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Roles of odorant receptors in projecting axons in the mouse olfactory system

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In the mouse olfactory epithelium, there are about ten million olfactory sensory neurons, each expressing a single type of odorant receptor out of ~1000. Olfactory sensory neurons expressing the same odorant receptor converge their axons to a specific set of glomeruli on the olfactory bulb. How odorant receptors play an instructive role in the projection of axons to the olfactory bulb has been one of the major issues of developmental neurobiology. Recent studies revealed previously overlooked roles of odorant receptor-derived cAMP signals in the axonal projection of olfactory sensory neurons; the levels of cAMP and neuronal activity appear to determine the expression levels of axon guidance/sorting molecules and thereby direct the axonal projection of olfactory sensory neurons. These findings provide new insights as to how peripheral inputs instruct neuronal circuit formation in the mammalian brain.

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Introduction

In the mouse, odorants are detected with ~1000 odorant receptors (ORs) expressed by olfactory sensory neurons (OSNs) [1–3]. ORs are G protein coupled receptors with seven-transmembrane domains and transduce odorant-binding signals to neuronal activity via cyclic-AMP (cAMP). The depolarization signals are transmitted to glomeruli in the olfactory bulb (OB) of the brain. There are two basic principles for organizing the peripheral olfactory system. One is the one neuron – one receptor rule: each OSN expresses only one functional OR gene in a mono-allelic manner, whereby establishing the neuronal identity of OSNs [4–8]. The other is the OR-instructed axonal projection: OSNs expressing the same OR converge their axons to a specific set of glomeruli in the OB [9–11]. Thus, the odor information detected in the olfactory epithelium (OE) by ~1000 different ORs are topographically represented by ~1000 pairs of glomeruli in each OB. This conversion of odor information to an odor map, consisting of a set of activated glomeruli that is specific for each odor, enables the mammalian brain to detect and discriminate various odorants. Remarkably, both basic rules mentioned above are maintained and regulated by the expressed OR protein itself. In this report, we will summarize the recent studies that have focused on the roles of ORs in the establishment of the glomerular map in the mouse.

Odorant receptor gene choice

The mammalian olfactory system is capable of detecting and discriminating a huge variety of airborne chemicals. To cover such a diverse array of odorants, ~1000 functional OR genes in the mouse, comprising ~4% of all protein-coding genes in the genome, are dedicated to olfaction [2]. To discriminate a variety of odorous information, the mammalian olfactory system takes a remarkable decoding strategy: the OE contains ~1000 types of OSNs, each expressing only one type of OR [5]. Furthermore, the OR gene is expressed from either maternal or paternal allele (allelic exclusion) [4,6], allowing the distinction of polymorphic allele differences. The monogenic and mono-allelic mode of OR gene expression is known as the 'one neuron – one receptor rule'. Because the exclusion is observed even among the same transgenes integrated in tandem on the same chromosome [12], the OR gene choice in mouse appears to be stochastic, in contrast to the genetically determined OR gene choice in the fly with only ~50 OR genes [13].

How is it, then, that a single OR gene is chosen and activated from a repertoire of 2000? Both positive and negative regulations appear to function for OR gene expression [14]. For positive regulation, a *cis*-acting enhancer '*H*' was proposed to activate one OR gene within the *MOR28* cluster by physically interacting with one particular OR gene promoter [7]. A recent study indicated that *H* may even activate in *trans* other OR gene loci on different chromosomes [15•]. However, more recent, targeted deletion of *H* in knockout mice contradicts the *trans*-acting enhancer model: *H* acts locally only in *cis*, but not in *trans* [16•,17•].

Possible involvement of negative feedback regulation in the maintenance of the one neuron – one receptor rule was demonstrated by the null or nonsense mutation of OR genes [7,8]. When the coding region of an OR gene was deleted, OSNs expressing the deletion allele were found

to co-express other members of OR genes. Similar results were obtained with naturally occurring frame-shift mutants of OR genes. However, the exact nature of the negative feedback signals is yet to be explored. Given that the genetic manipulation that uncouples ORs from G protein activation does not cause co-expression of multiple OR genes [18••], negative feedback may not require G protein – mediated signaling pathways. Targets of the feedback signals are also issues for future studies. Promoters of OR genes, enhancers of OR gene clusters, and/or protein factors binding them could be silenced by the OR-derived negative feedback signals.

Axonal convergence

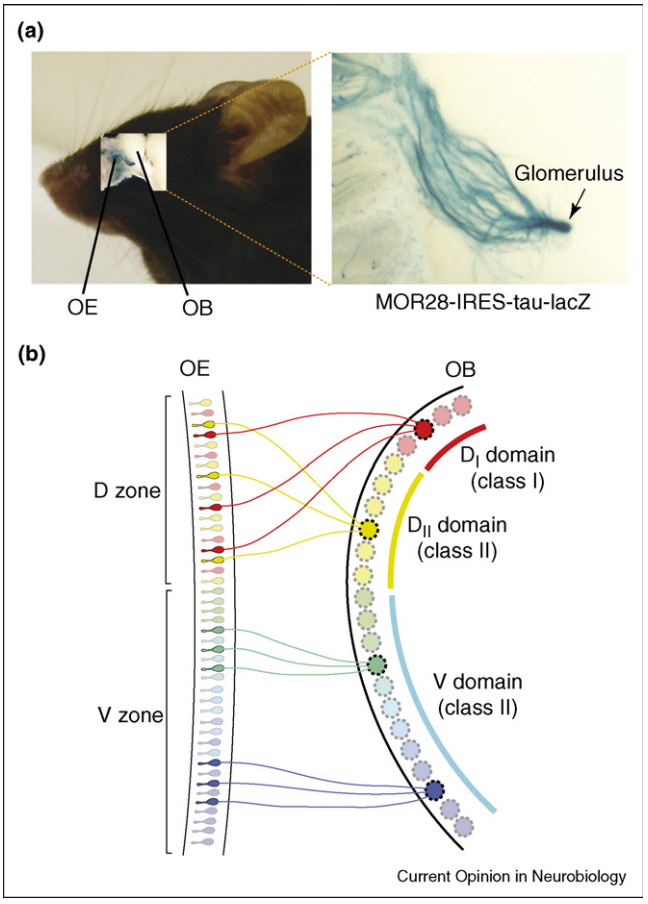
How is the odor information represented in the OB? *In situ* hybridization studies indicated that OSNs expressing the same OR converge their axons to specific sites on the OB; typically a pair of glomeruli per bulb [9,10]. These results were later confirmed with a genetic labeling method [11], which introduces an *IRES* (internal ribosome entry site) and an axonal marker gene, *tau-lacZ*, into the 3′ UTR of the *P2* OR gene. This technique has widely been used to visualize axonal projection for various OR genes (Figure 1a).

Because OSNs expressing the same OR are scattered in the OE, topographic reorganization must occur during the process of axonal projection to the OB. This contrasts axonal projection seen in other sensory systems, for example, visual, somatosensory, and auditory systems, where relative positional information is preserved between the periphery and the brain [19]. Since such reorganization could be the basis for the complex informational processing in the brain, axonal projection of OSNs may serve as an excellent model system to study the molecular basis of neuronal wiring in the brain.

Projection along the dorsoventral axis

What mechanisms could account for the striking axonal convergence of OSNs: OR-instructed glomerular positioning? One parameter used to define the axonal projection site is the spatial information in the OE. *In situ* hybridization demonstrated that each OR gene is expressed in a restricted area, or 'zone', in the OE [20,21]. Using zone-specific marker genes, the OE can be divided into the dorsomedial (D) and ventrolateral (V) zones. For example, the *O-MACS* and *NQO-1* genes are specifically expressed in the D zone of the OE [22,23], whereas the *OCAM* is expressed in the V zone [24]. Interestingly, phylogenetically distinct class I ORs are mostly expressed within the D zone [25,26•], although class II OR genes are expressed in both D and V zones [25,27•]. Within the D zone, class I-and class II-expressing cells are intermingled and there is no restricted distribution for both classes [26•]. In the V zone, however, each class II OR gene possesses its unique expression area, which is distributed in a continuous and overlapping manner along the dorsomedial–ventrolateral axis in the OE [27•,28].

Figure 1



Structure of the mouse peripheral olfactory system. **(a)** The transgenic mouse, *MOR28-IRES-tau-lacZ* [12], stained with X-gal (lateral view). OSNs expressing the *lacZ*-tagged *MOR28* gene project their axons to a specific site forming a glomerulus in the OB. **(b)** Spatial correlation between the OE and the OB in the mouse olfactory system. OSNs in the dorsomedial zone (D zone) in the OE project their axons to the dorsal domain of the OB. Class I ORs are mostly expressed by OSNs in the D zone in the OE, which target the anterodorsal cluster of the D domain in the OB. In the ventrolateral zone (V zone), each class II ORs possesses its own unique expression area, which is distributed in a continuous and overlapping manner along the dorsomedial–ventrolateral axis in the OE. The dorsomedial–ventrolateral expression area in the OE corresponds to the glomerular positioning along the D–V axis in the OB, thus, the D–V arrangement of glomeruli is roughly determined by the locations of OSNs in the OE. This positional information may be represented by the expression levels of guidance molecules, for example, neuropilin-2, that form gradients along the D–V axis.

It has been proposed that there is a spatial correlation between the OE and OB in the OSN projection. Antibody staining of zone-specific molecules demonstrated the 'zone-to-zone' correlation: OCAM-positive axons project to the ventral part of the OB (V domain), whereas

NQO-1-positive axons project to the dorsal part of the OB (D domain) [23,24]. Systematic *in situ* hybridization indicated that OSNs expressing class I ORs target their axons to the most anterodorsal areas within the D domain. Zone-to-zone projection along the D–V axis in the OB was also confirmed by dye tracing experiments [27•,29]. Good correlation was demonstrated between dorsal and ventral (D–V) positioning of glomeruli in the OB and dorsomedial–ventrolateral locations of OSNs in the OE, although no discernible correlation was found for anterior–posterior (A–P) positioning. These observations suggest that spatial information in the OE contributes to the D–V glomerular positioning in the OB (Figure 1b).

This notion was also supported by the analyses of some transgenic mice, in which the expression areas of particular ORs were genetically altered. When the expression areas of ORs were shifted or broadened, the projection sites were accordingly changed along the D–V axis in the OB [27•,30,31]. Neuropilin-2 (Nrp2) is a good candidate guidance molecule along the D–V axis, because it demonstrates a gradient of expression (dorsal-low and ventral-high) in the glomeruli in the OB [32,33]. However, the molecular logics that coordinate the zone-specific OR gene expression and axonal targeting are still unclear.

Projection along the anteroposterior axis

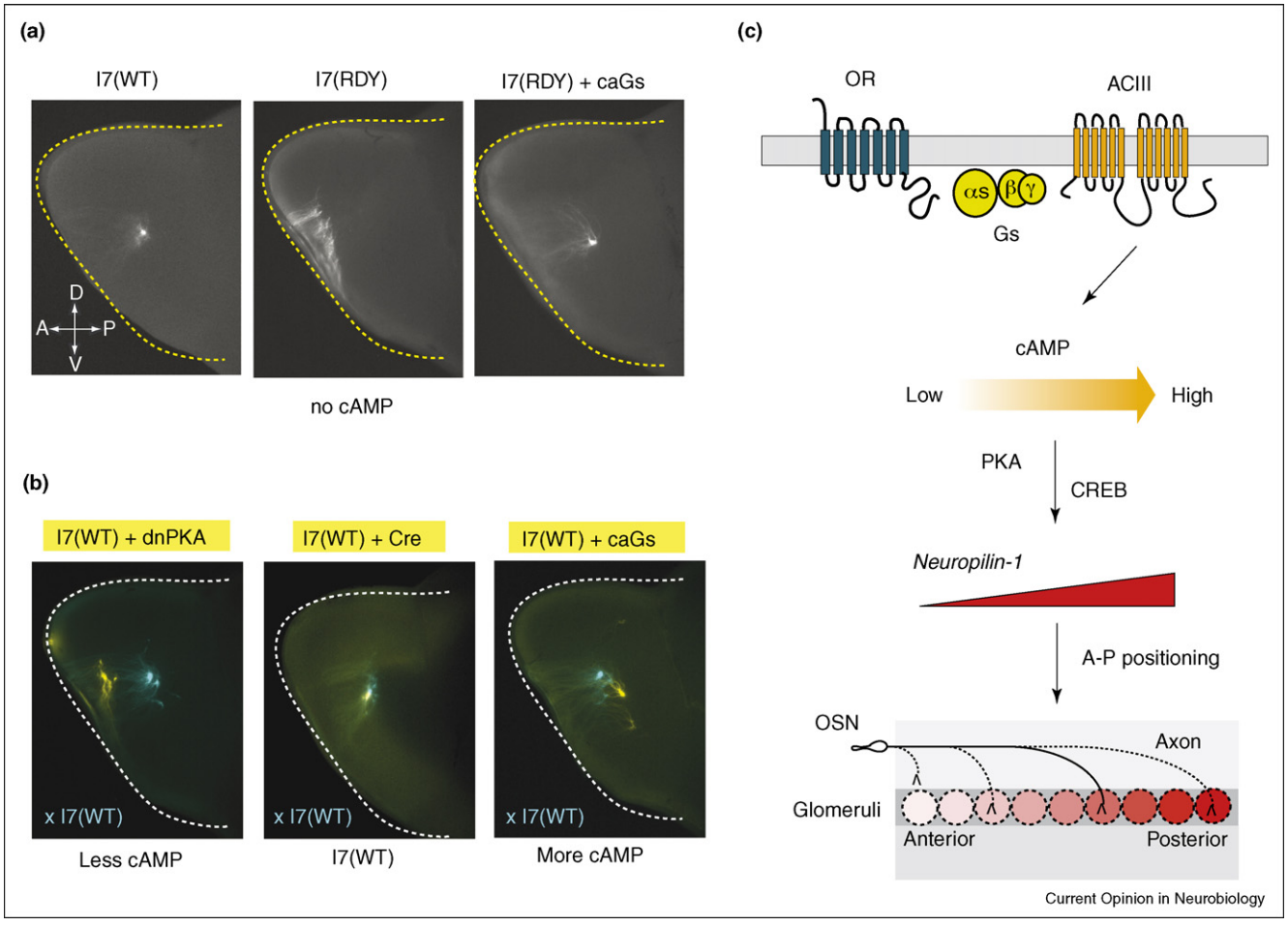
In contrast to the D–V arrangement of glomeruli, A–P positioning appears to be independent of the OE zone and more dependent on the expressed OR species. Possible involvement of OR protein in OSN projection was indicated by the coding-swap experiments of OR genes [11,34,35]. While it has been thought that OR molecules play an instructive role in forming the glomerular map, it has remained entirely unclear how this occurs at the molecular level [36]. It was also unclear if OR-derived signaling was involved during the process of OSN projection. In odor detection, binding of an odorant to an OR converts the olfactory-specific G protein, G_{olf}, from a GDP to a GTP-bound state. G_{olf} in turn activates adenylyl cyclase type III (ACIII), generating cAMP, which opens cyclic nucleotide gated (CNG) channels. The CNG channel, together with the chloride channels, induces the depolarization of membrane potentials. Targeted deletions of the G_{olf} and *CNGA2* genes cause severe anosmia. However, these knockouts do not demonstrate major defects in the initial process of glomerular map formation [37–39]. It was, therefore, assumed that OR-derived cAMP signals may not be needed for OSN projection.

Despite these observations, it was possible to assume that an alternate G protein mediates OR-instructed axonal projection. To examine this possibility, we generated a mutant OR, whose conserved Asp-Arg-Tyr (DRY) motif, which is essential for G protein coupling, was changed to RDY [18••]. Although null OR gene alleles, for example the coding deletion, allow secondary activation of other OR genes, the RDY mutant did not permit co-expression of other OR genes. Interestingly, axons of OSNs expressing the RDY mutant stayed in the anterior region of the OB, did not converge to a specific site, and failed to penetrate the glomerular layer in the OB (Figure 2a). However, co-expression of a constitutively active G_s rescued the defective wiring of the RDY mutant. Partial rescue of the RDY phenotype was also achieved either by the constitutively active PKA or CREB mutant. Thus, the cAMP-dependent transcriptional regulation appears to play major roles in establishing of the OR-instructed axonal projection [18••]. Although the functions of ORs at axon termini are yet to be clarified [40,41], it is possible that axonal cAMP/PKA may modulate growth cone navigation [42]. Our results are consistent with the previous observation that G_s-coupled β2 adrenergic receptor can instruct OSN projection, whereas a G_{i2}-coupled vomeronasal receptor V1rb2 cannot [43].

The ability to rescue of the RDY mutant by constitutively active G_s indicates that the receptor function of the OR is not required for the wiring specificity of OSN axons. How is it, then, that OR-derived cAMP signaling defines the wiring specificity? It was found that an increase in cAMP level by constitutively active G_s causes a posterior shift of glomeruli, whereas suppression of cAMP signals by dominant-negative PKA causes an anterior shift [18••] (Figure 2b). To screen for genes with expression levels correlated with cAMP signals, we performed single-cell microarray analysis of different transgenic OSNs, and identified a gene coding for Neuropilin-1 (Nrp1) [18••]. The *Nrp1* is expressed at elevated levels in the cAMP-high OSNs, and at low levels, if any, in the OSNs expressing the RDY mutant OR or dominant-negative PKA. Furthermore, the level of Nrp1, as a readout of cAMP signals, demonstrated an anterior-low/posterior-high gradient in the glomerular layer of the OB [18••]. Previous OR swapping experiments indicated that OR proteins may determine the projection sites along the A–P axis in the OB [11,34]. It was also found that expression levels of OR protein can affect OSN projection [43]. It is conceivable that different ORs generate different levels of cAMP, which in turn define the expression levels of axon guidance molecules (e.g. Nrp1), to determine the OR-specific projection sites (Figure 2c). Knockout of Sema3A, a repulsive ligand for Nrp1, alters the glomerular arrangement along the A–P axis [44,45], suggesting that Nrp1 is involved in the establishment of the A–P topography. Gain of function experiments with transgenic mice indicated that changes in the level of Nrp1 indeed affect the axonal projection of OSNs along the A–P axis (our unpublished data).

Knockout studies of ACIII also supported the involvement of cAMP signals in axonal projection of OSNs. A previous study demonstrated that the glomerular structure is severely disorganized in the mice deficient for

Figure 2



OR-instructed glomerular positioning [18••]. **(a)** OR-derived cAMP signals direct axonal targeting. A wild-type odorant receptor I7 navigated axons to a specific glomerulus in the OB. When the conserved Asp-Arg-Tyr (DRY) motif at the cytoplasmic end of transmembrane domain III was changed to RDY, G protein coupling was blocked, and accordingly, OSN axons failed to innervate the glomerular layer. Co-expression of a constitutively-active G_s mutant (caG_s) with the I7(RDY) mutant rescued the defective phenotype of I7(RDY) projection, although the glomerular position was not exactly the same as that for I7(WT). Axons were visualized with gap-ECFP or gap-EYFP fluorescence in the whole-mount medial OBs. **(b)** Changes in the cAMP signals affect glomerular positioning along the A–P axis. Co-expression of caG_s with I7(WT) caused a posterior shift of the target glomeruli. In contrast, co-expression of the dominant-negative mutant of PKA (dnPKA), which blocks cAMP signals, caused an anterior shift of glomeruli. **(c)** A model for the OR/cAMP-directed glomerular positioning along the A–P axis. We assume that each OR generates a unique level of cAMP signals, driven by its intrinsic activity at an earlier stage of OSN projection. The level of cAMP signals is converted to a relative expression level of axon guidance molecules (e.g. Neuropilin-1) via cAMP-dependent PKA and CREB. Neuropilin-1, together with other guidance molecules, navigates OSN axons along the A–P axis according to its expression level.

ACIII, a major adenylyl cyclase in OSNs [46]. Recent analyses of the ACIII-deficient mice demonstrated that Nrp1 expression is abolished in OSNs by the ACIII knockout and OR-specific glomerular map formation is perturbed along the A–P axis [47••,48•,49•]. Remarkably, two neighboring glomeruli for M71 and M72 no longer segregate into distinct glomeruli in the ACIII mutant mice [47••].

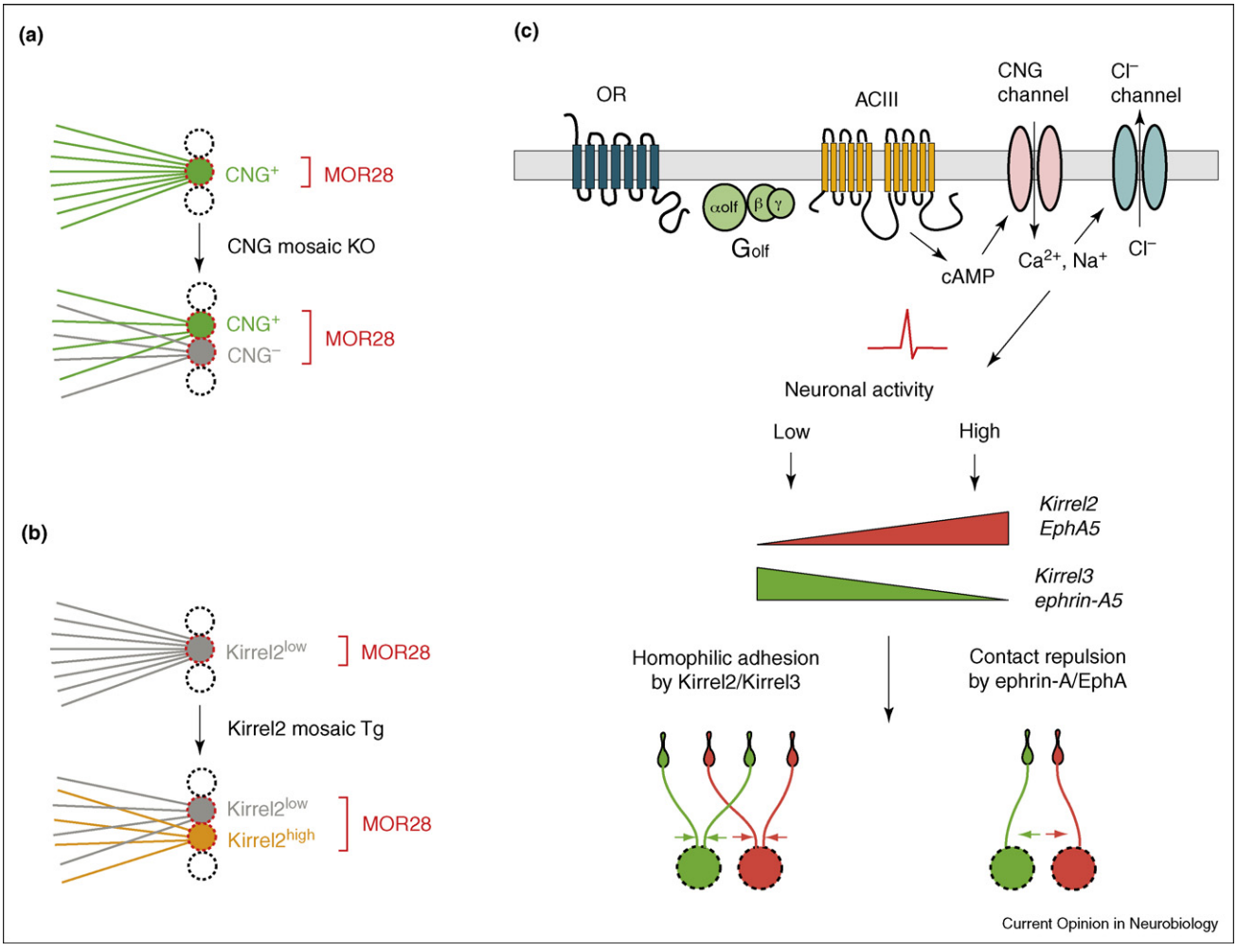
Axon sorting to form discrete glomeruli

It appears that a combination of D–V patterning, based on anatomical locations of OSNs, and A–P patterning, based on OR-derived cAMP signals, establishes a rough OB topography. After OSN axons reach their approximate destinations in the OB, further refinement of the glomerular map may occur through fasciculation and segregation of axon termini in an activity-dependent manner. The CNGA2-null mouse, which almost entirely lacks odor-evoked neuronal activity, demonstrates defects in axonal convergence for some ORs [39]. Segregation of glomeruli for the CNGA2-positive and negative OSNs has been reported in the mosaic knockout mice [39]. Genetic block of neuronal activity by the overexpression of the inward rectifying potassium channel, *Kir2.1*, severely affects

axonal convergence [50]. Developmental studies have shown that neighboring glomeruli are not well-separated before birth, and discrete glomeruli emerge only after the early neonatal period [51,52].

To study how OR-instructed axonal fasciculation is controlled, our group searched for genes whose expression profiles are correlated with the expressed ORs. Using the transgenic mouse in which the majority of OSNs express a particular OR, such genes were identified. These genes includes those that code for homophilic adhesive molecules Kirrel2/Kirrel3 and repulsive molecules ephrin-A5/EphA5 [53••]. In the *CNGA2* knockout mouse, *Kirrel2* and *EphA5* were downregulated, while *Kirrel3* and *ephrin-A5* were upregulated, indicating that these genes are transcribed in an activity-dependent manner. Heterozygous females of the X-linked *CNGA2* mutant mice generate CNG-positive and CNG-negative glomeruli even for the same OR (Figure 3a). Mosaic analysis demonstrated that gain of function of *Kirrel2*/*Kirrel3* genes also generates duplicated glomeruli (Figure 3b). It is possible that a specific set of adhesive/repulsive molecules, whose expression levels are determined by OR molecules, regulate the

Figure 3



OR-specific and activity-dependent axon sorting [53••]. **(a)** A mosaic analysis demonstrating the activity-dependency of axon sorting. Heterozygous females of the X-linked *CNGA2* mutant mice were used for the mosaic analysis, taking advantage of the random X chromosome inactivation. In these mice, CNG-positive and CNG-negative axons formed distinct, neighboring glomeruli even for the same OR (e.g. MOR28), suggesting that CNG-mediated neuronal activity has an instructive role in sorting axons. **(b)** A mosaic analysis demonstrating the Kirrel2-mediated axon sorting. Using the *H* enhancer and negative feedback by OR molecules, a transgenic system was devised, which generates an additional repertoire of OSNs expressing a particular gene of interest. In the Kirrel2 mosaic mice, a subset of MOR28-positive OSNs expressed Kirrel2 at an elevated level. In these mice, Kirrel2-low and Kirrel2-high MOR28 axons were segregated, forming separate glomeruli. **(c)** A model for the OR-specific and activity-dependent axon sorting to form a discrete map. We propose that different ORs generate different neuronal activities through the CNG channel, at a later stage of OSN projection. It appears that CNG-mediated neuronal activity upregulates *Kirrel2* and *EphA5* genes, and downregulates *Kirrel3* and *ephrin-A5*. We assume that homophilic cell adhesion molecules, for example, Kirrel2 and Kirrel3, induce homotypic axonal fasciculation, whereas ephrin-A5 and EphA5 may facilitate heterotypic axonal segregation through their contact-induced repulsive activities.

axonal fasciculation of OSNs during the process of glomerular map formation (Figure 3c).

In *Caenorhabditis elegans*, the Kirrel2/3 homolog SYG-1 plays a role in determining the location of specific HSNL synapses [54]. In our study, experiments using affinity probes *in situ* and on COS cells confirmed the homophilic, adhesive properties of Kirrel2 and Kirrel3 proteins [53••]. Homophilic interactions of these molecules at axon termini were also confirmed with the *H-Kirrel* transgenic mice, in which Kirrel2 or Kirrel3 was overexpressed in a mosaic manner. These observations indicate potential roles for Kirrel2 and Kirrel3 in segregating like axons via homophilic, adhesive interactions. In addition to *Kirrel2* and *Kirrel3* genes, several other genes are also transcribed in OSNs at various levels that correlate with the expressed OR species. Among them, the *ephrin-A/EphA* family genes are particularly interesting. In other tissues, ephrin-As and EphAs are known to interact with each other, causing repulsion of the interacting cells [19]. In the mouse olfactory system, expression of ephrin and Eph proteins has been analyzed with various antibodies [55]. It has been reported that OSNs expressing different ORs express different levels of ephrin-A proteins on their axons [56]. EphAs are also differentially expressed in different subsets of OSNs [53••]. Since ephrin-As and EphAs are expressed in a complementary manner in each subset of OSNs, the repulsive interaction between two different sets of axons, one that is ephrin-A^{high}/$EphA^{low}$ and the other that is ephrin-A^{low}/$EphA^{high}$, may be important in the segregation of OSN axons [53••].

It was demonstrated that the expression levels of OR-correlated cell-recognition molecules are affected by the *CNGA2* mutation in OSNs. Since the CNG channel converts OR activity to a change in membrane potential and calcium entry, an intriguing possibility is that the OR-mediated neuronal activity regulates the expression of cell recognition molecules (Figure 3c). OSNs may set the rate of neuronal activity, depending upon the expressed OR species. Neuronal activity and calcium influx can regulate the expression of a particular set of genes in other systems [57,58]. Similarly, in OSNs, neuronal activity, most likely set by the particular OR expressed, may also determine the expression pattern of cell-recognition molecules.

Refinement and maintenance of the map

Activity-dependent refinement, which follows the initial targeting processes, plays an important role in many sensory systems during development [59]. Activity inputs are also essential to maintain the neuronal map. In the mouse olfactory system, satellite glomeruli are ectopically formed in young animals. Such minor glomeruli are gradually and eventually eliminated with age, refining the glomerular map. In mice deficient for *CNGA2* or whose naris is surgically occluded, ectopic glomeruli are uneliminated or remain longer [31,39,60].

Taking advantage of the X-linked *CNGA2* mutant, it was demonstrated that neuronal activity is required for the maintenance of the glomerular map [61]. In the mosaic female mice, CNGA2-negative cells are eliminated in a competitive condition, but survive in a non-competitive condition without odors. The refinement and maintenance appears to be regulated at the level of cell survival, rather than axonal retraction/rewiring [31,50,60,61]. However, the exact molecular mechanisms for the selective elimination remains elusive.

Although higher-order olfactory circuits are beginning to be elucidated by recent pioneering studies [62,63••], the progress in wiring mechanisms is hampered by the lack of powerful genetic labeling/manipulation techniques. In *Drosophila*, wiring patterns of projection neurons, as well as OSNs, are genetically determined [64,65]. However, those of mice appear to be distinct and rely more on OR-derived inputs to instruct the OB circuitry [66]. Recently, it was reported that the odor-evoked neuronal activity is required for the maintenance of the precise intrabulber neuronal connections [67••]. Unlike in the visual system [68], no discernible critical period was found for the plasticity. How the higher-order circuits are organized and how information is extracted from the bulbar map in the olfactory cortex, are important issues for future studies.

Concluding remarks

Since the discovery of OR genes, it has remained entirely elusive how each OSN expresses only one OR gene, and how OSNs expressing the same OR converge their axons to a specific set of glomeruli [1]. In recent years, clear answers were provided to these problems. Singular OR gene choice appears to be ensured by the combination of a rate-limiting enhancer-promoter interaction and negative-feedback regulation by OR proteins [7,8,14]. For the OR-instructed axonal projection, OR molecules at axon termini have been assumed to recognize guidance cues on the OB and also mediate the homophilic interactions of like axons [34,35,40,41]. However, recent studies demonstrated that OR-instructed axonal projection is established by OR-derived cAMP signals. The levels of cAMP establish the A–P topography of axonal projection via cAMP-dependent PKA at an early stage of development [18••]. The neuronal activity generated by cAMP via CNG channels promotes the fasciculation of OSN axons at a later stage [53••].

For the D–V arrangement of glomeruli, locations of OSNs in the OE determine the target sites of OSN axons. This positional information may be represented by the expression levels of guidance molecules, for example Nrp2, forming a gradient along the D–V axis. Along the A–P axis, a different set of guidance molecules, for example, Nrp1, are involved, whose expression levels are correlated with the OR species via cAMP. After axons are guided to

approximate destinations in the OB, axon termini are further sorted based on the expressed OR species. It is conceivable that a unique combination of axon guidance/sorting molecules, whose expression levels are determined by OR molecules and neuronal activity, constitute the 'neuronal identity code', and contribute to the discrete glomerular map formation during the process of olfactory development in the mouse.

Recently, it has been suggested that the generation of the complex mammalian nervous system is controlled to a certain extent by peripheral inputs. The peripheral target-derived factors, for example, GDNF and BMP4, appear to control the transcriptional programs of the sensory-motor neuronal connections in the spinal cord and axonal projection of trigeminal sensory neurons, respectively [69,70]. The cone photoreceptor has been reported to have instructive roles in forming the neuronal circuit for trichromatic color vision [71••]. Although much remains to be investigated, the mammalian olfactory system will continue to provide insightful information to our understanding of the roles of peripheral inputs in the neuronal circuit formation in the brain.

Note added in proof

Recent knockout studies revealed the involvement of Slit-1 and Robo-2 in the OSN projection along the D-V axis [72]. In contrast to Nrp2, Robo-2 is expressed in a dorsomedial-high and ventrolateral-low gradient in the OE. Segregation of axonal projection sites for class I and class II ORs was clearly demonstrated with the zone-specific labeling/ablation mice in a recent literature [73].

Acknowledgement

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