Chem. Senses 27: 245–260, 2002

Axon Navigation in the Mammalian Primary Olfactory Pathway: Where to Next?

Brian Key[1,2] and James St John[1]

[1]Department of Anatomy and Developmental Biology, School of Biomedical Sciences and [2]Centre for Functional and Applied Genomics, University of Queensland, Brisbane 4072, Australia

Correspondence to be sent to: Brian Key, Department of Anatomy and Developmental Biology, School of Biomedical Sciences, University of Queensland, Brisbane 4072, Australia. e-mail: brian.key@uq.edu.au

Abstract

The process of establishing long-range neuronal connections can be divided into at least three discrete steps. First, axons need to be stimulated to grow and this growth must be towards appropriate targets. Second, after arriving at their target, axons need to be directed to their topographically appropriate position and in some cases, such as in cortical structures, they must grow radially to reach the correct laminar layer. Third, axons then arborize and form synaptic connections with only a defined subpopulation of potential post-synaptic partners. Attempts to understand these mechanisms in the visual system have been ongoing since pioneer studies in the 1940s highlighted the specificity of neuronal connections in the retino-tectal pathway. These classical systems-based approaches culminated in the 1990s with the discovery that Eph–ephrin repulsive interactions were involved in topographical mapping. In marked contrast, it was the cloning of the odorant receptor family that quickly led to a better understanding of axon targeting in the olfactory system. The last 10 years have seen the olfactory pathway rise in prominence as a model system for axon guidance. Once considered to be experimentally intractable, it is now providing a wealth of information on all aspects of axon guidance and targeting with implications not only for our understanding of these mechanisms in the olfactory system but also in other regions of the nervous system.

Introduction

In this commentary we will highlight insights into axon navigation within the mammalian primary olfactory system provided by recent molecular studies while at the same time controversies that surround some of the underlying data will be pointed out. We will finish our discussion with a description of a model that may help to explain axon targeting in the olfactory bulb. The pathway followed by olfactory sensory axons can be treated as consisting of three distinct sub-regions: the olfactory nerve connecting the olfactory neuroepithelium to the olfactory bulb; the outer olfactory nerve fibre layer; and finally the inner olfactory nerve fibre and glomerular layers. Since axons course in each of these regions they appear to depend on distinct molecular and cellular cues. For instance, in mice deficient in the SRC-family tyrosinase kinases, $p59^{fyn}$ and $pp60^{c\text{-}src}$, axons defasciculate in the olfactory nerve (Morse *et al*., 1998) while in N-CAM-180 null mutant mice, olfactory sensory axons do not correctly sort out in the nerve fibre layer (Treloar *et al*., 1997). In mice lacking the P2 odorant receptor gene, P2 axons fail to exit the nerve fibre layer and do not enter the underlying glomerular layer (Wang *et al*., 1998). Although we will examine each of these different sub-regions in turn, our main focus will be on the homing of axons to specific glomerular targets in the olfactory bulb.

Axon navigation to the telencephalon

Little is known about the signals that stimulate axon formation and outgrowth from olfactory sensory neurons. Early studies suggested that the mesenchyme lying between the presumptive placode and the rostral surface of the neural tube produced inductive signals. Retinoic acid is expressed by this mesenchyme (LaMantia *et al*., 1993) and it has been implicated in both olfactory sensory neuron generation and subsequent axon outgrowth (Whitesides *et al*., 1998; LaMantia *et al*., 2000). In the absence of appropriate olfactory neuronal cell lines, studies have focused on understanding olfactory sensory neuron-specific gene expression rather than describing mechanisms controlling the initiation of axon growth (Kudrycki *et al*., 1998; Qasba and Reed, 1998; Behrens *et al*., 2000). However, a recent study has identified NELF, a novel 50 kDa protein expressed by olfactory sensory axons which has neurite outgrowth promoting activity (Kramer and Wray, 2000). Inhibition of expression of this protein using antisense oligonucleotides demonstrated a remarkable attenuation of outgrowth of

olfactory axons from explant cultures of olfactory mucosa. An understanding of the function of this protein in development of the olfactory nerve pathway awaits the targeted mutagenesis of this molecule.

There is an unwritten law that once generated '*axons prefer to grow in a forward direction unless directed to do otherwise*'. This is clearly true *in vitro* where axons are observed to grow radially away from explants of neural tissue. Axons do not spontaneously turn-about, form whorls or make radical directional changes. In some cases the forward growth of an axon is restricted to a particular pathway by physical constraints. For instance, in the retino-tectal pathway axons are initially guided by the physical restraints imposed by the optic stalk. Although the olfactory system does not possess a similar conduit connecting the olfactory nasal pit to the rostral telencephalon it appears to generate its own conduit *de novo*. Olfactory axons navigate through the frontonasal mesenchyme by following a glial bridge that forms between the nasal pit and the rostral surface of the brain (Farbman and Squinto, 1985; Doucette, 1989; Tennet and Chuah, 1996). These glia are born in the olfactory neuroepithelium of the nasal pit and migrate towards the telencephalon probably under the influence of chemotropic molecules released by the presumptive olfactory bulb (Liu *et al*., 1995) or surrounding frontonasal mesenchyme (LaMantia *et al*., 2000). This distance is initially only 150–250 μm in mouse (Marin-Padilla and Amieva, 1989), which is appropriate for the action of soluble growth factors (Nellen *et al*., 1996).

The glia in the olfactory nerve pathway are the presumptive ensheathing cells of the olfactory nerve and nerve fibre layer of the olfactory bulb. These ensheathing cells are excellent promoters of olfactory axon growth *in vitro* (Ramón-Cueto and Valverde, 1995; Kafitz and Greer, 1998b, 1999). In fact, these cells are now credited with the extraordinary growth potential of olfactory axons throughout life and appear to facilitate regeneration of spinal axons when transplanted into lesions of the spinal cord (Ramón-Cueto and Avila, 1998). If given a choice of extracellular matrices or ensheathing cells, olfactory axons prefer to grow on ensheathing cells (Tisay and Key, 1999). Thus, the initial guidance of olfactory axons to the brain should really be thought of in terms of mechanisms guiding migratory glia. The frontonasal mesenchyme is rich in chondroitin sulphates whereas the olfactory nerve pathway is devoid of these molecules (Treloar *et al*., 1996a). As ensheathing cells do not migrate on a substrate of chondroitin sulphates (Tisay and Key, 1999), the chondroitin sulphates may act to restrict migration of glia and hence confine axons to defined channels just as the optic stalk provides a physical conduit for retinal axons. In comparison, ensheathing cells readily migrate away from explants of olfactory neuroepithelium plated on laminin and as the olfactory nerve pathway also contains laminin (Gong and Shipley, 1996; Treloar *et al*., 1996a; Kafitz and Greer, 1998a) it probably acts in an autocrine loop to stimulate both the migratory activity and axon-growth promoting properties of ensheathing cells (Tisay and Key, 1999). The dorso-lateral surface of the olfactory nerve is bounded by a region of neural crest-derived mesenchyme that selectively expresses Pax-7 (LaMantia *et al*., 2000). When the area of the Pax-7 expressing cells are experimentally reduced *in vitro* the trajectory of olfactory axons arising from neuroepithelial explants is severely disrupted (LaMantia *et al*., 2000). These results suggest that this specialized domain of mesenchyme provides specific guidance cues for the migratory mass, although the identity of these molecules remains to be determined. It is clearly not laminin or CS-56 chondroitin sulphates since this region of the frontonasal mesenchyme is devoid of these extracellular matrix molecules (Treloar *et al*., 1996a). The importance of the frontonasal mesenchyme in formation of the olfactory pathway was confirmed, at least *in vitro*, when this tissue was prevented from forming in explant cultures. In this case olfactory sensory axons failed to grow away from olfactory neuroepithelium and instead whorled on top of the explant (LaMantia *et al*., 2000). Interestingly, a similar phenotype is also observed when ensheathing cells fail to migrate away from explants grown on a chondroitin sulphate substrate (Tisay and Key, 1999).

Axon sorting in the nerve fibre layer

Upon reaching the rostral telencephalon the olfactory sensory axons and glial cells do not immediately enter into the neuroepithelium of the telencephalon, rather the axons defasciculate and branch (Whitesides and LaMantia, 1996) and form a transient tissue aggregate referred to as the migratory mass (Valverde *et al*., 1992). This mass is clearly separated from the telencephalon and its formation occurs at a time when the marginal zone and outer surface of the presumptive olfactory bulb expresses a 'wall' of CS-56 chondroitin sulphates (Treloar *et al*., 1996a). The migratory mass only fuses with the bulb when the expression of these chondroitin sulphates is downregulated. At this time the olfactory axons penetrate the marginal zone of the telencephalon and form the presumptive nerve fibre layer. Although the purpose of this delay in fusion of the migratory mass with the telencephalon is unknown, one possibility is that it may allow sufficient time for axon subpopulations to sort out into discrete fascicles as demonstrated in the insect olfactory system [(Rössler *et al*., 1999); see reviews by (Oland and Tolbert, 1996; Tolbert, 1998)]. However, in the absence of appropriate markers of axon subpopulations at these earlier ages in mammals, this hypothesis remains untested.

After the olfactory axons fuse with the telencephalon their tangential spread is restricted to the surface of the presumptive olfactory bulb. The mechanisms limiting the spread of olfactory axons on the telencephalic surface remains unknown. However, the territory on the rostral

surface of the telencephalon that is destined to become the olfactory bulb is delineated by the expression of two transcriptional factors, Brn-4 and Tst-1 (Alvarez-Bolado *et al*., 1995) and by anosmin-1, an extracellular matrix protein (Hardelin *et al*., 1999). The presumptive olfactory bulb division is also contained within a much broader domain of neuroepithelium expressing retinoic acid response elements which may activate specific differentiation events in response to retinoic acid in the surrounding frontonasal mesoderm (LaMantia *et al*., 1993). Although the significance of these transcriptional factors in development of the olfactory system remains unknown, retinoic acid has been implicated in a Pax-6 dependent signalling pathway associated with olfactory bulb formation (Anchan *et al*., 1997). Anosmin-1 is the protein encoded by the gene KAL-1 that is disrupted in the X chromosome-linked form of Kallmann syndrome (Hardelin, 2001). In Kallmann syndrome the olfactory bulbs fail to develop after the olfactory axons are unsuccessful in penetrating the presumptive olfactory bulb region of the telencephalon (Hardelin, 2001). Anosmin-1 has both neurite growth promoting as well as cell adhesion activities, the latter of which is dependent on trans-interactions with cell surface heparan sulphate and chondroitin sulphate glycosaminoglycans (Soussi-Yanicostas *et al*., 1996, 1998; Robertson *et al*., 2001). Since olfactory sensory axons are rich in heparan sulphate proteoglycans (Watanabe *et al*., 1996) as well as the chondroitin sulphate proteoglycan neurocan (Clarris *et al*., 2000), it is possible that the entry of axons into the presumptive olfactory bulb is dependent on interactions between these molecules and anosmin-1.

There is considerable defasciculation and sorting occurring in the olfactory nerve fibre layer, particularly in its outer region. Subpopulations of olfactory axons are typically intermixed in the olfactory nerve and only as they enter the nerve fibre layer do they sort out into molecularly distinct bundles such as those expressing cell surface carbohydrates (Figure 1a) (Key and Akeson, 1993; Riddle *et al*., 1993; St John and Key, 2001a). One possibility is that the nerve fibre layer is a sorting zone for olfactory axons in the same way that there is a sorting zone in the olfactory pathway of insects (Rössler *et al*., 1999). This sorting seems to be a necessary prerequisite for subsequent homing to specific glomerular targets. For instance, the failure of distinct subpopulations of axons to correctly fasciculate in the nerve fibre layer of either N-CAM-180 (Treloar *et al*., 1997) or galectin-1 (Puche *et al*., 1996) deficient mice leads to aberrant glomerular formation. Evidence from other systems indicates that chemorepulsive forces play an important role in sorting axons into specific bundles (Simpson *et al*., 2000) or defasciculating axons at specific sites in pathways (Hentschel and van Ooyen, 1999). Little attention has been paid to the role of chemorepulsive molecules and their receptors in the defasciculation and sorting of axons in the nerve fibre layer. Defasciculation of axons, for instance at target sites, is often thought to be mediated by the presence of local chemorepulsive ligands acting on receptors present on the axons. One possibility in the olfactory pathway is that chemorepulsive cues may be expressed by ensheathing cells in the nerve fibre layer and that these molecules act on receptors expressed by the axons. For instance, the chemorepulsive receptor neuropilin-1 is expressed by a subpopulation of olfactory sensory axons that appears to be diverted from ensheathing cells residing in the ventral olfactory nerve fibre layer which selectively express the secreted neuropilin ligand Sema3A (Crandall *et al*., 2000). There is still some debate concerning the precise expression pattern of Sema3A in rat with reports that it is either expressed solely by olfactory sensory neurons (Williams-Hogarth *et al*., 2000) or also by both plial cells and second-order mitral cells in the bulb (Pasterkamp *et al*., 1998). Nonetheless, olfactory sensory axons are clearly responsive to Sema3A and display chemorepulsive behaviour to substrate-bound Sema3A *in vitro* (Crandall *et al*., 2000; William-Hogarth *et al*., 2000). A subsequent and more detailed analysis of expression of Sema3A in embryonic mice revealed that this ligand was expressed by mitral cells as well as by ensheathing cells in the outer region of the nerve fibre layer, in the region where olfactory axons are known to sort out (Schwarting *et al*., 2000). More importantly, analysis of Sema3A homozygous mutant mice revealed that the sorting of axons within the nerve fibre layer was disrupted and axons terminated in topographically inappropriate glomeruli (Schwarting *et al*., 2000). Thus, chemorepulsive interactions between axons and ensheathing cells in the outer region of the nerve fibre layer mediate sorting out of axons and this sorting is a necessary prerequisite for subsequent homing of axons to correct glomerular targets. These results are consistent with the aberrant targeting in N-CAM-180 mutant mice where the loss of polysialic acid (which regulates the degree of axon fasciculation) in the nerve fibre layer inhibited sorting of axons in the nerve fibre layer (Treloar *et al*., 1997).

While axon sorting is clearly modulated by interactions between axon receptors and chemorepulsive ligands in the local environment it is also possible that axons co-express both ligand and receptor. By regulating the activity of these molecules either directly or indirectly, it is possible to control the extent of defasciculation in axon tracts (Lin *et al*., 1994; Tang *et al*., 1994; Yu *et al*., 2000). Interestingly, olfactory sensory neurons express the chemorepulsive receptor Robo1 and its ligands slit1, slit2 and slit3 (Ba-Charvet *et al*., 1999; Yuan *et al*., 1999). The relative levels of these molecules are known to influence the fasciculation patterns of axons (Simpson *et al*., 2000). The role of these molecules and of other co-expressed chemorepulsive receptors and ligands such as the Ephs and ephrins (St John *et al*., 2000; St John and Key, 2001b) needs to be better understood in the olfactory system.

It is becoming clear in other systems that the combinatorial role of multiple cues is essential for axon navigation (Winberg *et al*., 1998). The relative balance between

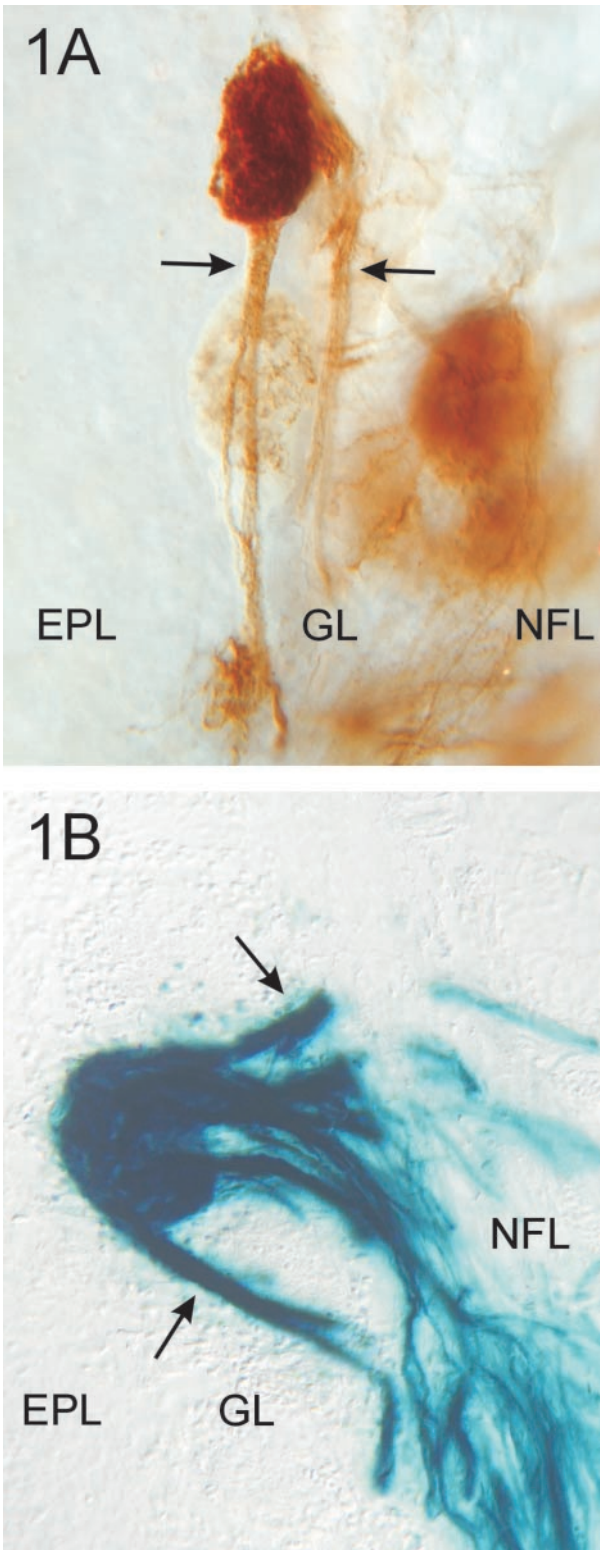

Figure 1 Targeting of defined subpopulations of axons to glomeruli in the adult mouse olfactory bulb. **(A)** Axons expressing cell surface carbohydrate ligands for the lectin *Dolichos biflorus* agglutinin (DBA) sort out and self-fasciculate in the nerve fibre layer (NFL) before terminating in discrete glomeruli in the glomerular layer (GL). These DBA-stained axons (arrows) target select glomeruli, which suggests that cell surface carbohydrates play a role in axon targeting. **(B)** Axons expressing the P2 odorant receptor were engineered to co-express LacZ (Mombaerts *et al*., 1996) and were visualized by enzyme histochemistry. These axons sort out into numerous small fascicles in the NFL before coalescing into larger bundles (arrows) that converge and terminate in a single glomerulus. The glomerular layer lies between the nerve fibre layer and the external plexiform layer (EPL).

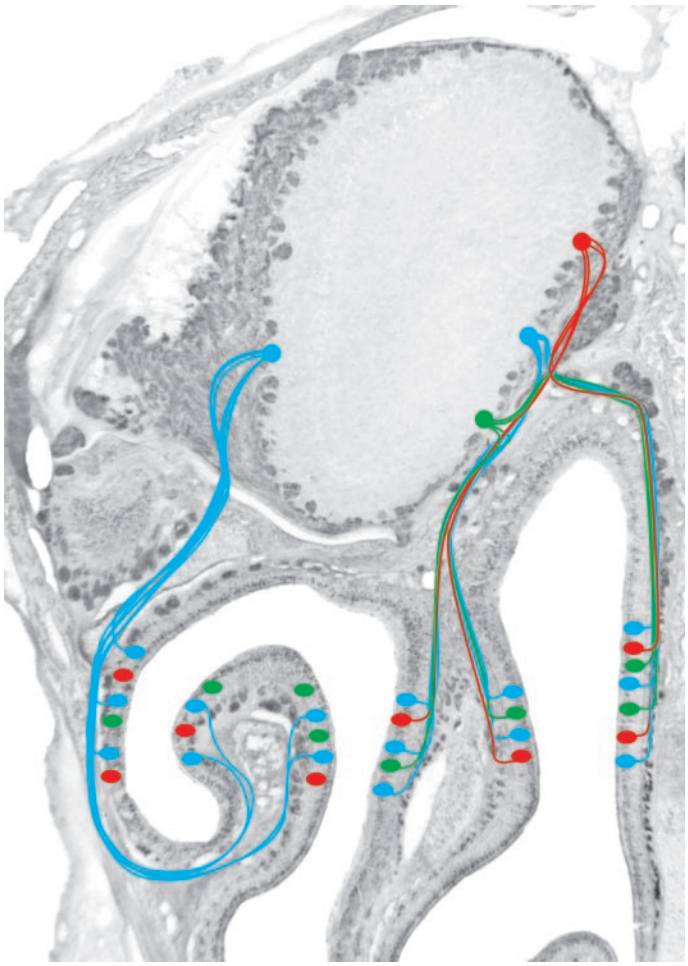

Figure 2 Schematic representation of axon targeting in the olfactory pathway. Olfactory sensory neurons expressing three different odorant receptors (represented as green, red and blue) are located in a specific semi-annular zone of olfactory neuroepithelium lining the nasal cavity. The axons of these neurons are interspersed in peripheral olfactory nerve bundles. When these axons enter the nerve fibre layer of the bulb they sort out and converge to glomeruli that occupy similar topographical positions in different animals. Identical glomeruli are located on both the medial and lateral surfaces of the olfactory bulb, although at different topographical positions.

repulsive and attractive cues is important for correct guidance and this is achieved by cis-interactions between receptor domains both at the cell surface and at cytoplasmic face of the plasma membrane as well as by cross-talk

between transmembrane signalling pathways. For instance, chemoattractive responses mediated by interactions between the axon receptor DCC and its secreted ligand netrin-1 are silenced by slit activation of Robo through a Robo/DCC complex (Stein and Tessier-Lavigne, 2001). Since DCC is expressed by olfactory neurons early in development (Schwarting *et al.*, 2001) it is possible that this receptor modulates Robo mediated behaviour. The role of these and other interactions such as those occurring in NCAM/axonin-1/NgCAM (Rutishauser, 2000) and neuropilin/plexin/sema (Takahashi and Strittmatter, 2001) complexes needs to be clarified in the olfactory pathway. It is intriguing that binding of EphB receptors to ephrin-B ligands can silence G protein signalling downstream of seven transmembrane G-coupled receptors (Schmucker and Zipursky, 2001). One of the consequences of this blockage is the silencing of signalling downstream of the CXCR4 cytokine receptor, resulting in abnormal granule cell migration in the developing cerebellum (Lu *et al.*, 2001). It is tempting to speculate that EphB–ephrin-B interactions in olfactory axons could modulate signalling downstream of the G-coupled odorant receptors in growth cones and affect olfactory sensory axon navigation.

Axon homing to glomerular targets

The degree to which distinct subpopulations of olfactory sensory axons converge to specific glomeruli in the olfactory bulb was not fully realized until after the cloning of olfactory receptor genes. By using *in situ* hybridization for detection of receptor mRNA in axons it was possible to show that neurons expressing the same receptor projected specifically to a small number of glomeruli in each olfactory bulb (Figure 2) (Vassar *et al.*, 1994). Axons typically terminated in a glomerulus on both the medial and lateral surfaces of the olfactory bulb. The positions of these glomeruli appeared to be highly conserved between bulbs, both within the same animal as well as between different animals. This raised the possibility that odorant receptors were subserving two distinct roles, one at the level of the nasal cavity in signal transduction and the other in the growth cone as a guidance receptor to enable axons to target specific glomeruli. The targeting of axons expressing the same receptor to individual glomeruli was subsequently more clearly defined using mice engineered to express reporter molecules specifically in a subpopulation of olfactory sensory neurons (Figure 1b) (Mombaerts *et al.*, 1996). By using homologous recombination, the P2 coding region was replaced with a construct consisting of the P2 coding region linked to a tandem array of an internal ribosome entry site recognition (IRES) sequence, a signalling sequence from the tau gene that traffics proteins into axons, and finally the LacZ gene. In this P2-IRES-tau-LacZ line of mice, β-galactosidase was expressed in the axons of neurons expressing the P2 receptor (P2 axons). Thus, enzyme histochemical staining enabled the complete trajectory of P2 axons from the olfactory neuroepithelium to two principal glomeruli in each olfactory bulb to be clearly depicted.

A knock-in approach was subsequently used to directly test the hypothesis that receptor proteins were involved in axon targeting to specific glomeruli. By replacing the P2 coding region with the M12 coding region it was possible to examine whether axon targeting was dependent on the type of receptor a neuron expressed (Mombaerts *et al.*, 1996). A line of mice was generated in which the P2 coding region was replaced by an M12-IRES-tau-LacZ construct (abbreviated as M12⇒P2). If the receptor was instructing the axons to target a specific glomerulus in the olfactory bulb then P2 neurons should inappropriately project to the M12 glomerulus in these M12⇒P2 mice. If the receptor was not involved in targeting then the P2 neurons in the M12⇒P2 mice should project to the P2 glomerulus regardless of the fact that they were expressing M12 rather than P2. Instead, it was found that P2 neurons projected somewhere in-between the P2 and M12 glomeruli, indicating that receptors play a role in axon targeting but that there were certainly other molecules that also contributed to the guidance of olfactory axons.

There was controversy concerning the interpretation of these results. The very notion that receptors involved in transduction of sensory stimuli could also be involved in targeting was unsettling. Questions were raised as to whether the knock-in approach disrupted the regulatory sequences which non-specifically or indirectly affected axon targeting. This was of particular concern since the insertion of IRES-tau-LacZ into the M12 locus caused aberrant axon targeting of M12 axons (Mombaerts *et al.*, 1996). However, these concerns were partly allayed following multiple repeats of these swap experiments using both distant and closely related members of receptor sub-families (Wang *et al.*, 1998). Altogether four distinct receptor lines were generated and in all cases axons targeted inappropriate glomeruli. However, all lines were generated by modifying the P2 locus and as yet these results have not yet been confirmed with knock-ins into other receptor genes.

It is still not clear from these experiments whether the odorant receptors are either directly or indirectly involved in axon guidance. One possibility is that the choice of receptor expressed by an olfactory neuron could determine the expression of other guidance receptors. Several possible mechanisms could explain this regulation. First, the coding sequence of receptor genes may contain remote enhancer elements that act at a distance with promoters for guidance genes. These enhancers would have to be present in the coding region since in the receptor knock-in mice only the coding regions were replaced. Second, the odorant receptor mRNA itself may directly modulate expression of other guidance cues, either at the transcriptional or post-transcriptional level. Third, odorant receptor protein or its signalling pathways may regulate guidance receptor activity.

However, none of these mechanisms seem very likely and axon targeting is probably best explained by a direct role of the odorant receptor as a guidance receptor at the cell surface. To date, there is still no evidence that odorant receptors are expressed on the growth cones where they would be expected to be functionally active as guidance receptors nor are there any reports that these molecules are neurite-outgrowth promoting using any of the classical substrate-bound *in vitro* assays. Just how these odorant receptors act as guidance receptors is not at all clear. If they act as receptors for ligands expressed in the olfactory bulb then mutational analysis of extracellular domains in transgenic knock-in mice might provide a means of identifying the binding sites.

The importance of an odorant receptor in axon convergence and glomerular formation was clearly demonstrated in transgenic mice whose P2 coding region was either replaced by an IRES-tau-LacZ construct or contained three point mutations which resulted in expression of truncated receptors (Wang *et al*., 1998). In these mice the axons of P2 neurons lacking a functional P2 receptor were easily visualized by LacZ histochemical staining. P2 axons in these mice failed to form glomeruli. They appeared to reach the vicinity of their topographically correct target site and only then failed to converge, instead remaining in the nerve fibre layer. Similar results were noted with deletion of the M12 receptor although this data was not published (Wang *et al*., 1998). Interestingly, the P2 axons in the P2 deletion mice were confined to the nerve fibre layer in 2-week-old knockout mice in a similar distribution to that displayed by P2 axons during normal embryonic development (Royal and Key, 1999). At E15.5, P2 axons are diffusely distributed in the nerve fibre layer before they begin to condense and form presumptive glomerular-like loci at E17.5 (Royal and Key, 1999). In the absence of the P2 receptor, the P2 axons appear to fail to converge and form these protoglomeruli. It would seem that the receptor protein is critical at least for the latter stages of axon targeting and convergence. However, it was never resolved whether the failure of P2 axons to converge was a result of the upregulation of inappropriate receptors following either deletion of P2 or expression of truncated receptor.

Although we are concerned here only with axon navigation in the main olfactory system it is important to briefly examine the effect of mis-expression of odorant receptors in the vomeronasal sensory neurons on axon targeting in the accessory olfactory bulb. Deletion of the putative pheromone receptor VR_i2 causes the axons of neurons that should be expressing this receptor to fail to converge and form specific glomeruli (Rodriguez *et al*., 1999). Instead these axons now broadly terminate throughout the glomerular layer of the accessory olfactory bulb. When the VR_i2 receptor coding region is replaced by the coding region of the unrelated M71 odorant receptor the convergence of axons to specific glomeruli is restored. Although in this case the glomeruli are distinct from the wild-type VR_i2 glomeruli, they are restricted to a topographically appropriate zone in the accessory olfactory bulb (Rodrigeuz *et al*., 1999). The important point here is that the coding region of M71 is sufficient to allow convergence of axons albeit to the wrong target sites. These results suggest that the receptor protein is autonomously responsible for convergence, most likely through receptor–receptor interactions. It is hard to imagine that the M71 receptor could be binding heterophically with molecules selectively expressed by subpopulations of axons present in both the main and accessory olfactory systems. Both odorant receptor and other cues appear to be necessary for correct targeting after convergence. If odorant receptor type was not involved in targeting then one would predict that these axons would have innervated their correct glomeruli after they had converged. Thus, the odorant receptor appears to have a dual role in both convergence and targeting. It would be interesting to see whether a $VR_i2 \Rightarrow$P2 swap would both maintain convergence and perturb targeting of P2 axons in the main olfactory system.

What is the cellular source of putative guidance cues for targeting in the olfactory bulb? One possibility is the undifferentiated neuroepithelial cells in the presumptive olfactory bulb. When the migratory mass fuses with the telencephalon, olfactory sensory axons penetrate into the outer telencephalic wall through holes that emerge in its basement membrane (Treloar *et al*., 1996a). Axons then interact directly with the endfeet of undifferentiated neuroepithelial cells that span the width of the telencephalic wall at this stage (Marin-Padilla and Amieva, 1989). These neuroepithelial cells are prime candidates for providing topographical cues. Another possibility is that the cues are present on early differentiating projection neurons, the mitral cells. To begin to address the role of mitral cells and interneurons in axon targeting Bulfone *et al*. examined the trajectory of P2 axons in two lines of mice lacking either Tbr-1 or Dlx-1 and Dlx-2 (Bulfone *et al*., 1998). The Tbr-1 deficient mice had a reduced number of mitral/tufted cells while the Dlx-1/Dlx-2 deficient mice had a reduced complement of interneurons. In both mouse lines P2 axons continued to converge and form glomeruli at positions similar to those of wild-type animals. Although it is clear that interneurons do not play a role in axon targeting since they are not generated until well after P2 axons have begun to converge (Hinds, 1968), the role of mitral cells still remains uncertain. The Tbr-$1^{-/-}$ mice had a reduced number of mitral cells that still may have been sufficient to produce appropriate guidance cues.

Three recent papers (Bailey *et al*., 1999; Treloar *et al*., 1999; Puche and Shipley, 2001) have examined in detail the cellular interactions between olfactory sensory axons and bulb cells occurring during glomerular formation. These studies have demonstrated that glomerular formation precedes the compartmentalization of dendritic arborizations of second-order mitral/tufted cells into glomeruli. Bailey *et*

al. revealed that olfactory axon arborization appears to be secondary to the formation of tufts of radial glial cell processes (Bailey *et al*., 1999). They proposed that radial glial cells induced the formation of protoglomeruli independent of any involvement of mitral cells. However, this observation does not preclude the role of second-order neurons in the initial targeting events but only in the process of glomerularization. The cellular basis of targeting remains to be clarified and increasing attention should be directed at deciphering the role of undifferentiated neuroepithelial cells, radial glia and mitral cells in this process.

How specific is targeting by olfactory axons? The view that is generally exhorted is that all axons expressing the same odorant receptor typically project to two glomeruli, one on the medial surface and one on the lateral surface of each olfactory bulb (Mombaerts *et al*., 1996; Wang *et al*., 1998). The positions of these glomeruli are considered fixed between bulbs within the same animal as well as between different animals. Convergence of axons begins as early as E16.5 (Mombaerts *et al*., 1996), which led to the idea that olfactory axons form glomeruli without error. This has been supported by evidence from time-lapse imaging of single axons arborizing without extensive overshooting in the zebra fish olfactory bulb (Dynes and Ngai, 1998). Interestingly, while Golgi staining in the early postnatal rat olfactory bulb failed to detect exuberant axon growth or extra-glomerular branching (Klenoff and Greer, 1998) a similar analysis in rabbit showed extra-glomerular branching to inappropriate glomeruli (Yilmazer-Hamke *et al*., 2000). Some of the differences in results obtained between these studies could be attributed to the species analysed as well as to problems with the use of Golgi staining to observe thin immature axons. The view that axon targeting is initially highly specific is not supported by dye-tracing experiments. Detailed analysis of the projections of olfactory axons in the early postnatal period in both rat and mouse revealed pre-glomerular branching, axons passing through the glomerular layer without branching, axon branching into two glomeruli and axons arborizing in glomeruli as well as growing into deeper layers (Tenne-Brown and Key, 1999). This overshooting and arborization in more than one glomerulus was also observed for P2 axons by LacZ histochemistry (Royal and Key, 1999). Moreover, P2 axons were found in a second extra glomerulus at one surface of the olfactory bulb in 85% of adult animals. The prevalence of these extra glomeruli was more marked on the medial surface of the olfactory bulb, an observation subsequently verified in a different colony of P2-IRES-tau-LacZ mice (Ebrahimi and Chess, 2000). The position of extra glomeruli was highly variable as opposed to the more constant location of the principal glomerulus (Royal and Key, 1999; Schaeffer *et al*., 2001). In addition, P2 axons sometimes only partially innervated the extra glomerulus. This result clearly revealed that glomeruli can be innervated by axons expressing more than a single receptor protein, an idea initially suggested several years earlier from observations of a line of transgenic mice expressing LacZ in a subpopulation of olfactory neurons (Treloar *et al*., 1996b) which appears to be under the control of an odorant receptor gene promoter (Pyrski *et al*., 2001). These extra glomeruli are not only formed by P2 neurons; they have also been observed for neurons expressing M72 (Zheng *et al*., 2000), M50 and M71 (Lin *et al*., 2000), OR-Z6 (Pyrski *et al*., 2001) and mOR37 (Strotmann *et al*., 2000). Taken together, the overshooting of axons and the innervation of more than one glomerulus by axons expressing the same receptor suggests that once axons have reached the vicinity of their target their ultimate site of convergence can be modulated by additional mechanisms. One possibility is that neuronal activity and/or level of receptor protein may influence this process. In transgenic mice, olfactory sensory neurons mis-expressing an exogenous copy of the odorant receptor MOR28 innervated an independent glomerulus located near to the wild-type MOR28 glomerulus (Serizawa *et al*., 2000). The distance separating these two glomeruli was determined by both the genetic background of the endogenous and transgene alleles and by whether the alleles were tagged with reporter molecules (Ishii *et al*., 2001). For instance, when endogenous alleles were homozygous from the 129 strain and the transgene was from a C57/Bl6 background the glomeruli were ~300–400 μm apart. This phenotype is similar to that observed for the P2 extra glomeruli when P2 axons are tagged with LacZ (Royal and Key, 1999). However, the modification of the P2 locus by the knock-in of LacZ is not solely responsible for the formation of extra glomeruli since these structures are also present in wild-type animals as revealed by *in situ* hybridization (Pyrski *et al*., 2001). It seems that differences in genetic background between the two alleles contributes to the formation of these extra glomeruli. Interestingly, when one endogenous MOR28 allele was modified to co-express GFP, axons expressing this allele segregated from axons expressing the unmodified endogenous allele within the same target glomerulus (Ishii *et al*., 2001). This segregation became more prominent when the maternal and paternal alleles were from different genetic backgrounds. Thus, it appears that the final target site is fine tuned according to the genetic background of the receptor allele which may reflect either differences in amino acid sequence and/or differences in level of receptor expressed. In each case glomerular targeting could be affected by resultant differences in either target recognition and/or neuronal activity dependent mechanisms.

It is now clear that during the early stages of glomerular formation both olfactory sensory axons (Tenne-Brown and Key, 1999) and the dendrites of mitral cells (Malun and Brunjes, 1996) branch and contribute to multiple glomeruli. Over a period of several days these branches are withdrawn until each axon and dendrite terminates in a single glomerulus. Although little attention has been directed towards understanding the molecular basis of these rearrangements

it has been suggested that interactions between the chemorepulsive Eph receptors and their ephrin ligands may be involved (St John *et al*., 2000). During this period of plasticity, EphA5 is differentially expressed by mitral cells while its ligands are differentially expressed by olfactory sensory axons leading to the presence of subpopulations of glomeruli with high and low levels of these molecules (St John *et al*., 2000). It was proposed that axons expressing high levels of ligands innervated glomeruli containing dendrites expressing low levels of EphA5 and vice versa (St John *et al*., 2000). However, without knowing the full complement of Ephs and ephrins expressed by these neurons this hypothesis will be difficult to test.

What is the role of neuronal activity in axon targeting? This question was recently addressed in two studies that examined the targeting of P2, M50 and M72 olfactory neurons in mice deficient in the olfactory cyclic nucleotide-gated channel OCNC-1 (Lin *et al*., 2000; Zheng *et al*., 2000). While the trajectory of P2 and M50 axons were unaffected by loss of neuronal activity there were modest defects in the targeting of M72 axons (Zheng *et al*., 2000). In the absence of OCNC-1 there was an increase in the number of small ectopic glomeruli innervated by M72 axons in comparison to control littermates. It remains to be determined whether this activity-dependent fine tuning of targeting occurs for many different olfactory neuron subpopulations or whether it is instead restricted to only a small subpopulation. Nonetheless, the take-home lesson appears to be that not all neurons are equally dependent on neuronal activity for correct targeting. One therefore has to be careful when extrapolating from mechanisms identified for either P2 or M72 to the whole population of olfactory sensory neurons. Zheng *et al*. (Zheng *et al*., 2000) extended their analysis from complete loss of neuronal activity to a paradigm where M72 neurons with different levels of neuronal activity were forced to compete for the same glomerular space. By taking advantage of the phenomenon of monoallelic inactivation of odorant receptor genes by olfactory sensory neurons, Zheng *et al*. (Zheng *et al*., 2000) were able to generate mice with M72 olfactory neurons that were either OCNC1-negative and tagged with GFP or OCNC1-positive and tagged with LacZ in an OCNC1$^{-/-}$ background. In these mice the GFP and LacZ positive axons segregated into distinct glomeruli indicating that the activity of the M72 neurons governed their final targeting. Whether this is the case for all olfactory neuron subpopulations remains to be determined. Considering that addition of reporter tags can affect axon targeting it is possible that this separate innervation of glomeruli occurs independently of differences in activity. In an important control, Zheng *et al*. examined the targeting of M72 alleles tagged with either LacZ or GFP (Zheng *et al*., 2000). Despite the presence of these reporters, M72 axons converged to a single glomerulus, indicating that the level of neuronal activity was the important determining factor in the segregation of axons. Thus, differences in neuronal activity may explain why olfactory sensory neurons expressing an odorant receptor MOR28 transgene innervate a glomerulus adjacent to the glomerulus innervated by wild-type MOR28 neurons when all alleles are from the same genetic background (Serizawa *et al*., 2000).

The role of neural activity in organization of the olfactory pathway was also demonstrated in females heterozygous for a mutant allele of the X-linked OCNC1 (Zhao and Reed, 2001). In these mosaic animals approximately half of the olfactory sensory neurons would be expected to express wild-type OCNC1 due to random inactivation of the gene on one of the two X chromosomes. In neonates, two distinct populations of glomeruli innervated by either wild-type or OCNC1 negative neurons co-existed. In contrast, essentially all the glomeruli in adult female heterozygous mice were wild type and contained OCNC1. There were few OCNC1-negative glomeruli (which were tagged with LacZ) since the OCNC1-negative neurons were selectively lost with increasing age. Thus, competition between subpopulations of neurons with either all or no activity also influences neuronal survival. However, one should remember that the all-or-none effect of neural activity in these mice models is artificial. Perturbations to targeting and survival were only demonstrated in an extreme competitive environment; these results may not be extrapolated to variations in physiological activity that may arise during normal experience. It remains unclear whether different physiologically relevant odour environments can modulate the activity of subpopulations of neurons sufficiently to affect targeting.

Working models of axon navigation

Taken together, the current available data support a hierarchical model of axon navigation to explain formation of the olfactory pathway (Figure 3). First, widely expressed molecules such as N-CAM and TAG-1 probably ensure fasciculation along the olfactory nerve and in the olfactory nerve fibre layer. Second, molecules such as OCAM (Alenius and Bohm, 1997; Yoshihara *et al*., 1997; Nagao *et al*., 2000), which are expressed in several zones of the olfactory neuroepithelium, may be involved in the partitioning of axons into large spatially defined bundles (e.g. ventrolateral vs ventromedial). Third, adhesion molecules may also be expressed by all neurons in a single zone where they could contribute to a zonal partitioning of the olfactory pathway. Although such zone-specific adhesion molecules have not yet been identified, there is evidence of the restricted expression of other molecules (Miyawaki *et al*., 1996). However, such zonal-specific expression may be unnecessary since it is possible that combinations of several adhesion molecules distributed across several zones could generate sufficient specificity for zonal identity. The whole purpose of this hierarchy of cell adhesion molecules is to ensure that olfactory sensory axons arising from one region of the nasal cavity are guided to a complementary region in

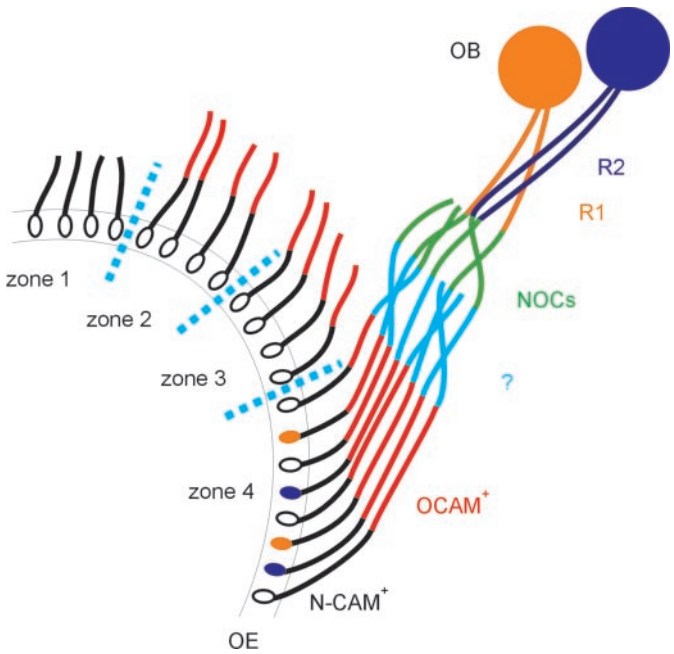

Figure 3 Hierarchical model of axon targeting in the olfactory pathway. The nasal cavity is divided into four zones according to the expression of specific odorant receptor proteins. Each zone expresses a unique complement of receptors. All axons ubiquitously express cell adhesion molecules (CAMs) such as N-CAM, L1 and TAG-1. These CAMs are involved in fasciculating olfactory axons into large bundles. Expression of other CAMs such as OCAM causes axons to sort out into non-overlapping bundles. In the case of OCAM, axons are partitioned into two discrete bundles—those arising from zone 1 and those from zones 2–4. Further sorting into zone-specific bundles could be mediated either by zone-specific CAMs, or by CAMs expressed at varying levels in different zones, or by several CAMs expressed in different combinations of zones. After these zone-specific bundles enter the nerve fibre layer they sort out according to the expression of cell surface carbohydrates such as NOCs. Soluble lectins expressed by ensheathing cells cross-link and self-fasciculate axons expressing the same cell surface carbohydrates, referred to as a glycocode. These cell surface carbohydrates do not have to be expressed by neurons in a single zone since the axons are already sub-partitioned into zone-specific bundles. As long as they are expressed mosaically they would facilitate the sorting of axons within zone-specific bundles. Similarly, lectins do not have to be restricted to a specific region of the bulb. The homogeneous expression of lectins by all ensheathing cells would still cause cross-linking of axons within the different zone-specific bundles. Finally, axons are directed to their topographically correct position according to the expression of odorant receptor proteins. Axons recognize cues initially laid down in the early developing telencephalon according to the *two point* source model of guidance cues as outlined in Figure 4.

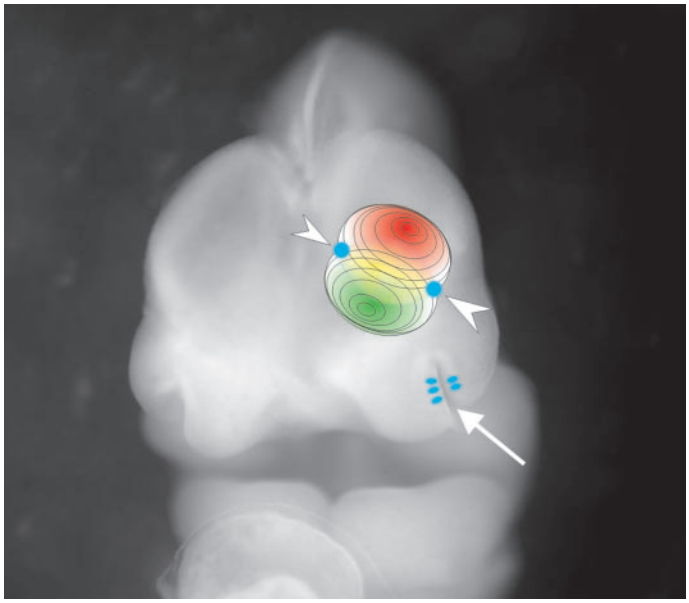

Figure 4 *Two point* source model of guidance cues on the rostrolateral surface of the forebrain. Olfactory sensory neurons expressing a specific odorant receptor are represented as blue neurons in the nasal pit (arrow). The axons of these neurons target discrete glomeruli lying on both the medial and lateral surfaces of the olfactory bulb. The positions of these glomeruli are represented on the rostrolateral surface of each telencephalic hemisphere (arrowheads) prior to the evagination of the olfactory bulb. These positions are fixed according to the levels of two overlapping gradients of guidance cues (represented as green and red). Because the gradients are emanating from *two point* sources they produce identical levels of guidance cues on both the medial and lateral surfaces of the hemisphere. Although the absolute position of glomeruli will depend on the final shape of the bulb as it evaginates from the telencephalon, the relative positioning of glomeruli will be similar on both its medial and lateral surfaces.

the olfactory bulb. Superimposed on this selective sorting through adhesion interactions are chemorepulsive interactions that drive the growth of axons away from inappropriate bulbar regions (Nagao *et al.*, 2000; Schwarting *et al.*, 2000). Fourth, subpopulations of axons in each zone-specific bundle express distinct cell surface carbohydrates such as NOCs (Dowsing *et al.*, 1997; Pays and Schwarting, 2000; St John and Key, 2001a). NOCs could form a glycocode which would enable sorting of axons into discrete smaller fascicles in the nerve fibre layer (St John and Key, 2001a) through interactions with carbohydrate-binding proteins such as galectin-1 (Puche *et al.*, 1996; St John and Key, 1999; Tenne-Brown *et al.*, 1998). This step in the hierarchy of interactions would ensure finer partitioning of the axons, a necessary prerequisite for their subsequent convergence. Finally, subpopulations of axons expressing specific receptor proteins then converge and target specific glomeruli.

This model of hierarchical sorting of axons does not require all axons expressing the same receptor to selectively fasciculate into a single bundle before entering its target glomerulus. What this model predicts is that axons expressing other cell surface recognition molecules (e.g. carbohydrates) will target topographically fixed glomeruli. This prediction was recently confirmed for NOC-3, a unique glycoform of N-CAM (St John and Key, 2001a). Although axons expressing the same receptor must interact there is no *a priori* reason to suppose that these axons must self-fasciculate. In fact, during embryonic development the glomerulus forms over several days as the arbors of loosely dispersed and intermixed subpopulations of axons sort

out on the basis of receptor expression and condense at a specific locus (Royal and Key, 1999). As a result of the dynamics of this condensation, axons enter the glomerulus from multiple points. As development proceeds, axons appear to self-organize into fascicles as they continue to enter glomeruli. The spatial arrangement of axon entry points into a glomerulus appears stochastic and hence different for every glomerulus and for the same glomerulus in different bulbs.

What is the nature of the cue in the bulb that determines the target site? It is unlikely that there will be 1000 unique molecular markers for each of the glomerular pairs within the bulb. It is not that this number is too large since there are already 1000 odorant receptors—it is just that these molecules would then need to be expressed at topographically invariant points in each bulb, a highly uneconomical scenario in terms of molecular interactions. The molecular markers of glomerular position could not be stochastically expressed in the bulb, like the receptors are in the olfactory neuroepithelium, since glomerular position is highly stereotypical. By observing the massive convergence of P2 axons in adult animals it is tempting to speculate the presence of a soluble tropic substance emanating from glomeruli and attracting axons to their target. However, as discussed above, axons do not home to a point source during embryonic development but rather reach a general address and then condense gradually into a glomerulus. We instead favour the postulate that glomerular target sites are defined by the expression of two overlapping gradients of ligands or cues that are distributed over the glomerular surface of the olfactory bulb (Gierer, 1998).

In the retino-tectal pathway, target cues exist as linear gradients across the ventrodorsal and anterioposterior axes of the optic tectum. If a similar gradient model was present on the olfactory bulb, duplicate gradients would need to exist on both the medial and lateral surfaces since olfactory axons expressing the same receptor target glomeruli on both of these surfaces. We propose a variant of this model based on gradients originally set up from point sources. In this model, *two point* sources positioned above one another in the dorsoventral axis on either side of the rostrolateral surface of the telencephalon would create a symmetrical coordinate system of radially dispersed cues, with lower levels of cues present at positions more distant from the source (Figure 4). Every glomerular position on the surface of the presumptive olfactory bulb (e.g. see arrowheads pointing to blue glomeruli in Figure 4) would correspond to a particular level of each of the two cues (coloured red and green in Figure 4). Since the cues are radially dispersed, there would be two points in space represented for every combination of the two cues, one on the medial surface and one on the lateral surface of the presumptive olfactory bulb (see arrowheads pointing to blue glomerular targets in Figure 4). This two-dimensional topographical map of glomerular targets is initially present on the telencephalic surface but is converted into a three-dimensional map as the bulb evaginates. If the midline of the bulb evaginates from a line connecting the *two point* sources, near identical gradient cues would be present on both the medial and lateral surfaces of the bulb. In this way, complementary blue glomeruli would be present on either surface of the bulb.

The final stereotactic position of glomeruli would depend on the ultimate shape of the bulb. If one side of the bulb grew disproportionately more than the other, there would be a skew in the absolute glomerular positioning between the medial and lateral surfaces. This appears to be the case since identified glomeruli never appear to be at the same rostrocaudal or dorsoventral position on the medial and lateral surfaces of the bulb. In fact, for all glomeruli examined to date [P2 (Mombaerts *et al*., 1996); M72 (Zheng *et al*., 2000); MOR10, MOR18, MOR28, MOR83 and A16 (Tsuboi *et al*., 1999); P3 (Wang *et al*., 1998)], the medial glomerulus is always located slightly more caudally along the rostrocaudal axis than its counterpart on the lateral surface. It appears that the gradient has uniformly shifted caudally on the medial surface. The *two point* source gradient model also predicts that the relative positioning of identified glomeruli will be the same on either surface of the bulb, which is supported by the detailed mapping of five glomeruli in the mouse olfactory bulb (Tsuboi *et al*., 1999). In addition, this model predicts that the midline will contain glomeruli which are not duplicated since at this position the intersection of gradients between the *two point* sources produces only a single level of each ligand. To date, available data confirms this prediction. Two distinct subpopulations of olfactory sensory neurons expressing either mOR37 (Strotmann *et al*., 2000) or OR-Z6 (Pyrski *et al*., 2001) innervate single glomeruli in the ventral midline of the olfactory bulb. There was some variability in the number of these glomeruli and for mOR37A ~20% of bulbs contained two glomeruli. This would be expected for axons targeting the midline since small variations in the morphology of the bulb could easily lead to a flattening of the gradient in this midline region and hence result in multiple glomeruli. Consequently, one would also expect to have some variability in the relative positioning of glomeruli in the midline, which proved to be the case when glomerular targets of several members of the mOR37 receptor subfamily were mapped (Strotmann *et al*., 2000). All these observations are consistent with the duplication of gradients on both the medial and lateral surfaces of the bulb as predicted by the hypothesis of radial gradients arising initially from *two point* sources. Is there any evidence for point sources of gene expression on the rostrolateral surface of the telencephalon? Two transcriptional factors Brn-4 and Tst-1 are both expressed as discrete patches in the region of the presumptive olfactory bulb in the early embryonic telencephalon (Alvarez-Bolado *et al*., 1995). However, it remains to be determined whether Brn-4 and Tst-1 are expressed in overlapping gradients or whether they regulate the release of factors that set up gradients across the surface of the

telencephalon, either of which would support the *two point* source hypothesis.

The hierarchical and *two point* source models of guidance attempt to explain the ability of olfactory sensory axons to sort out and converge to form specific glomeruli. Although correct sorting of axons in the nerve fibre layer appears to be necessary for the subsequent receptor-mediated homing of axons to their topographic target site in the olfactory bulb (Puche *et al*., 1996; Treloar *et al*., 1997; Schwarting *et al*., 2000), it is interesting to ask why this sorting is necessary in the first place. It could be argued that sorting is not needed and is merely a passive by-product of axons actively homing to target signals present in the bulb. This appears to be the case at least in the retino-tectal pathway where fibre order in the optic nerve and tract as well as their point of entry of retinal axons into the tectum does not affect the topographic targeting of these axons (DeLong and Coulombre, 1967; Fujisawa, 1981; Trowe *et al*., 1996). Provided retinal axons reach the tectum they seem capable of correct targeting due to the existence of complementary gradients of guidance receptors on retinal axons and ligands on tectal cells. Thus, retinal axons expressing a distinct level of guidance receptor will either migrate up or down concentration gradients of ligands in the tectum with little concern for their point of entry into that gradient in order to reach their target site.

If there is a ligand gradient over the glomerular surface of the bulb why then do axons depend on prior sorting in the nerve fibre layer to reach their target? Why don't olfactory axons behave like retinal axons which do not require sorting cues to correctly home in on their target site in the tectum? One possibility is that guidance receptors on olfactory axons are only able to respond to a narrow range of ligand levels in this gradient. Hence axons need to be sorted and directed to the vicinity of the target site first by other guidance cues. Thus, when axons find themselves in the wrong region they are incapable of navigating back to their correct target. This could explain why in the receptor swap experiments (Mombaerts *et al*., 1996) M12⇒P2 axons never reach the M12 glomerulus but instead converge prematurely at inappropriate sites. Since the hierarchical sorting of these axons is dictated by their original P2 identity, M12⇒P2 axons will be guided to the broad vicinity of the P2 glomerulus. The receptor swap experiments in the accessory olfactory system have taught us that receptors act autonomously to cause convergence of axons expressing the same receptor. Hence, M12⇒P2 axons will converge but at inappropriate sites. However, if the M12⇒P2 axons could not read the local ligand gradient why do they still terminate in topographically fixed positions? In fact, these inappropriately targeted glomeruli appear to be in the same relative position with respect to the P2 glomerulus on both the medial and lateral surface of the olfactory bulb (Mombaerts *et al*., 1996; Wang *et al*., 1998). Thus, it is most likely that the M12⇒P2 axons probably can read the local gradient but are forced to adopt a new position due to the confines placed on them by other guidance molecules expressed by the P2 neurons.

An alternative explanation for why axons need to be presorted in the olfactory nerve fibre layer in order to reach their correct target has to do with the location of the putative guidance cues in the olfactory bulb. In the tectum the guidance cues seem to be present on at least the astroglial cells present in the outer plexiform layer—a location that facilitates direct interactions with retinal axons (Davenport *et al*., 1996; Braisted *et al*., 1997; Stier and Schlosshauer, 1999). However, the outer olfactory nerve fibre layer of the olfactory bulb, which is derived from the olfactory neuroepithelium, seems to lack astroglial cells during targeting (Bailey *et al*., 1999; Treloar *et al*., 1999; Puche and Shipley, 2001). Within the olfactory nerve, axons expressing the same odorant receptor are widely separated across different filia olfactoria (bundles of axons enwrapped by the processes of individual ensheathing cells) as well as across different fasciculi (bundles of filia olfactoria). These fasciculi pass through the numerous foramina in the roof of the nasal cavity and then enter the olfactory bulb at multiple points along its rostrocaudal length. As the olfactory nerve fibre layer grows in thickness the later growing axons would reach the surface of the bulb lacking any guidance cues associated with astroglia, unlike in the tectum where axon–glial interactions continue throughout formation of the retino-tectal pathway (Vanselow *et al*., 1989). Therefore defasciculation and sorting of olfactory sensory axons would be essential for widely dispersed axons to reach cues lying deeper in the bulb.

Where to next?

Although most of the cellular and molecular events underlying axon targeting in the mammalian olfactory system remain unknown, the models outlined here at least provide a framework to formulate predictions that can be empirically tested. For instance, what is now needed is a systematic search for molecules expressed from point sources on the surface of the rostral telencephalon. To start with, Brn-1 and Tst-1 are two possible candidates and mis-expression of these molecules should alter the topography of the olfactory projection if they are somehow involved in the formation of gradients of guidance cues. Alternatively, the *two point* source hypothesis predicts that there will be differences in expression of regulatory molecules between the ventral and dorsal regions of the presumptive olfactory bulb region in the telencephalon so it may be possible to examine for differential expression across this axis. The role of putative targeting cues in the retino-tectal pathway has been tested by mis-expressing molecules in the tectum using microinjected retroviruses (Nakamoto *et al*., 1996). A similar approach could be used in the adult olfactory bulb by taking advantage of the unique regenerative capacity of olfactory neurons. There are now genetic means of synchronizing the ablation and subsequent regeneration of select sub-

populations of olfactory sensory neurons expressing reporter molecules (Gogos *et al*., 2000). Since these regenerating olfactory axons are able to enter the adult olfactory bulb and target the site of their original glomerulus they could be used as an assay system to examine the role of numerous target derived guidance molecules. The alternative approach is to mis-express putative guidance receptors or dominant negative forms of these molecules on the surface of olfactory axons in transgenic mice using a global promoter such as that for OMP (Walters *et al*., 1996). Promoters for zone-specific molecules such as OCAM and neuropilin-1 have yet to be defined but when available they should provide alternate means of regulating the spatial expression pattern of guidance molecules in the olfactory system. This approach could be used to test the role of candidate guidance molecules such as cell surface carbohydrates, OCAM and neuropilin-1. Ideally, it would be more appropriate if promoters for various odorant receptors were available so that molecules could be mis-expressed in specific subpopulations of olfactory neurons by transgenesis. Evidence to date indicates that the most of the regulatory elements are within 7 kb upstream of the coding region of the odorant receptor (Qasba and Reed, 1998). Although this fragment recapitulates random expression in a subpopulation of olfactory neurons, it does not correctly specify zonal expression and it does not limit expression to the correct subpopulation. Because of this unpredictability in expression pattern this 7 kb promoter region is not appropriate for studies looking at targeting of specific subpopulations of axons.

Interestingly, *in ovo* electroporation was recently used to mis-express dominant negative neuropilin-1 in chick olfactory axons (Renzi *et al*., 2000). Although this technique in chick does not provide the same level of resolution capable by transgenesis in mouse, it at least demonstrates proof of principle for the mis-expression approach. The advantage of using a knock-in strategy rather than transgenesis (apart from the obvious fact that promoters do not have to be defined) is that it is possible to create a competitive environment where wild-type axons are competing with genetically modified axons for the same glomerular space. Since odorant receptors are monoallelically expressed (Chess *et al*., 1994), each olfactory neuron subpopulation consists of a mosaic of neurons randomly expressing either one allele or the other but never both. Consequently in mice heterozygous for a knock-in in one receptor gene, approximately half of the neurons from that subpopulation would be expressing the wild-type gene while the other half would be expressing from the genetically modified locus. In this way it is possible to knock-in dominant negative forms of guidance molecules in half of the subpopulation of, for example, P2 neurons. Such a competitive environment is advantageous since it also allows one to determine whether axon guidance molecules act cell autonomously. By directly comparing axon trajectories between wild-type and mutated axons in the same animal critical guidance points could also be readily identified. One could extend this approach to begin a systematic structure–function analysis of the role of odorant receptors in axon guidance. For example, the P2 coding region could be replaced with a P2 allele modified by site-directed mutagenesis. The P2 gene is a prime candidate for such an analysis since P2 axon targeting is independent of electrical activity (Lin *et al*., 2000; Zheng *et al*., 2000) and hence would be unaffected by activity changes arising from modifications of the P2 receptor. In addition to the examination of molecules on the surface of olfactory axons, it should also be possible to mis-express molecules within the olfactory pathway in transgenic mice using promoters specific for ensheathing cells and not for axons. In this way the contribution of these cells to growth and guidance of olfactory axons could be revealed.

The above genetic manipulations should be complemented by *in vitro* culture approaches that have typically been so fruitful in other systems. Ideally what is needed is a slice culture of the embryonic olfactory pathway that includes the nasal pit, olfactory nerve and presumptive olfactory bulb so that the activity of specific molecules can be modified with function-blocking antibodies. This approach is particularly important for molecules such as those in the extracellular matrix whose spatio-temporal expression cannot be easily manipulated using genetic techniques or for molecules whose mis-expression is embryonic lethal. It should be pointed out however that early embryonic lethals can be circumvented by using conditional expression constructs in transgenic animals. In this approach, the expression of specific genes is dependent on the presence of exogenous transcriptional activators such as doxycycline (Gogos *et al*., 2000). Culture systems will also be important for examining the role of ensheathing cells in stimulating axon growth. These cells have proven useful in the regeneration of spinal cord axons and yet we still do not understand why they are so effective as substrates for axon growth in the olfactory system (Tisay and Key, 1999). A culture assay system will prove invaluable as more of these cells are subjected to genetic manipulation (Imaizumi *et al*., 2000). In fact, the co-culture of ensheathing cells (purified either from transgenic animals globally mis-expressing molecules or from knock-out animals) with neurons from wild-type neurons provides another means of assessing their role in development of the olfactory pathway. Alternatively, a similar approach can be used where neurons are derived from transgenic or knock-out animals while the ensheathing cells are from wild-type animals. Analysis of the interactions of these cells and axons in real time at the single-cell level should provide a unique insight into the cellular interactions occurring in the olfactory pathway which would not be achieved from *post hoc* analysis *in situ*. Progress in understanding the role of odorant receptors in axon growth has been hindered by difficulties in expressing these molecules *in vitro* so that they can be used as substrates in neurite

outgrowth assays. However, recent advances in the expression of these molecules in heterologous systems should facilitate structure–function analyses in axon growth (Krautwurst *et al*., 1998; Yasuoka *et al*., 2000; Gimelbrant *et al*., 2001; Wetzel *et al*., 2001). Moreover, the generation of olfactory neuron cell lines with *in vivo*-like characteristics (Murrell and Hunter, 1999) will prove invaluable for analyses of neurite outgrowth and will provide a rapid means of identifying potentially interesting guidance molecules.

Finally, it is interesting to consider whether the regenerating olfactory system will provide clues as to the nature of targeting mechanisms. A select subpopulation of olfactory sensory neurons which are conditionally ablated by genetic techniques are able to regenerate and correctly target their appropriate glomerulus (Gogos *et al*., 2000). In contrast, when all olfactory neurons are chemically ablated, larger subpopulations of axons identified by expression of a transgene do not appear to fully recapitulate their endogenous projection pattern to the bulb (Cummings *et al*., 2000). The genetic ablation experiments indicate that the appropriate targeting cues must be present in the adult olfactory bulb. The complete chemical ablation paradigm suggests that if axon organization is severely disrupted in the olfactory nerve fibre layer then axons may not reach their correct target site. More severe targeting defects are observed when the olfactory nerve pathway is physically lesioned (Costanzo, 2000). In this case, axons continue to converge but do so at multiple sites. These results implicate either axon–axon and/or axon–glial interactions in sorting axons as a necessary prerequisite for targeting. By comparing and contrasting mechanisms of axon targeting during development and different regeneration paradigms, it may be possible to identify important cell–cell interactions. What is currently emerging from analyses of normal development and regeneration is that glial cells in the olfactory pathway may be providing critical cues for the guidance of axons to their correct target. This is certainly consistent with results from analyses in the moth *Manduca sexta* where specialized glia are essential for olfactory axon targeting (Rössler *et al*., 1999).

Acknowledgements

This work was supported by Australian National Health and Medical Research Council grants to J.S.J. and B.K., by a research grant from the Mituzanti Foundation for Glycoscience to B.K. and by a Rodney Williams and Garnett Passe Memorial Foundation research grant to J.S.J. J.S.J. was supported by a National Health and Medical Research Council Peter Doherty Fellowship.

References

Alenius, M. and **Bohm, S.** (1997) *Identification of a novel neural cell adhesion molecule-related gene with a potential role in selective axonal projection.* J. Biol. Chem., 272, 26 083–26 086.

Allen, W.K. and **Akeson, R.** (1985) *Identification of a cell surface glycoprotein family of olfactory receptor neurons with a monoclonal antibody.* J. Neurosci., 5, 284–296.

Alvarez-Bolado, G., Rosenfeld, M.G. and **Swanson, L.W.** (1995) *Model of forebrain regionalization based on spatiotemporal patterns of POU-III homeobox gene expression, birthdates, and morphological features.* J. Comp. Neurol., 355, 237–295.

Anchan, R.M., Drake, D.P., Haines, C.F., Gerwe, E.A. and **LaMantia, A.S.** (1997) *Disruption of local retinoid-mediated gene expression accompanies abnormal development in the mammalian olfactory pathway.* J. Comp. Neurol., 379, 171–184.

Ba-Charvet, T.T.N., Brose, K., Marillat, V., Kidd, T., Goodman, C.S., Tessier-Lavigne, M., Sotelo, C. and **Chédotal, A.** (1999) *Slit2-mediated chemorepulsion and collapse of developing forebrain axons.* Neuron, 22, 463–473.

Bailey, M.S., Puche, A.C. and **Shipley, M.T.** (1999) *Development of the olfactory bulb: evidence for glia–neuron interactions in glomerular formation.* J. Comp. Neurol., 415, 423–448.

Behrens, M., Venkatraman, G., Gronostajski, R.M., Reed, R.R. and **Margolis, F.L.** (2000) *NFI in the development of the olfactory neuroepithelium and the regulation of olfactory marker protein gene expression.* Eur. J. Neurosci., 12, 1372–1384.

Braisted, J.E., McLaughlin, T., Wang, H.U., Freidman, G.C. and **Anderson, D.D.** (1997) *Graded and lamina-specific distributions of ligands of EphB receptor tyrosinse kinases in the developing retinotectal system.* Dev. Biol., 191, 14–28.

Buck, L. and **Axel, R.** (1991) *A novel multigene family may encode odorant receptors: a molecular basis for odor recognition.* Cell, 65, 175–87.

Bulfone, A., Wang, F., Hevner, R., Anderson, S., Cutforth, T., Chen, S., Meneses, J., Pedersen, R., Axel, R. and **Rubenstein, J.L.** (1998) *An olfactory sensory map develops in the absence of normal projection neurons or GABAergic interneurons.* Neuron, 21, 1273–1282.

Chess, A., Simon, I., Cedar, H. and **Axel, R.** (1994) *Allelic inactivation regulates olfactory receptor gene.* Cell, 78, 823–834.

Clarris, H. Rauch, U. and **Key, B.** (2000) *Dynamic spatiotemporal expression patterns of neurocan and phosphacan indicate diverse roles in the developing and adult mouse olfactory system.* J. Comp. Neurol., 46, 113–125.

Crandall, J.E., Dibble, C., Butler, D., Pays, L., Ahmad, N., Kostek, C., Puschel, A.W. and **Schwarting, G.A.** (2000) *Patterning of olfactory sensory connections is mediated by extracellular matrix proteins in the nerve fibre layer of the olfactory bulb.* J. Neurobiol., 45, 195–206.

Costanzo, R.M. (2000) *Rewiring the olfactory bulb: changes in odor maps following recovery from nerve transection.* Chem. Senses, 25, 199–205.

Cummings, D.M., Emge, D.E., Small, S.L. and **Margolis, F.L.** (2000) *Pattern of olfactory bulb innervation returns after recovery from reversible peripheral deafferentation.* J. Comp. Neurol., 421, 362–373.

Davenport, R.W., Thies, E. and **Nelson, P.G.** (1996) *Cellular localization of guidance cues in the establishment of retinotectal topography.* J. Neurosci., 16, 2074–2085.

DeLong, G.R. and **Coulombre, A.J.** (1967) *The specificity of retinotectal connections studied by retinal grafts onto the optic tectum in chick embryos.* Dev. Biol., 16, 513–531.

Doucette, R. (1989) *Development of the nerve fiber layer in the olfactory bulb of mouse embryos.* J. Comp. Neurol. 285, 514–527.

Dowsing, B., Puche, A.C., Hearn, C. and **Key, B.** (1997) *The presence of novel N-CAM glycoforms in the rat olfactory systerm.* J. Neurobiol., 32, 659–670.

Dynes, J.L. and **Ngai, J.** (1998) *Pathfinding of olfactory neuron axons to stereotyped glomerular targets revealed by dynamic imaging in living zebrafish embryos.* Neuron, 20, 1081–1091.

Ebrahimi, F.A. and **Chess, A.** (2000) *Olfactory neurons are interdependent in maintaining axonal projections.* Curr. Biol., 10, 219–222.

Farbman, A.I. and **Squinto, L.M.** (1985) *Early development of olfactory receptor cell axons.* Brain Res., 351, 205–213.

Gierer, A. (1998) *Possible involvement of gradients in guidance of receptor cell axons towards their target position on the olfactory bulb.* Eur. J. Neurosci., 10, 388–391.

Gimelbrant, A.A., Haley, S.L. and **McClintock, T.S.** (2001) *Olfactory receptor trafficking involves conserved regulatory steps.* J. Biol. Chem., 276, 7285–7290.

Gogos, J.A., Osborne, J., Nemes, A., Mendelsohn, M. and **Axel, R.** (2000) *Genetic ablation and restoration of the olfactory topographic map.* Cell, 103, 609–620.

Gong, Q. and **Shipley, M.T.** (1996) *Expression of extracellular matrix molecules and cell surface molecules in the olfactory nerve pathway during early development.* J. Comp. Neurol., 366, 1–14.

Hardelin, J.-P. (2001) *Kallmann syndrome: towards molecular pathogenesis.* Mol. Cell. Endocrinol., 179, 75–81.

Hardelin, J.-P., Julliard, A.K., Moniot, B., Soussi-Yanicostas, N., Verney, C., Schwanzel-Fukuda, M., Ayer-Le Lievre, C. and **Petit, C.** (1999) *Anosmin-1 is a regionally restricted component of basement membranes and interstitial matrices during organogenesis: implications for the developmental anomalies of X chromosome-linked Kallmann syndrome.* Dev. Dyn., 215, 26–44.

Hentschel, H.G.E. and **Van Ooyen, A.** (1999) *Models of axon guidance and bundling during development.* Proc. R. Soc. Lond. B, 266, 2231–2238.

Hinds, J.W. (1968) *Autoradiographic study of histogenesis in the mouse olfactory bulb.* I. Time of origin of neurons and neuroglia. J. Comp. Neurol., 134, 287–304.

Imaizumi, T., Lankford, K.L., Burton, W.V., Fodor, W.L. and **Kocsis, J.D.** (2000) *Xenotransplantation of transgenic pig olfactory ensheathing cells promotes axonal regeneration in rat spinal cord.* Nat. Biotechnol., 18, 949–953.

Ishii, T., Serizawa, S., Kohda, A., Nakatani, H., Shiroishi, T., Okumura, K., Iwakura, Y., Nagawa, F., Tsuboi, A. and **Sakano, H.** (2001) *Monoallelic expression of the odorant receptor gene and axonal projection of olfactory sensory neruones.* Genes Cells, 6, 71–78.

Kafitz, K.W. and **Greer, C.A.** (1998a) *Differential expression of extracellular matrix and cell adhesion molecules in the olfactory nerve and glomerular layers of adult rats.* J. Neurobiol., 34, 271–282.

Kafitz, K.W. and **Greer, C.A.** (1998b) *The influence of ensheathing cells on olfactory receptor cell neurite outgrowth* in vitro*.* Ann. N.Y. Acad. Sci., 855, 266–269.

Kafitz, K.W. and **Greer, C.A.** (1999) *Olfactory ensheathing cells promote neurite extension from embryonic olfactory receptor cells* in vitro*.* Glia, 25, 99–110.

Key, B. and **Akeson, R.A.** (1993) *Distinct subsets of sensory olfactory neurons: possible role in the formation of the mosaic olfactory projection.* J. Comp. Neurol., 335, 355–368.

Klenoff, J.R. and **Greer, C.A.** (1998) *Postnatal development of olfactory receptor cell axonal arbors.* J. Comp. Neurol., 390, 256–267.

Kramer, P.R. and **Wray, S.** (2000) *Novel gene expressed in nasal region influences outgrowth of olfactory axons and migration of luteinizing hormone-releasing hormone (LHRH) neurons.* Genes Dev., 14, 1824–1834.

Krautwurst, D., Yau, K.W. and **Reed, R.R.** (1998) *Identification of ligands for olfactory receptors by functional expression of a receptor library.* Cell, 95, 917–926.

Kudrycki, K.E., Buiakova, O., Tarozzo, G., Grillo, M., Walters, E. and **Margolis, F.L.** (1998) *Effects of mutation of the Olf-1 motif on transgene expression in olfactory receptor neurons.* J. Neurosci. Res., 52, 159–172.

LaMantia, A.S., Colbert, M.C. and **Linney, E.** (1993) *Retinoic acid induction and regional differentiation prefigure olfactory pathway formation in the mammalian forebrain.* Neuron, 10, 1035–1048.

LaMantia, A.S., Bhasin, N., Rhodes, K. and **Heemskerk, J.** (2000) *Mesenchyme/epithelial induction mediates olfactory pathway formation.* Neuron, 28, 411–425.

Lin, D.M., Fetter, R.D., Kopczynski, C., Grenningloh, G. and **Goodman, C.S.** (1994) *Genetic analysis to Fasciclin II in* Drosophila*: defasciculation, refasciculation, and altered fasciculation.* Neuron, 13, 1055–1069.

Lin, D.M., Wang, F., Lowe, G., Gold, G.H., Axel, R., Ngai, J. and **Brunet, L.** (2000) *Formation of precise connections in the olfactory bulb occurs in the absence of odorant-evoked neuronal activity.* Neuron, 26, 69–80.

Liu, K.L., Chuah, M.I. and **Lee, K.K.** (1995) *Soluble factors from the olfactory bulb attract olfactory Schwann cells.* J. Neurosci., 15, 990–1000.

Lu, Q., Sun, E.E., Klein, R.S. and **Flanagan, J.G.** (2001) *Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction.* Cell, 105, 69–79.

Malun, D. and **Brunjes, P.C.** (1996) *Development of olfactory glomeruli: temporal and spatial interactions between olfactory receptor axons and mitral cells in opossums and rats.* J. Comp. Neurol., 368, 1–16.

Marin-Padilla, M. and **Amieva, M.R.** (1989) *Early neurogenesis of the mouse olfactory nerve: Golgi and electron microscopic studies.* J. Comp. Neurol. 288, 339–352.

Miyawaki, A., Homma, H., Tamura, H., Matsui, M. and **Mikoshiba, K.** (1996) *Zonal distribution of sulfotransferase for phenol in olfactory sustentacular cells.* EMBO J., 15, 2050–2055.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Nemes, A., Mendelsohn, M., Edmondson, J. and **Axel, R.** (1996) *Visualizing an olfactory sensory map.* Cell, 87, 675–686.

Morse, W.R., Whiteshead, J.G., LaMantia, A.S. and **Maness, P.F.** (1998) *$p59^{fyn}$ and $pp60^{c\text{-}src}$ modulate axonal guidance in the developing mouse olfactory pathway.* J. Neurobiol., 36, 53–63.

Murrell, J.R. and **Hunter, D.D.** (1999) *An olfactory sensory neuron line, odora, properly targets olfactory proteins and responds to odorants.* J. Neurosci., 19, 8260–8270.

Nagao, H., Yoshihara, Y., Mitsui, S., Fujisawa, H. and **Mori, K.** (2000) *Two mirror-image sensory maps with domain organization in the mouse main olfactory bulb.* Neuroreport, 11, 3023–3027.

Nakamoto, M., Cheng, H.J., Friedman, G.C., McLaughlin, T., Hansen, M.J., Yoon, C.H., O'Leary, D.D. and **Flanagan, J.G.** (1996) *Topographically specific effects of ELF-1 on retinal axon guidance* in vitro *and retinal axon mapping* in vivo*.* Cell, 86, 755–766.

Nellen, D., Burke, R., Struhl, G. and **Basler, K.** (1996) *Direct and long-range action of a DPP morphogen gradient.* Cell, 85, 357–68.

Oland, L.A. and **Tolbert, L.P.** (1996) *Multiple factors shape development of*

olfactory glomeruli: insights from an insect model system. J. Neurobiol., 1, 92–109.

Pasterkamp, R.J., de Winter, F., Holtmaat, A.J.G.D. and **Verhaagan, J.** (1998) *Evidence for a role of the chemorepellent Semaphorin III and its receptor neuropilin-1 in the regeneration of primary olfactory axons.* J. Neurosci., 18, 9962–9976.

Pays, L. and **Schwarting, G.** (2000) *Gal-NCAM is differentially expressed marker for mature sensory neurons in the rat olfactory system.* J. Neurobiol., 43, 173–185.

Puche, A.C. and **Shipley, M.T.** (2001) *Radial glia development in the mouse olfactory bulb.* J. Comp. Neurol., 434, 1–12.

Puche, A.C., Poirier, F., Hair, M., Bartlett, P. and **Key, B.** (1996) *Role of galectin-1 in the developing mouse olfactory system.* Dev. Biol., 179, 274–287.

Pyrski, M., Zheng, X., Walters, E., Gilbert, D.J., Jenkins, N.A., Copeland, N.G. and **Margolis, F.L.** (2001) *The OMP-LacZ transgene mimics the unusual expression pattern of OR-Z6, a new odorant receptor gene on mouse chromosome 6: implication for locus-dependent gene expression.* J. Neurosci., 21, 4637–4648.

Qasba, P. and **Reed, R.R.** (1998) *Tissue and zonal-specific expression of an olfactory receptor transgene.* J. Neurosci., 18, 227–236.

Ramón-Cueto, A. and **Avila, J.** (1998) *Olfactory ensheathing glia: properties and function.* Brain Res. Bull., 46, 175–187.

Ramón-Cueto, A. and **Valverde, F.** (1995) *Olfactory bulb ensheathing glia: a unique cell type with axonal growth-promoting properties.* Glia, 14, 163–73.

Renzi, M.J., Wexler, T.L. Raper, J.A. (2000) *Olfactory sensory axons expressing a dominant-negative semaphorin receptor enter the CNS early and overshoot their target.* Neuron, 28, 437–447.

Riddle, D.R., Wong, L.D. and **Oakley, B.** (1993) *Lectin identification of olfactory receptor neuron subclasses with segregated central projections.* J. Neurosci., 7, 3018–3033.

Robertson, A., MacColl, G.S., Nash, J.A., Boehm, M.K., Perkins, S.J. and **Bouloux, P.M.** (2001) *Molecular modelling and experimental studies of mutation and cell-adhesion sites in the fibronectin type III and whey acidic protein domains of human anosmin-1.* Biochem. J., 357, 647–659.

Rodriguez, I., Feinstein, P. and **Mombaerts, P.** (1999) *Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system.* Cell, 97, 199–208.

Rössler, W., Oland, L.A., Higgins, M.R., Hildebrand, J.G. and **Tolbert, L.P.** (1999) *Development of a glia-rich axon-sorting zone in the olfactory pathway of the moth* Manduca sexta*.* J. Neurosci., 19, 9865–9877.

Royal, S.J. and **Key, B.** (1999) *Development of P2 olfactory glomeruli in P2-IRES-tau-LacZ transgenic mice.* J. Neurosci., 19, 9856–9864.

Rutishauser, U. (2000) *Defining a role and mechanism for IgCAM function in vertebrate axon guidance.* J. Cell Biol., 149, 757–759.

Schaefer, M.L., Finger, T.E. and **Restrepo, D.** (2001) *Variability of position of the P2 glomerulus within a map of the mouse olfactory bulb.* J. Comp. Neurol., 436, 351–362.

Schmucker, D. and **Zipursky, S.L.** (2001) *Signaling downstream of Eph receptors and ephrin ligands.* Cell, 105, 701–704.

Schwarting, G.A., Kostek, C., Ahmad, N., Dibble, C., Pays, L., Puschel, A.W. (2000) *Semaphorin 3A is required for guidance of olfactory axons in mice.* J. Neurosci., 20, 7691–7697.

Schwarting, G.A., Kostek, C., Bless, E.P., Ahmad, N. and **Tobet, S.A.** (2001) *Deleted in colorectal cancer (DCC) regulates the migration of luteinizing hormone-releasing hormone neurons to the basal forebrain.* J. Neurosci., 21, 911–919.

Serizawa, S., Ishii, T., Nakatani, H., Tsuboi, A., Nagawa, F., Asano, M., Sudo, K., Sakagami, J., Sakano, H., Ijiri, T., Matsuda, Y., Suzuki, M., Yamamori, T., Iwakura, Y. and **Sakano, H.** (2000) *Mutually exclusive expression of odorant receptor transgenes.* Nat. Neurosci., 3, 687–693.

Simpson, J.H., Bland, K.S., Fetter, R.D. and **Goodman, C.S.** (2000) *Short-range and long-range guidance by Slit and its Robo receptors: a combinatorial code of Robo receptors controls lateral position.* Cell, 103, 1019–1032.

Soussi-Yanicostas, N., Hardelin, J.-P., del Mar Arroyo-Jimenez, M., Ardouin, O., Legouis, R., Levillers, J., Traincard, F., Betton, J.-M., Cabanie, L. and **Petit, C.** (1996) *Initial characterization of anosmin-1, a putative extracellular matrix protein synthesized by definite neuronal cell populations in the central nervous system.* J. Cell Sci., 109, 1749–1757.

Soussi-Yanicostas, N., Faivre-Sarrailh, C., Hardelin, J.-P., Levillers, J., Rougon, G. and **Petit, C.** (1998) *Anosmin-1 underlying the X-chromosome-linked Kallmann syndrome is an adhesion molecule that can modulate neurite growth in a cell-type specific manner.* J. Cell Sci., 111, 2953–2965.

Sperry, R.W. (1944) *Optic nerve regeneration with return of vision in anurans.* J. Neurophysiol., 7, 57–69.

Stier, H. and **Schlosshauer, B.** (1999) *Cross-species collapse activity of polarized radial glia on retinal ganglion cell axons.* Glia, 25, 143–153.

Stein, E. and **Tessier-Lavigne, M.** (2001) *Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex.* Science, 291, 1928–1938.

St John, J. and **Key, B.** (1999) *Expression of galectin-1 in the olfactory nerve pathway of rat.* Dev. Brain Res., 117, 171–178.

St John, J. and **Key, B.** (2001a) *Chemically and morphologically identifiable glomeruli in the rat olfactory bulb.* J. Comp. Neurol., 436, 497–507.

St John, J. and **Key, B.** (2001b) *EphB2 and two of its ligands have dynamic expression patterns in the developing olfactory system.* Dev. Brain Res., 126, 43–56.

St John J.A., Tisay K.T., Caras I.W. and **Key, B.** (2000) *The expression of EphA5 during development in the olfactory nerve pathway of rat.* J. Comp. Neurol., 416, 540–50.

Strotmann, J., Conzelmann, S., Beck, A., Feinstein, P., Breer, H. and **Mombaerts, P.** (2000) *Local permutations in the glomerular array of the mouse olfactory bulb.* J. Neurosci., 20, 6927–6938.

Takahashi, T. and **Strittmatter, S.M.** (2001) *Plexinal autoinhibition by the plexin sema domain.* Neuron, 29, 429–439.

Tang, J., Rutishauser, U. and **Landmesser, L.** (1994) *Polysialic acid regulates growth cone behavior during sorting of motor axons in the plexus region.* Neuron, 13, 405–414.

Tenne-Brown, J. and **Key, B.** (1999) *Errors in lamina growth of olfactory axons in the rat and mouse olfactory bulb.* J. Comp. Neurol., 410, 20–30.

Tenne-Brown, J., Puche, A. and **Key, B.** (1998) *Expression of the â-galactose binding lectin, galectin-1, in the mouse olfactory system.* Int. J. Dev. Biol., 42, 791–799.

Tennent, R. and **Chuah, M.I.** (1996) *Ultrastructural study of ensheathing cells in early development of olfactory axons.* Dev. Brain Res., 95, 135–139.

Tisay, K.T. and **Key, B.** (1999) *The extracellular matrix modulates olfactory neurite outgrowth on ensheathing cells.* J. Neurosci., 19, 9890–9899.

Tolbert, L.P. (1998) *Olfactory development in invertebrates.* On the scent of central developmental issues. Ann. N.Y. Acad. Sci., 855, 95–103.

Treloar, H.B., Nurcombe, V. and **Key, B.** (1996a) *Expression of extracellular matrix molecules in the embryonic rat olfactory pathway.* J. Neurobiol., 31, 41–55.

Treloar, H.B., Walters, T., Margolis, F. and **Key, B.** (1996b) *Olfactory glomeruli are innervated by more than one distinct subset of primary sensory olfactory neurons in mice.* J. Comp. Neurol., 367, 550–562.

Treloar, H.B., Tomasiewicz, H., Magnuson, T. and **Key, B.** (1997) *The olfactory nerve pathway is abnormal in mice lacking the N-CAM-180 isoform.* J. Neurobiol., 32, 643–658.

Treloar, H.B., Purcell, A.L. and **Greer, C.A.** (1999) *Glomerular formation in the developing rat olfactory bulb.* J. Comp. Neurol., 413, 289–304.

Trowe, T., Klostermann, S., Baier, H., Granato, M., Crawford, A.D., Grunewald, B., Hoffmann, H., Karlstrom, R.O., Meyer, S.U., Müller, B., Richter, B., Nüsslein-Volhard, C. and **Bonhoeffer, F.** (1996) *Mutations disrupting the ordering and topographical mapping of axons in the retinotectal projection of the zebrafish,* Danio rerio*.* Development, 123, 439–450.

Tsuboi, A., Yoshihara, S., Yamazaki, N., Kasai, H., Asai-Tsuboi, H., Komatsu, M., Serizawa, S., Ishii, T., Matsuda, Y., Nagawa, F. and **Sakano, H.** (1999) *Olfactory neurons expressing closely linked and homologous odorant receptor genes tend to project their axons to neighboring glomeruli on the olfactory bulb.* J. Neurosci., 19, 8409–8418.

Valverde, F., Santacana, M. and **Heredia, M.** (1992) *Formation of an olfactory glomerulus: morphological aspects of development and organization.* Neuroscience, 49, 255–275.

Vanselow, J., Thanos, S., Godement, P., Henke-Fahle, S. and **Bonhoeffer, F.** (1989) *Spatial arrangement of radial glia and ingrowing retinal axons in the chick optic tectum during development.* Brain Res. Dev. Brain Res., 45, 15–27.

Vassar, R., Chao, S.K., Sitcheran, R., Nunez, J.M., Vosshall, L.B. and **Axel, R.** (1994) *Topographic organization of sensory projections to the olfactory bulb.* Cell, 79, 981–991.

Walters, E., Grillo, M., Tarozzo, G., Stein-Izsak, C., Corbin, J., Bocchiaro, C. and **Margolis, F.L.** (1996) *Proximal regions of the olfactory marker protein gene promoter direct olfactory neuron-specific expression in transgenic mice.* J. Neurosci. Res., 43, 146–160.

Wang, F., Nemes, A., Mendelsohn, M. and **Axel, R.** (1998) *Odorant receptors govern the formation of a precise topographic map.* Cell, 93, 47–60.

Watanabe, E., Matsui, F., Keino, H., Ono, K., Kushima, Y., Noda, M. and Oohira, A. (1996) *A membrane-bound heparan sulfate proteoglycan that is transiently expressed on growing axons in the rat brain.* J. Neurosci Res., 44, 84–96.

Wetzel, C.H., Behrendt, H.J., Gisselmann, G., Stortkuhl, K.F., Hovemann, B. and **Hatt, H.** (2001) *From the cover: functional expression and characterization of a* Drosophila *odorant receptor in a heterologous cell system.* Proc. Natl Acad. Sci. USA, 98, 9377–9380.

Whitesides, J. and **LaMantia, A.S.** (1996) *Differential adhesion and the initial assembly of the mammalian olfactory nerve.* J. Comp. Neurol., 373, 240–254.

Whitesides, J., Hall, M., Anchan, R., LaMantia, A.S. (1998) *Retinoid signaling distinguishes a subpopulation of olfactory receptor neurons in the developing and adult mouse.* J. Comp. Neurol., 394, 445–461.

Williams-Hogarth, L.C., Puche, A.C., Torrey, C., Cai, X., Song, I., Kolodkin, A.L., Shipley, M.T. and **Ronnett, G.V.** (2000) *Expression of semaphorins in developing and regenerating olfactory epithelium.* J. Comp. Neurol., 423, 565–578.

Winberg, M., Mitchell, K.J. and **Goodman, C.S.** (1998) *Genetic analysis of the mechanisms controlling target selection: complementary and combinatorial functions of netrins, semaphorins, and IgCAMs.* Cell, 93, 581–591.

Yasuoka, A., Emori, Y. and **Abe, K.** (2000) *Addition of signal sequences to the N-termini of olfactory receptor proteins enhances their expression in* Xenopus *oocytes.* Biosci. Biotechnol. Biochem., 64, 1688–1695.

Yilmazer-Hanke, D.M., Hudson, R. and **Distel, H.** (2000) *Morphology of developing olfactory axons in the olfactory bulb of the rabbit (*Oryctolagus cuniculus*): a Golgi study.* J. Comp. Neurol., 426, 68–80.

Yoshihara, Y., Kawasaki, M., Tamada, A., Fujita, H., Hayashi, H., Kagamiyama, H., and **Mori, K.** (1997) *OCAM: A new member of the neural cell adhesion molecule family related to zone-to-zone projection of olfactory and vomeronasal axons.* J. Neurosci., 17, 5830–5842.

Yu, H.H., Huang, A.S. and **Kolodkin, A.L.** (2000) *Semaphorin-1a acts in concert with cell adhesion molecules Fasciclin II and connectin to regulate axon fasciculation in* Drosophila*.* Genetics, 156, 723–731.

Yuan, W., Zhou, L., Chen, J., Wu, J.Y., Rao, Y. and **Ornitz, D.M.** (1999) *The mouse SLIT family: secreted ligands for ROBO expressed in patterns that suggest a role in morphogenesis and axon guidance.* Dev. Biol., 212, 290–306.

Zhao, H. and **Reed, R.R.** (2001) *X inactivation of the OCNC1 channel gene reveals a role for activity-dependent competition in the olfactory system.* Cell, 104, 651–660.

Zheng, C., Feinstein, P., Bozza, T., Rodriguez, I. and **Mombaerts, P.** (2000) *Peripheral olfactory projections are differentially affected in mice deficient in a cyclic nucleotide-gated channel subunit.* Neuron, 26, 81–91.

Accepted November 29, 2001