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Development of wiring specificity in the olfactory system

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The olfactory system discriminates a large number of odorants using precisely wired neural circuits. It offers an excellent opportunity to study mechanisms of neuronal wiring specificity at the single synapse level. Each olfactory receptor neuron typically expresses only one olfactory receptor from many receptor genes (1000 in mice). In mice, this striking singularity appears to be ensured by a negative feedback mechanism. Olfactory receptor neurons expressing the same receptor converge their axons to stereotypical positions with high precision, a feature that is conserved from insects to mammals. Several molecules have recently been identified that control this process, including olfactory receptors themselves in mice. The second order neurons, mitral cells in mammals and projection neurons in insects, have a similar degree of wiring specificity: studies in *Drosophila* suggest that projection neuron-intrinsic mechanisms regulate their precise dendritic targeting. Finally, recent studies have revealed interactions of different cell types during circuit assembly, including axon–axon interactions among olfactory receptor neurons and dendro–dendritic interactions of projection neurons, that are essential in establishing wiring specificity of the olfactory circuit.

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Introduction

Spatial representation of information is a common feature of the nervous system. Examples include topographical representation of sensory information in the somatosensory and visual systems. A prerequisite for such spatial representation is specific connectivity among neurons participating in the neuronal circuit that ensures correct relay of information. The olfactory system is a dramatic example of specific connectivity of the nervous system. The identification of olfactory receptors by Buck and Axel in 1991 [1], which led to their Nobel prize in 2004, has started a series of studies revealing the organization and striking wiring specificity of the olfactory system. The olfactory system has attracted researchers to use it as a model system to study the development of neuronal wiring specificity using molecular genetic approaches. Here, we review recent literature — published in the past two years — on advances in this field, focusing on genetic organisms, primarily the mouse and fly (Figure 1). For a more comprehensive review of the olfactory systems of other organisms, see, for example, Hildebrand and Shepherd [2].

Wiring specificity in the olfactory system

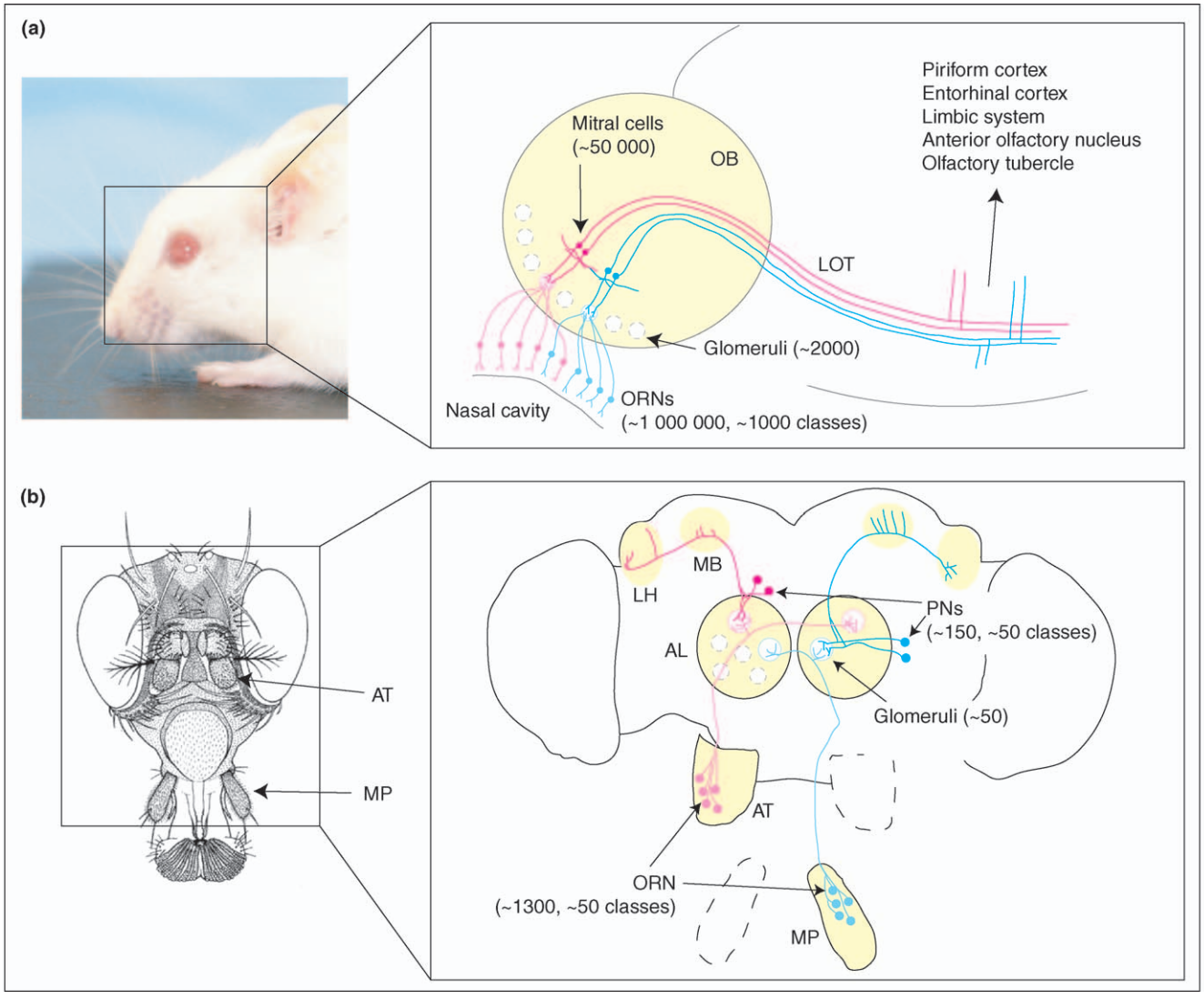
Information about chemical odorants is transformed into electrical activity when odorants bind to olfactory receptors (ORs) expressed on the dendrites of olfactory receptor neurons (ORNs). Mice have ~1000 functional OR genes in the genome, whereas flies have ~60. Each of these ORs is thought to be expressed mutually exclusively in ORNs (one neuron–one receptor rule), and ORNs expressing the same OR target their axons to single glomeruli in the mammalian olfactory bulb or the insect antennal lobe. Therefore, odorants that activate a set of ORs will activate a specific set of glomeruli, potentially enabling chemical information of odorants to be spatially represented in the brain. At the level of the glomeruli, ORN axons synapse with dendrites of the second order mitral cells or projection neurons. Axons of at least some second order neurons send olfactory information to stereotypical positions of higher olfactory centers in the brain. In the olfactory bulb and antennal lobe, there are also glial cells and local interneurons that could contribute to wiring specificity of the system, but relatively little is known about these cell types, thus we will not discuss these in detail.

One neuron–one receptor rule

The olfactory system is capable of identifying the quality and quantity of a large variety of odorants. For olfactory information to be represented in the brain in an interpretable fashion, it seems essential for specific odorants to activate specific sets of ORNs, thus activating specific sets of neurons in the brain. Therefore, OR expression in ORNs must be precisely regulated. Since the work of Buck and Axel [1], the olfactory system has been thought to deal with this problem in a remarkably simple manner: each ORN expresses only one OR [3–6].

How is this one neuron–one receptor rule achieved? Recent studies in mice have shed light on this matter. Previously, researchers entertained the idea that ORNs

Figure 1



Schematics of the olfactory systems of **(a)** the mouse and **(b)** the fly. Olfactory receptor neurons (ORNs) expressing the same receptor (shown in the same color) target their axons to the same glomerulus in the olfactory bulb (a) and antennal lobe (b). The dendrites of fly projection neurons (PNs) and the apical dendrites of mouse mitral cells also target to single glomeruli, and their axons project to specific parts of higher olfactory centers. Numbers in parentheses refer to numbers of neurons and glomeruli. Note the striking similarity of organizational principles between these two organisms despite large differences in cell numbers. Abbreviations: AL, antennal lobe; AT, 3rd antennal segment; LH, lateral horn; LOT, lateral olfactory tract; MB, calyx of the mushroom body; MP, maxillary palp; OB, olfactory bulb; ORN, olfactory receptor neuron; PN, projection neuron. Not shown in this figure are the local interneurons and glial cells in the OB or AL. The mouse head picture is courtesy of J Zhang. The fly head drawing is courtesy of Academic Press (taken with permission from Bryant [62]).

might undergo a somatic DNA rearrangement, analogous to regulation of immunoglobulin expression, allowing only one OR to be in an active locus for expression. However, two groups have successfully created mice cloned from nuclei of ORNs, and these mice recovered the diversity of OR expression [7,8]. The ORN genome, therefore, does not undergo an irreversible rearrangement. Rather, there appears to be a feedback mechanism that keeps other ORs from being expressed once a functional OR is chosen for stable expression. When an OR coding sequence is replaced with a reporter gene, the reporter gene is co-expressed with other ORs [9–12], indicating that expression of an OR normally prevents co-expression of others. The molecular nature of this feedback signal, however, remains unknown.

In addition to this feedback regulation that ensures singularity, the initial choice of which OR to express is not entirely stochastic. For more than a decade it has been believed that the mouse olfactory epithelium is divided into four discrete zones, and a given OR is expressed only in one of the four zones [3,4]. However, recent extensive

expression studies show that OR expression patterns do not divide the olfactory epithelium into discrete zones; rather, expression domains of ORs form a continuous gradient [13,14]. This suggests that ORN fate determinants are expressed in gradients, and that each OR is expressed at an optimal level of these determinants, resulting in unique expression domains.

In *Drosophila*, OR expression does not depend on negative feedback. When an OR gene is deleted, ORNs that usually express that OR lose responsiveness to all tested odorants [15,16], suggesting that there is no expression of a second functional OR in response to OR deletion. A study in *Drosophila* [17] shows that a group of ORNs in the maxillary palp expresses two ORs, both of which have been demonstrated to be functional by electrophysiological recording. Two groups have recently published papers describing a nearly complete OR–ORN mapping in *Drosophila* [18••,19••], and found a few more examples of OR co-expression. However, although *Drosophila* does not have a feedback mechanism, the one neuron–one receptor rule appears to be maintained in most cases in *Drosophila*. It remains to be seen how OR expression is precisely regulated, and whether feedback-independent mechanisms are evolutionarily conserved.

Olfactory receptor neuron axon targeting

ORNs expressing the same OR target their axons to the same glomeruli in the olfactory bulb and antennal lobe. This creates the challenging task of correlating OR expression with axon targeting specificity. In mice, this problem is ameliorated by the dual functions of ORs: ORs not only detect odors but also regulate axon targeting specificity. This has been demonstrated by experiments showing that ORN axon targeting specificity changes when an OR coding region is swapped with that of another OR [20,21]. A study with chimera proteins of two very closely related ORs shows that OR residues that contribute to axonal identity are scattered all over the protein [22••]. This finding argues against the possibility that the OR protein has 'axonal identity domains' that are physically separate from the domains that function as a receptor for odorants. It remains to be seen where and how ORs function directly to regulate ORN axon targeting. ORs could function directly in axon targeting by signaling in ORN axon growth cones, alternatively they could act indirectly, for example, through neuronal activity or transcriptional regulation. Recent studies lend support to the first model, that is, direct signaling in growth cones. Use of specific OR antibodies [23,24] and a GFP-fused OR [12] enabled visualization of OR localization not only in ORN dendrites but also in axon termini. This is a prerequisite for an extreme model in which ORs act as axon guidance receptors, which is not yet proven.

In contrast to the situation in mice, ORs do not affect axon targeting in *Drosophila* [15,25]. This difference between the mouse and the fly has an interesting evolutionary implication. When an organism acquires a new OR through gene duplication and subsequent mutations, the organism needs to evolve a mechanism to confer ORNs expressing the new OR with new axon targeting specificity. In mice, this task is much simpler, because a new OR by definition means new targeting specificity. Taken together with the feedback regulation of OR expression described above, this flexibility might have enabled mice to accumulate a large number of ORs relatively easily, eventually devoting now ~3% of the genome to encode OR genes.

Even in mice, ORs are not the sole determinant of ORN axon targeting. In most cases, when ORNs that normally express OR X are forced to express OR Y, their axons target novel glomeruli that are distinct from targets of endogenous X or Y ORNs [20,21]. These experiments indicate that there are other instructive forces in mice for ORN targeting. Perhaps these OR-independent mechanisms are evolutionarily conserved.

Indeed, several classical axon guidance molecules have been implicated in ORN axon targeting in various organisms. Ephrin-As are expressed at different levels in different ORN classes in mice, and are required for correct targeting of their axons [26]. Furthermore, an expression study in the moth revealed that ephrins and Eph receptors are differentially expressed in different ORN classes [27], raising the possibility that control of ORN axon targeting by Eph–Ephrin signaling might be conserved from insects to mice. The Robo receptors are also implicated in ORN axon targeting in both zebrafish [28] and flies [29]. Additionally, several Semaphorins have been implicated in different aspects of ORN targeting in mice. Semaphorin3F–Neuropilin2 signaling is required for restricting ORN axon termination to the glomerular layer, instead of it overshooting into the deeper layers of the olfactory bulb [30–32]. Moreover, Semaphorin3A–Neuropilin1 contributes to the broad organization of ORN axon targeting [33–35]. Semaphorin3A is expressed in a broad compartment of the olfactory bulb by glial cells, and Neuropilin1+ ORNs avoid this area. By contrast, the Sema3A–Neuropilin1 signaling appears to have a different function in chick ORN targeting: it prevents ORNs from prematurely entering, and subsequently overshooting, the olfactory bulb [36].

Recent genetic studies in *Drosophila* have identified several proteins that affect different aspects of ORN axon targeting. In addition to the Robo receptors mentioned above, the immunoglobulin (Ig)-superfamily cell-surface receptor Dscam (for Down syndrome cell adhesion molecule) is required in ORNs to regulate their axon targeting specificity [37]. Potential downstream signaling partners of Dscam, Dock and Pak, are also required for ORN axon targeting [38]. *Drosophila* N-cadherin plays a permissive

role in the formation or stabilization of the protoglomerulus [39]. Although these proteins are required for axon targeting of many, and perhaps all, ORN classes, the POU-domain transcription factor Acj6 (for abnormal chemosensory jump 6) is required for targeting of a specific subset of ORN classes, suggesting that it might participate as part of a transcription factor code that regulates ORN axon targeting [40••].

These studies are just the beginning of the 'molecule mining'. A deeper understanding of the logic of ORN axon targeting requires identifying other molecules and determining the context of cellular communications in which these molecules exert their functions (see the last section below).

Does neuronal activity affect ORN axon targeting? Previously, odor-induced neuronal activity was thought to play only a minor role in ORN axon targeting, because mice deficient for olfactory cyclic nucleotide gated channels (OCNC), the ORNs of which lack odor-induced activity, have only mild targeting defects [41,42]. However, a recent careful developmental study suggests that odor-induced activity-dependent mechanisms function to prune 'incorrect' targeting that ORNs make during early development [43]. In addition, when activity was blocked in a competitive environment either by specific expression of a potassium channel [44] or by mosaic analyses of OCNC using random X-chromosomal inactivation of OCNC [45], inactive ORNs were eventually eliminated. Therefore, neuronal activity also plays a permissive role in maintaining correct targeting, and ORNs devoid of activity in the presence of active ORNs are at a disadvantage. It is debatable whether spontaneous activity is sufficient for this permissive role. In summary, these studies suggest that neuronal activity does play a role in ensuring high precision of ORN axon targeting, but it might be secondary to activity-independent hardwiring mechanisms.

Wiring specificity of the second order neurons

In the olfactory bulb and antennal lobe, ORN axons form synapses with the second order mitral cells and projection neurons, respectively. Dendritic targeting of second order neurons has been best studied in *Drosophila*. Most projection neurons have uniglomerular targeting, exhibiting the same degree of specificity as ORN axons. Interestingly, the targeting specificity of a projection neuron is determined by lineage and birth order [46,47], suggesting specification independent of ORN axons. It has recently been demonstrated that targeting specificity of projection neurons is instructed by cell-autonomous functions of POU-domain transcription factors such as Acj6 and Drifter [48], further indicating that projection neurons have intrinsic targeting specificity.

Mitral cells and projection neurons send olfactory information through their axons to higher brain centers (Figure 1). Genetic tracing experiments in mice have provided evidence that mitral cell axons have stereotypical projections in the higher olfactory centers where olfactory information from the olfactory bulb both converges and diverges [49]. Similar anatomical studies of axon projections of second order neurons with a single cell resolution were performed in the fly [50–52]. These studies showed that projection neuron axons have stereotypical branching patterns and terminal areas according to the glomeruli that their dendrites innervate, suggesting that olfactory information might be spatially represented in the higher centers. Recently, expression of the immediate early gene c-Fos was used to monitor neuronal activity in response to different odorants in mice, providing a clue to understanding how olfactory information carried by mitral cell axons might be represented in the cortex [53,54].

Cellular communications during circuit assembly

Assembly of neuronal circuits relies on extensive cell–cell interactions. The precise wiring exemplified in the olfactory system relies on intrinsic properties of axons and dendrites and spatial cues in their environments. The olfactory system affords an excellent opportunity to study, with available genetic tools, how different cell types interact with each other to ensure correct wiring specificity.

Which cells instruct ORN axons to converge onto glomeruli in stereotypical locations? Accumulating evidence suggests that ORNs might talk among themselves. In mice, when ORNs were forced to express different artificial chimeric ORs, their axon targeting phenotypes were dependent on which other ORNs were present [22••]. One interpretation is that ORN axons monitor 'axonal identity' of neighboring axons, to make the decision of which axons to converge with. In *Drosophila*, mosaic analyses of the transcription factor Acj6 showed that it is cell-autonomously required for axon targeting of a subset of ORNs. In addition, in the presence of mistargeted *acj6* mutant ORNs, some wild type ORNs also exhibited targeting defects [40••]. Some ORN classes are dependent on others, but not vice versa, suggesting hierarchical interactions of ORN classes. It is possible that some ORN classes use other ORN classes as a guiding cue to navigate their way to the correct target. Thus, in both the fly and the mouse, direct axon–axon interactions are proposed to play a key role in ORN axon targeting. However, it is important to note that these observations do not preclude other alternative explanations: for example, 'axonal identity' could be monitored by the target area of ORN axons that sorts the axons into different glomeruli. Identification of cell surface molecules that directly mediate axon–axon interactions will be essential. In mice, ORs could play this direct role, but this needs to be tested by future studies.

The idea of axon–axon interactions has led some researchers to propose that ORNs might be autonomous,

and that the olfactory bulb has no intrinsic spatial information until instructed by ORN axons (for example, see [22••,55]). However, a detailed developmental study in *Drosophila* indicates that this is not the case, at least in the fly [56••]. In *Drosophila*, ORN axons do not reach the antennal lobe until ~18 h after puparium formation, and do not invade the lobe until later. Projection neuron dendrites, however, have already been targeted to appropriate locations in the developing antennal lobe before contact with ORNs, therefore excluding the possibility that the antennal lobe is devoid of spatial organization until ORN axons arrive [56••]. This prototypic organization of projection neuron dendrites appears to be mediated partially by interactions among projection neuron dendrites [56••,57], and perhaps also by interactions of projection neurons and other cell types. Taken together with the actions of intrinsic transcription factors controlling their targeting [40••], it indicates that projection neurons have intrinsic and autonomous information for dendrite targeting independent of ORN axons.

Do mammalian mitral cells have a similar degree of autonomy? When considering this question, it is important to note that the organization of second order neurons is significantly different between insects and mammals (Figure 1). Mammalian mitral cells send their apical dendrites to one of the nearest glomeruli, whereas in *Drosophila* there is no obvious correlation between the cell body position of a projection neuron and its glomerular target [46]. Therefore, insect projection neurons might require a more active dendritic targeting program than that present in mammalian mitral cells. This difference, however, does not preclude the need for mammalian mitral cells to be patterned independent of ORN axons. Different classes of mitral cells have stereotyped axonal projections [49]. Thus, mitral cells might require intrinsic wiring specificity to correlate the cell body position, and hence the glomerular class, with the axonal wiring specificity. It is difficult to imagine that ORNs can solely determine the wiring specificity of 1000 classes of mitral cell axons in higher olfactory centers through OR-dependent neuronal activity. In addition, even though convergence of like ORN axons might not require specific cell types in the target [58,59], self-organized ORN axons are likely to use spatial information in the olfactory bulb to decide the location of convergence. These lines of evidence lead us to suspect that mitral cells might possess intrinsic wiring specificity independent of ORNs.

In vitro studies show that ORN axon trajectory in olfactory epithelium explants is influenced by co-cultured olfactory bulbs [60], and mitral cell dendrites in bulb explants are attracted by co-cultured olfactory epithelia [61]. Future studies should identify the molecules mediating various cellular interactions, including those between ORNs, between second order neurons, and between ORNs and second order neurons. It will be interesting to determine to what extent targeting of ORNs and of second order neurons are dependent on each other, and the potential contribution of other cell types such as glia and local interneurons. The olfactory system offers an opportunity to pin down precisely the complex cellular communications required during circuit assembly.

Concluding remarks

The olfactory system is a dramatic example of wiring specificity in the nervous system. It presents a unique opportunity for precise genetic labeling and manipulations to test the requirement of specific cell types and molecules for correct wiring. Recent studies have revealed that interactions between ORN axons and those between projection neuron dendrites help to determine proper targeting. Future studies should elucidate the precise cellular and molecular mechanisms for establishing connections between first and second order neurons, and the development of cortical representations of sensory information. This system will continue to be an excellent model system to discover the principles governing the precise assembly of neuronal circuits, which form the basis for all brain functions.

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