vertebrate OR repertoires could be explained by changes in living environment or the emergence of novel sensory capabilities, such as trichromatic vision in primates [21]. Given the mechanistic ease of gene gain and loss, OR repertoire evolution is described as a continuous 'birth-and-death' process [21,25]. Although this process might in part reflect evolutionarily neutral changes [26], recent evidence points to a generally adaptive profile of a species' receptor repertoire [27]. Neutral genetic changes are not necessarily unimportant, however, because they offer a rich genetic substrate upon which natural selection might, in time, act.

Diversifying OR function

New receptors are likely to be closely related if not identical in amino acid sequence to their parental receptors and often – though not necessarily – in their expression pattern. Duplicated loci can follow several fates [28]. If the genes are redundant, one could degenerate. The presence of many pseudogenes in tandem arrays of recently duplicated OR genes indicates that this is a common terminal fate [21,29]. A more interesting outcome is one in which a duplicated gene, free of selective pressures to maintain a redundant function, mutates and acquires new properties (Figure 3b). If advantageous, perhaps by providing new odour detection capabilities, this receptor gene could be subject to unique selective pressures and maintained in the genome.

Evidence for functional divergence of recently duplicated receptors is starting to emerge from comparative

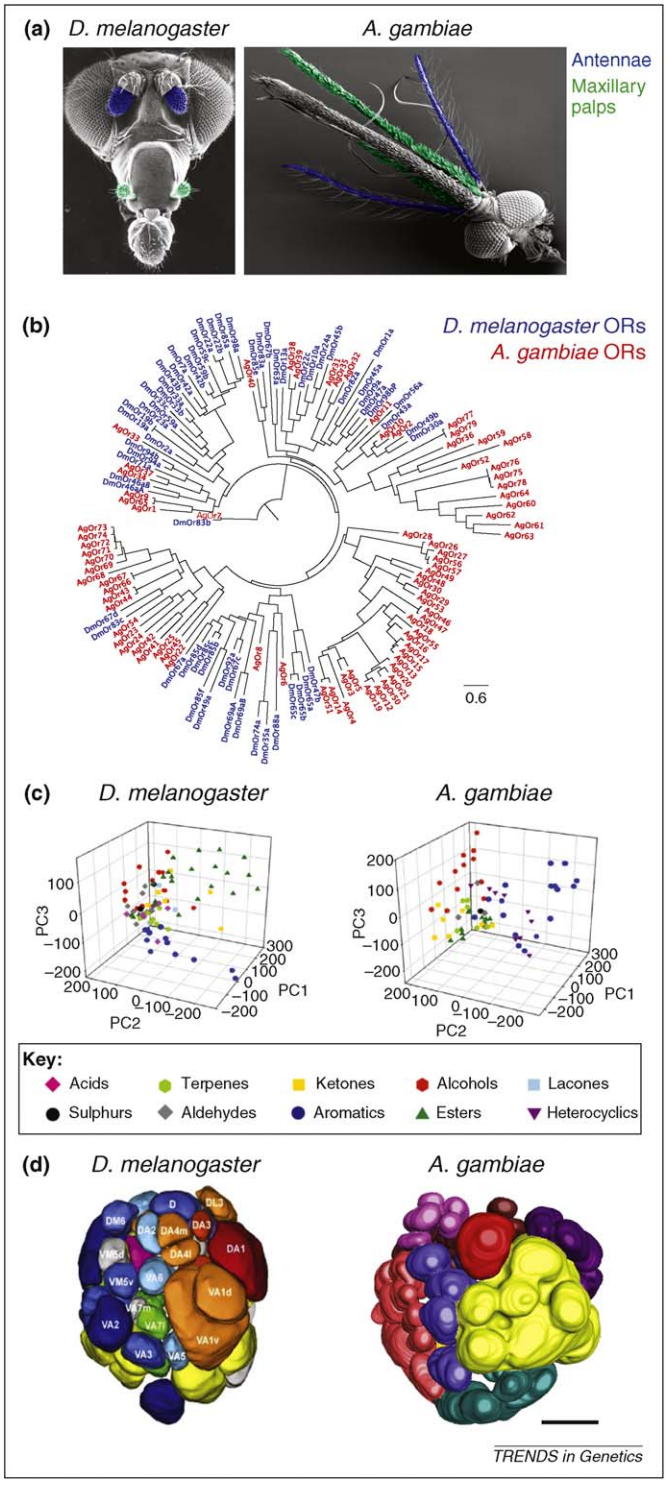

Figure 2. Neuroanatomical, molecular, functional and wiring divergence in two insect olfactory systems. **(a)** Scanning electron micrographs of the peripheral olfactory organs of the fruit fly, *Drosophila melanogaster*, and the malaria mosquito, *Anopheles gambiae*. Antennae (blue) and maxillary palps (green) have similar locations but dramatically distinct morphologies. Modified from Refs [100,101], with permission. **(b)** Phylogenetic tree of odorant receptor (OR) repertoires in *D. melanogaster* (DmOr) and *A. gambiae* (AgOr) reveals an absence of orthologous sequences, except for the OR co-receptor DmOr83b/AgOr7. The tree was generated in RaxML and visualised in FigTree v1.1.2. The scale bar indicates the number of substitutions per site. **(c)** Representations of receptor activity-based odour spaces for *D. melanogaster* and *A. gambiae* OR repertoires, plotted using the first three components derived from principal component analysis, a dimensional reduction of the responses (spikes per second) of ORs presented with a panel of odorants containing a variety of functional groups (colour-coded). Pairs of odours that lie close to one another in this space elicit similar responses across the olfactory receptor repertoire whereas those with distinct response profiles are more distant. Esters (green triangles) and aromatics (blue circles) are more dispersed in the fruit fly and mosquito receptor spaces respectively, possibly reflecting corresponding distinctions in odour discriminability. These differences may be associated with the ecological importance of these chemical classes: esters are found in many fruits and several aromatics are present in human odours. Reproduced from Ref. [18], with permission. **(d)** Three dimensional graphical reconstructions of antennal lobes from *D. melanogaster* and *A. gambiae* illustrating the divergent number and morphology of olfactory glomeruli. For *D. melanogaster*, glomeruli are colour-coded according to sensillum type (blue, antennal basiconic; red or orange, trichoid; yellow, coeloconic; green, maxillary palp basiconic). For *A. gambiae*, glomeruli are colour-coded according to topographical position in the antennal lobe. Both are oriented with the suboesophageal ganglion located ventrally. Reproduced from Ref. [19] (*D. melanogaster*) and Ref. [20] (*A. gambiae*; scale bar, 25 μm) with permission.

studies in drosophilids. For example, *D. melanogaster* antennal basiconic 3A (ab3A) (Glossary) OSNs co-express the tandem duplicate genes *OR22a* and *OR22b* [30]. OR22a detects several fruit-derived esters, whereas OR22b does not respond to these odours, suggesting a change in ligand-binding properties. ab3A neurones in other drosophilids display significant species-specific responses to different esters, in marked contrast to the conserved tuning of many other OSN populations [31,32]. This response diversity might reflect the variable nature of the *OR22* gene cluster in drosophilids, which is represented by just a single gene in several species but six (including two pseudogenes) in *D. ananassae* [29,33]. Although it is unknown whether all of these *OR22* genes are co-expressed in ab3A neurones as in *D. melanogaster*, the intriguing correlation between receptor and functional diversity suggests that OR22 receptors are in the first stages of divergence. Receptor diversity might also underlie specific olfactory preferences of extant drosophilids. *D. sechellia* ab3A neurones are several orders of magnitude more sensitive than their *D. melanogaster* counterparts to the volatile methyl hexanoate of noni fruit, and this could partly explain the increased attractivity of this food substrate for *D. sechellia* [34]. It is unknown, however, to what extent this heightened physiological sensitivity is due to perireceptor olfactory proteins, OR expression levels in ab3A neurones, or by the nine residues that distinguish *OR22a* orthologues in these species [34].

How many amino acid substitutions are required to alter a receptor's specificity? This question cannot yet be answered in insects, whose ORs have very diverse protein sequences that are unrelated to G protein-coupled receptors (GPCRs) [35], and for which little is known about odour recognition. However, a recent modelling approach that integrated OR odour–response profiles [14], odour-ligand structures and comparative OR sequence analysis identified a number of candidate 'specificity-determining residues' [36]. These findings hint at the existence of a binding pocket within the extracellular halves of the transmembrane domains, consistent with a recent experimental study [37]. Other studies on the molecular evolution of insect ORs have identified a number of sites within specific receptor sequences under positive selection that potentially contribute to their functional diversification in different species [29,38,39]. These bioinformatic data might provide a useful foundation for future experimental work.

More is known concerning the ligand-binding properties of vertebrate GPCR-family ORs, where several cross-species evolutionary analyses and computational models

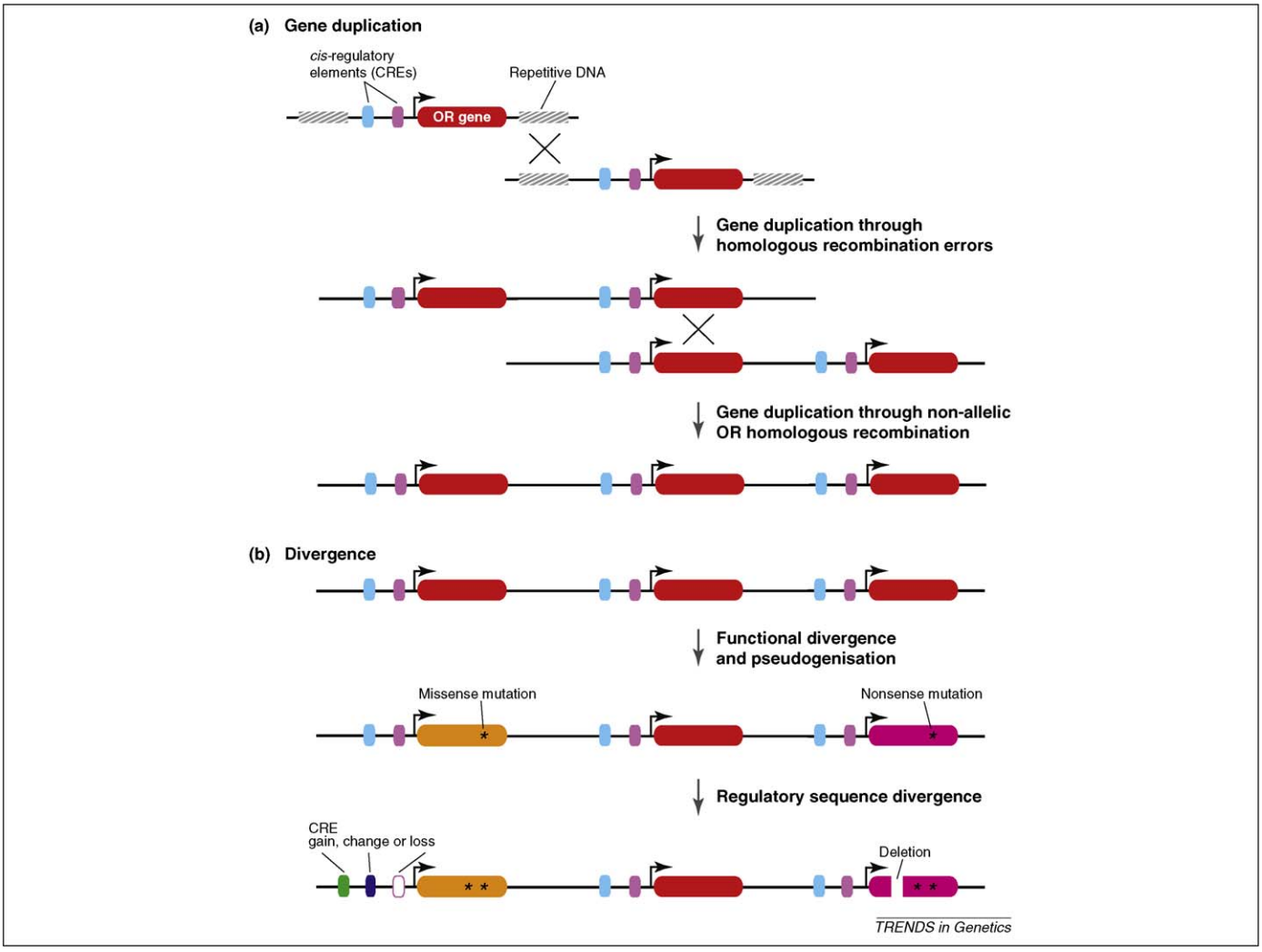

Figure 3. Expanding and diversifying OR gene repertoires. **(a)** A schematic model illustrating the genetic steps involved in the duplication of OR genes. A single odorant receptor (OR) gene (red box) whose expression is controlled by specific *cis*-regulatory elements (CREs) could initially be duplicated through recombination errors due, for example, to misalignment of flanking repetitive DNA sequences. The resulting tandem array of OR genes might then further expand through non-allelic homologous recombination. **(b)** Concomitant with duplication events, OR genes can undergo diversification. Missense mutations can change receptor function and nonsense mutations result in OR pseudogenisation. CRE divergence can change the expression of receptors. The acquisition of distinct transcriptional control sequences could therefore lead to discrete expression patterns of duplicated and divergent OR genes.

of OR structures (using the bovine rhodopsin X-ray crystal structure as a template) have defined an odour-binding cavity formed by residues in four different transmembrane domains [40]. Site-directed mutagenesis and functional analyses of this binding pocket in the human citronellic terpenoid receptors, OR1A1 and OR1A2 [41], and the mouse eugenol receptor, mOR-EG [42], have validated and extended these predictions, revealing that odours are bound mainly through hydrophobic and van der Waals interactions rather than via ionic or hydrogen bonds. These properties account for the lower affinity of ORs for their ligands than other GPCRs, as well as their broad but selective tuning properties. Importantly, rational design of mOR-EG revealed that single point mutations could predictably change ligand affinity, typically altering the EC50 value a few-fold [42]. This is an encouraging start, but more analysis is clearly required to understand how many natural mutations are required to more profoundly transform the specificity of an olfactory receptor.

Segregating OR expression

The expression of a single OR type in individual OSNs seems hard to reconcile with the dynamic nature of OR gene evolution [43]. How does a new OR acquire a unique expression pattern from both its parental ancestor and other existing OR genes? An answer to this question will require detailed knowledge of the developmental mechanisms specifying receptor expression [11]. Some principles are emerging in *D. melanogaster*: transgenic analyses have demonstrated that relatively short DNA sequences (a few hundred to a few thousand base pairs) are often sufficient to recapitulate endogenous receptor gene expression [44]. Bioinformatic screens for conserved sequences in putative orthologous drosophilid OR gene promoters have identified a number of novel DNA sequence motifs, or *cis*-regulatory elements (CREs), which are present in various combinations upstream (or occasionally downstream) of different OR coding sequences [44,45]. At least some of these motifs are necessary and partially sufficient to recapitulate OR gene expression, and these represent candidate binding

sites for transcription factors [45]. Thus, OR genes might acquire unique expression patterns through a purely deterministic mechanism, whereby a specific combination of modular CREs in their promoters are activated (or repressed) by transcription factors expressed in developing OSNs [11,44–46]. Mammalian OR gene expression might also rely on deterministic mechanisms involving combinations of short-range CREs and sequence-specific transcription factors [11]. Several additional regulatory mechanisms are important, including long-range locus control regions [47–49], and negative-feedback regulation [49,50]. For the generally much larger OR repertoires in mammals these mechanisms might simplify the process of ensuring that a single OR is expressed in each OSN.

The relatively short nature of many promoters in both *D. melanogaster* and mammalian OR genes means that new loci arising through gene duplication might also contain most or all of the CREs of its parent (Figure 3a). Indeed, this feature probably accounts for why ORs in tandem arrays are co-expressed [19,30,51], although there are other potential mechanisms, such as dicistronic transcripts [44]. Conserved CREs might even be maintained when the genes become cytologically separated, as potentially illustrated by the duplicate and co-expressed *D. melanogaster OR49a* and *OR85f* genes [19]. How do co-expressed genes ultimately segregate to distinct OSN populations? One possibility is that these loci undergo sub-functionalisation of their expression patterns by the complementary loss of CREs derived from the parental locus. This would result in the transcriptional activation of each gene in a subset of the original pattern. The modular and combinatorial nature of CREs makes this mechanistically plausible. Because OR genes only have a finite number of CREs, new elements must also presumably be generated (Figure 3b). Several mechanisms are known for CRE evolution [52]. Most transcription factors have relatively short recognition sequences, opening the possibility that new binding sites arise *de novo* by random mutation. Alternatively, new motifs might be acquired when OR genes move to other genomic locations. How likely is it that transposed OR genes acquire new expression patterns? This is difficult to answer because non-expressed loci might rapidly be pseudogenised and lost from the genome, leaving only functional, expressed genes in extant genomes. However, systematic analysis of mammalian OR gene expression across multiple tissues has revealed widespread, locus-dependent and heterogeneous 'ectopic' expression of ORs outside the olfactory system [53]. These observations indicate that OR basal promoters might easily become sensitive to enhancers in their genomic environment, making it possible for new regulatory motifs that promote expression in OSNs to be recruited as the repertoire expands.

Making room for new ORs

New ORs must ultimately be housed in new OSNs, requiring the modification of olfactory organ developmental programmes. In *D. melanogaster*, OSNs are organised into sensilla that comprise clusters of 1–4 neurones whose dendrites innervate porous hairs on the antennal/maxillary palp surfaces and whose cell bodies are surrounded by glia-like support cells [54]. Both neuronal and non-neuronal cells arise from divisions of sensory organ precursors (SOPs) that are specified in the antennal imaginal disc. Individual sensilla develop under precise genetic control resulting in an almost completely stereotypical number of OSNs of defined OR identity in a characteristic position on the antennal surface [19,55,56]. Developmental decisions in these lineages are controlled by a hierarchy of transcription factors. At the top of the cascade are the proneural basic helix–loop–helix proteins Atonal and Amos, which specify morphologically distinct classes of sensilla: coeloconic, or basiconic and trichoid, respectively [54]. Closer to the bottom are factors such as the POU-domain proteins Acj6 and Pdm3, which control the expression of specific subsets of OR genes [46,57,58]. In addition to cell-autonomous lineage specification mechanisms, cell–cell interactions mediated by Notch signalling contribute to the diversification of sibling OSNs within individual sensilla [59].

Although populations of OSNs expressing the same receptor are generally believed to be genetically homogeneous – that is, with identical gene expression profiles – this might not necessarily be the case. Several OR promoter reporters only incompletely recapitulate endogenous OR expression, presumably reflecting a lack of all necessary regulatory elements [19,30,56]. Importantly, the activity of these promoter elements in only a subset of the neurones that normally all express the same OR argues that these OSNs are not identical in their complement and/or levels of gene regulatory factors. Such OSN heterogeneity might result from asynchronous and spatially dispersed development of individual OR lineages from SOPs across the antennal disc [54].

The diversity in the cellular (neurone number) and molecular (transcription factor and OR gene expression) properties – and perhaps 'hidden' heterogeneity – of different sensilla that originate from a common antennal primordium suggests that minor changes to the genetic programme might easily create or remove OSNs lineages. Although a SOP can give rise to up to four OSNs, most sensilla contain only two or three neurones due to programmed cell death [60]. It is plausible that minor modifications to the gene regulatory network of specific sensilla could prevent neuronal death, giving rise to a new OSN population capable of housing a new OR gene. In addition, increases in the number of different types of sensilla might be achieved by single mutations: loss of the microRNA *miR-9a*, for example, results in the formation of additional SOPs in several peripheral sensory tissues (olfactory organs were not examined in this study) through reduced suppression of Senseless, a key transcriptional regulator of SOP specification [61]. Is there evidence for developmental diversity in sensilla patterning? Although fruit fly and mosquito olfactory organs clearly have dramatically different morphologies and sensilla numbers (Figure 2a), more subtle distinctions have also been observed in individual basiconic sensilla lineages of drosophilids. In *D. sechellia* the proportion of ab3 sensilla is increased at the expense of ab1 and ab2. Importantly, because the ab3A OR22a-expressing neurone is sensitive to an attractive noni odour, this specialisation could represent an adaptation to noni fruit detection [32,34].

Vertebrate olfactory system development might be even more flexible in accommodating new OR genes into new neurones because, in contrast to insects (and most of the vertebrate brain), neurogenesis continues in the adult olfactory system [62]. Nevertheless, some common developmental themes might exist in invertebrate and vertebrates, such as the role of proneural genes and microRNAs in regulating olfactory neurogenesis [63,64].

Emerging olfactory circuits

The generation of new olfactory circuits in some cases might be necessary for the meaningful utilisation of novel olfactory information. The one-to-one correspondence between individual ORs and glomerular targets within the antennal lobe indicates that new glomeruli are created during OR repertoire expansion. On a broad anatomical level, in both insects and vertebrates, the major classes of OSNs (defined by sensilla type in insects [55] and by phylogenetic relationships in vertebrates [65]) innervate physically segregated regions of the antennal lobe and olfactory bulb respectively [19,66]. These observations suggest the existence of lineage constraints on OSN projection patterns, and might correlate with coarse odotopic maps [67,68]. Within individual domains, OR genes with highest sequence similarity tend to be expressed in OSNs that project to neighbouring glomeruli [19,69]. These observations suggest a model of olfactory circuit evolution by 'budding': new glomeruli arise through the segregation of axon termini within a parental glomerulus.

What molecular mechanisms might drive this process? Screens in *D. melanogaster* have identified a number of developmental genes involved in OSN axon targeting [54,70], and these represent potential loci whose expression or functional modification could contribute to the formation of new glomeruli. Some of these are transcription factors, such as Pdm3 [57] and Acj6 [71], consistent with the existence of lineage constraints on axon targeting. Notably, the requirement for these factors in both OR expression and OSN axon guidance suggests that these processes are developmentally coupled, opening the possibility for coordinated evolution. Many other factors required for glomerular targeting are broadly expressed neural guidance receptors and ligands (e.g. Robo/Slit, Plexin/Semaphorin, N-Cadherin and Dscam1) or their downstream signalling components (e.g. Dock, Pak) [54,70]. These factors are thought to act in a hierarchical manner to direct OSNs axons to glomerular target regions with ever-increasing precision. For example, *Dscam1* mutant OSNs have abnormal projections that can form ectopic 'glomeruli' both within and outside the antennal lobe [72]. By contrast, *N-cadherin* mutant neurones project normally but their axons fail to form mature glomeruli [73]. In the context of a 'budding' model for new glomerulus formation, genes that control the later steps in this process, such as the convergence of neurones into discrete compact glomerular structures, might well be important for evolutionary adaptation. At present we can only speculate on how this occurs. Putative CREs of OR genes are also found upstream of genes encoding guidance molecules [45]. This suggests that neuronal populations containing a lineage-specific combination of transcription factors that induce expression of a particular OR might coordinately express a unique combination (or level) of guidance factors, leading to unique targeting properties. Classical loss-of-function genetics, however, would probably be insufficient to identify the relevant loci underlying relatively subtle evolutionary adaptations. Therefore, to understand how one glomerulus splits into two, it might be more fruitful to use single-cell gene profiling of OSNs that express recently duplicated receptors and now project to neighbouring glomeruli (e.g. *Or98a/Or98b* [19]).

Genetic studies of vertebrate OSN targeting have also identified several important transcription factors and axon-guidance molecules [13,74]. However, central to this process in these species is a beautifully simple mechanism to couple the evolution of new ORs and glomeruli by employing OR proteins in axon guidance [75]. ORs are thought to act by regulating direct or indirect homotypic interactions between like OSNs [76,77] and/or controlling neurone-specific expression levels of other transmembrane guidance receptors (e.g. Ephrins) through OR-dependent cyclic AMP signals [78–80]. The demonstration that single amino acid changes in ORs, whether engineered or representing naturally occurring polymorphisms, are sufficient to partially or completely segregate axon termini in glomeruli [77] is a compelling demonstration of the mechanistic simplicity by which new glomerular circuits arise.

Conceptualising the integration of novel glomeruli into circuits beyond the antennal lobe is currently difficult. Developmental genetic analyses in *D. melanogaster* have identified transcription factors, guidance molecules and microRNA-processing machinery involved in projection neurone development [54,70,81,82], and we extract from these studies three observations that might be generally relevant to how these circuits incorporate new antenna lobe projections. First, many molecules have roles in both projection neurone and OSN targeting, such as Acj6, N-Cadherin, Dscam1 and Semaphorin, indicating that novel innervation patterns of these populations could be genetically coupled. This might be particular important for matching new innervations of projection neurones to new populations of OSNs because projection neurones pattern the antennal lobe first [70]. Second, many (but not all [83]) mutations affecting projection neurone dendrite targeting to glomeruli also affect their stereotyped axonal projections to higher olfactory centres [84,85]. Thus, adaptive genetic changes leading to innervation of new glomeruli by projection neurone dendrites could also be accompanied by changes in synaptic partners of third order neurones. Finally, some projection neurones have multiglomerular innervations [16,86], perhaps reflecting antennal lobe output pathways that can easily 'capture' new sensory innervations. This is even more plausible in vertebrates where the pruning of otherwise broad innervations accompanies glomerular circuit maturation [87].

Evolving behaviour: capacitance and switching

How changes in wiring result in changes in olfactory behaviours requires consideration of the different types of circuitry subserving olfactory behaviours and their receptivity to the types of evolutionary changes described. Substantial evidence suggests that olfactory processing

might be combinatorial in nature, a mechanism that is thought to confer massive discriminatory power [15]. Many individual odorants or behaviourally-important odour blends are detected by combinations of receptors [14,88]. For these stimuli, genetic changes resulting in loss of one receptor gene or modifications to one glomerular structure would probably have little impact on perception and behaviour, a proposition supported by the absent or minimal behavioural phenotypes of several single *OR* gene mutants in *D. melanogaster* [89,90]. Redundant coding therefore might buffer important behaviours against an accumulation of genetic and wiring variation [16,91,92]. By comparison, if genetic changes result in a selective advantage (e.g. detection of a new odour stimulus guiding an animal to an unexplored food source), they could rapidly spread throughout a population.

In contrast to combinatorial coding, many of the best-characterised olfactory behaviours are those triggered by single chemical stimuli that activate 'labelled line' circuits to induce a stereotyped, innate response. In *D. melanogaster*, this coding strategy has been observed for sex, aggression and alarm pheromones [3,93,94]. Labelled-line and combinatorial coding might represent opposite ends of a continuous spectrum. Intermediate odour processing mechanisms have recently been highlighted through analysis of *D. melanogaster*'s innate behavioural responses to a complex volatile blend, apple-cider vinegar, and these are largely ascribed to individual glomerular circuits amongst the six physiologically activated by this stimulus [95].

Although narrowly tuned olfactory circuits coupled to robust innate behavioural responses at first appear resistant to evolutionary change, these circuits might actually provide a mechanism by which olfactory behaviours can be dramatically and relatively easily re-programmed. Switched circuit connections at any one of several different synapses along these labelled lines (or switches in OR expression) could conceivably modify responses to odour stimuli. Indeed, the expansion and divergence of peripheral sensory inputs as well as changes in OR expression might not necessarily be coupled with evolution of new central circuitry, but rather feed into pre-existing pathways already wired to particular behavioural outputs. A recent genetic study offers a potential insight into how the opposing behavioural responses of fruit flies and mosquitoes to CO_2 could have arisen through changes in existing circuitry [96] (Figure 4). In *D. melanogaster*, CO_2-sensing OSNs expressing the receptor pair GR21a and GR63a are

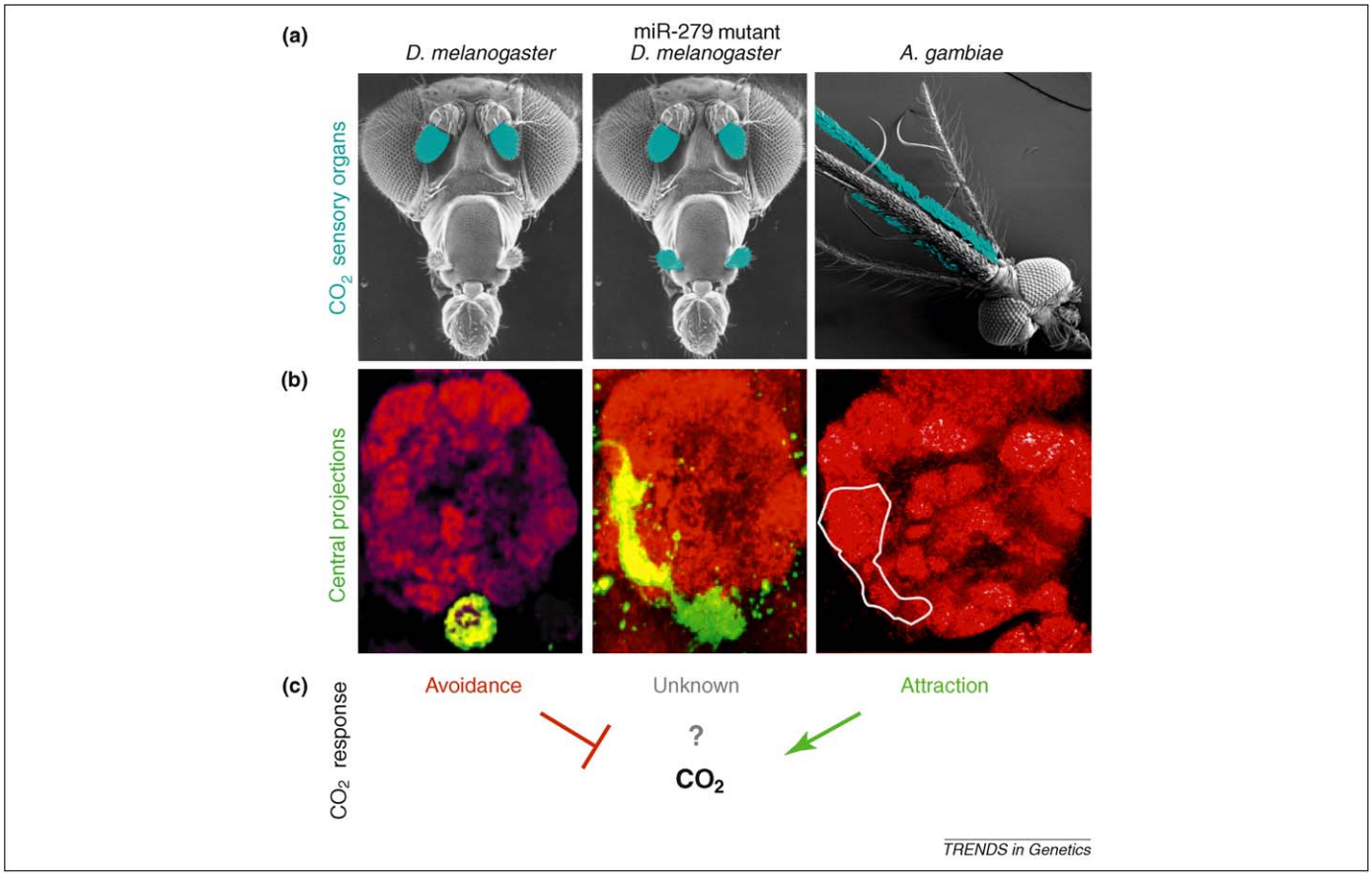

Figure 4. A potential evolutionary intermediate between CO_2-sensing circuits in the fruit fly and mosquito. **(a)** Scanning electron micrographs schematising the distinct appendages housing CO_2 sensory receptors (cyan) of the fruit fly *D. melanogaster*, the malaria mosquito, *A. gambiae*, and a *D. melanogaster* mutant for the microRNA *miR-279*. Whereas CO_2 sensory receptors are expressed in only the antennae of *D. melanogaster* and only the maxillary palps of *A. gambiae*, *miR-279* mutant *D. melanogaster* exhibit ectopic CO_2-receptor expression in the maxillary palps. Modified from Refs [100,101], with permission. **(b)** Ectopic CO_2-receptor-expressing sensory neurones in *miR-279* mutants project to both the ventral V glomerulus and medial glomeruli (green, or yellow in overlay with red) of the antennal lobe (red), representing an intermediate developmental phenotype of the wild type *D. melanogaster* V glomerulus projection and the maxillary palp medial projections in *A. gambiae* (white outline; the specific glomeruli innervated by CO_2-sensing neurones within this group of glomeruli have not been identified). Modified with permission from Refs [20,96]. **(c)** These differences in antennal lobe projections could in part explain the opposing behavioural valence of the fruit fly and mosquito away from and towards CO_2, respectively. Behavioural responses to CO_2 have not been examined in *miR-279* mutant flies.

found in the antenna and project to a single ventral 'V' glomerulus [3,97,98]. By contrast, *A. gambiae* CO_2 neurones, expressing orthologous receptor genes, are located in their maxillary palps whose axons are thought to project medially within the antennal lobe [20,99,100]. A forward genetic screen in *D. melanogaster* identified a loss-of-function microRNA mutant, *miR-279*, that exhibits ectopic expression of GR21a and GR63a in maxillary palp OSNs (in part through upregulation of the transcription factor Nerfin-1) [96]. These ectopic GR21a/GR63a neurones respond physiologically to CO_2, but also express other ORs and display hybrid axonal projections to both V and medial glomeruli. Although behavioural responses to CO_2 were not studied in these animals, the intriguing cellular phenotypes of this mutant might represent an evolutionary 'intermediate' between *D. melanogaster* and *A. gambiae* CO_2 olfactory circuits. They also highlight how single mutations in gene-regulatory molecules can coordinate changes in both receptor expression and OSN wiring.

Concluding remarks and future perspectives

Olfactory circuits, characterised by unparalleled diversity and species specificity, provide an excellent model for exploring the fundamental problem of how animal brains evolve. We have outlined recent data suggestive of mechanisms whereby new olfactory circuits and behaviours might appear but resolution of this question will depend on important advances in several areas, in particular neural development. Understanding how complex neural circuits are established is likely to provide crucial insights into brain evolution just as decades of research on the development of animal form is bearing fruit for our understanding of the evolution of morphological diversity [52]. Moreover, continued comparative studies on the olfactory systems of closely related species will be essential to identify the perhaps subtle but important distinctions that mark the birth of new olfactory genes, circuits and behaviours.

Acknowledgements

We are grateful to Hugh Robertson for providing insect OR sequences, Vincent Croset for generating the phylogenetic tree and Allison Carey, John Carlson, Africa Couto, Barry Dickson, Rickard Ignell, Larry Zwiebel, Pelin Cayirlioglu Volkan and Larry Zipursky for providing images. We thank Mattias Alenius, Henrik Kaessmann, Gaby Maimon, Sophie Martin, Marcus Stensmyr and members of the Benton laboratory for comments on the manuscript. We apologise to colleagues for being unable to cite all relevant primary literature due to space constraints. P.R. is supported by a Human Frontier Science Program Long-Term Fellowship. Research in R.B.'s laboratory is supported by the University of Lausanne, a European Research Council Starting Independent Researcher Grant and the Swiss National Science Foundation.

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