Axonal Ephrin-As and Odorant Receptors: Coordinate Determination of the Olfactory Sensory Map

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Summary

Olfactory sensory neurons expressing a given odorant receptor (OR) project with precision to specific glomeruli in the olfactory bulb, generating a topographic map. In this study, we demonstrate that neurons expressing different ORs express different levels of ephrin-A protein on their axons. Moreover, alterations in the level of ephrin-A alter the glomerular map. Deletion of the ephrin-A5 and ephrin-A3 genes posteriorizes the glomerular locations for neurons expressing either the P2 or SR1 receptor, whereas overexpression of ephrin-A5 in P2 neurons results in an anterior shift in their glomeruli. Thus the ephrin-As are differentially expressed in distinct subpopulations of neurons and are likely to participate, along with the ORs, as one of a complement of guidance receptors governing the targeting of like axons to precise locations in the olfactory bulb.

Introduction

Neurons connect to one another with remarkable precision. In most sensory systems, peripheral neurons project axons to precise locations in the central nervous system to create an internal representation of the sensory world that translates stimulus features into neural information. In the mammalian olfactory system, the repertoire of odorants is detected by a large family of olfactory receptors (ORs). Individual olfactory sensory neurons express only one of 1000 OR genes such that each neuronal subtype is functionally distinct and recognizes a restricted array of odorant molecules (Mombaerts, 2001). Neurons expressing the same OR are widely distributed in the olfactory epithelium, yet their axons converge to precise glomerular positions in the olfactory bulb (Mombaerts et al., 1996; Ressler et al., 1994; Vassar et al., 1994). The bulb therefore contains a spatial map that identifies which of the numerous receptors have been activated. The quality of an olfactory stimulus may

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therefore be encoded by the specific combination of glomeruli activated by a given odorant.

The spatial map present in the olfactory system differs in character from the representation of sensory cells in the auditory, somatosensory, and visual systems (Rubel and Fritzsch, 2002; Killackey et al., 1995; O'Leary et al., 1999). In these sensory systems, neurons within the peripheral receptor sheet project to the central nervous system in a continuous manner such that neighbor relations in the periphery are maintained in the brain. Topographically ordered sensory cells can therefore exploit spatial information inherent in the periphery to direct their projections to the brain. This organization is in sharp contrast to the discontinuous map present in the olfactory system. Peripheral olfactory neurons are unlikely to acquire a positional identity that guides their projections to the brain since the neurons expressing a given receptor are randomly dispersed within broad zones in the sensory epithelium (Ressler et al., 1993; Vassar et al., 1993). Spatial order is imposed more centrally, where similar axons converge on two positionally invariant glomeruli to create a map of receptor activation in the olfactory bulb. These data suggest that individual olfactory sensory neurons expressing a given receptor possess an identity independent of their position in the epithelium that dictates the pattern of their projections to the olfactory bulb.

The association between the choice of an OR and the site of axonal convergence has suggested that the OR might also function as a guidance receptor. In such a model, the OR expressed on dendrites would recognize odors in the environment, while the same protein expressed on axon termini would recognize a distinct set of guidance cues present in the bulb. Genetic experiments that result in either deletions or substitutions of specific receptor genes strongly suggest that the OR plays an instructive role in directing axons to precise glomerular positions (Wang et al., 1998). Substitution of the P2 coding sequence with the M71 OR gene, for example, results in convergence of axons at a glomerular target that differs from that of either M71 or P2 neurons. These observations suggest that guidance receptors other than the ORs also contribute to precise glomerular targeting. Moreover, neurons expressing different ORs are likely to express different levels of these guidance molecules.

What is the identity of the guidance molecules that collaborate with the ORs to establish a topographic map in the olfactory system? The large family of Eph receptor tyrosine kinases and their membrane-associated ligands, the ephrins, have been implicated in the formation of topographic maps in other sensory and motor systems (reviewed in Wilkinson, 2001). In the visual system, for example, a nasotemporal gradient of EphA receptors on retinal axons, combined with an overlapping gradient of two ephrin ligands with repellent activities (ephrin-A5 and ephrin-A2) in the tectum or superior colliculus, regulates the continuous mapping of retinal ganglion cells to brain centers (Brown et al., 2000; Feldheim et al., 2000; Hornberger et al., 1999). In the somatosensory

system, EphA/ephrin-A interactions are involved in the targeting of axons from the ventrobasal complex of the thalamus to appropriate targets in the cortex (Prakash et al., 2000; Vanderhaeghen et al., 2000). Ephrin-As have also been implicated in regulating anteroposterior patterning of vomeronasal sensory axons in the accessory olfactory bulb (Knöll et al., 2001).

In this study, we demonstrate that members of the ephrin-A subfamily play an instructive role in the formation of a precise topographic map in the olfactory bulb. Sensory neurons express both ephrin-A3 and ephrin-A5, whereas target cells in the olfactory bulb express EphA receptors (Knöll et al., 2001; St. John et al., 2000, 2002; Zhang et al., 1996, 1997). If ephrin-A levels play an instructive role in the migration and convergence of like axons to appropriate glomerular targets, we might expect that neurons expressing different ORs would express different levels of ephrin-A on their axons. In accordance with this model, we detect a mosaic distribution of ephrin-A protein on sensory axon termini, such that different glomeruli contain different levels of ephrin. The level of ephrin-A in axons correlates with the identity of the OR gene expressed by those neurons. In mutant mice lacking both ephrin-A3 and ephrin-A5, neurons expressing two different ORs exhibit a significant displacement of their glomerular targets in the olfactory bulb. Conversely, increased levels of ephrin-A5 in a subset of neurons expressing the same OR result in a displacement of the glomerular target in the opposite direction. These data suggest that ephrin-A molecules act together with ORs as guidance molecules that direct the formation of a sensory map in the olfactory bulb.

Results

Differential and Conserved Ephrin-A Protein Expression on Olfactory Neurons

We have characterized the expression of the vertebrate Eph and ephrin genes in the developing olfactory system. During embryonic and early postnatal stages, sensory neurons transiently express several members of the ephrin-A and ephrin-B gene families, along with their cognate Eph receptors (St. John et al., 2000, 2002; St. John and Key, 2001; Zhang et al., 1996). At postnatal day 3, a period of intense neurogenesis and axon outgrowth, the sensory neurons express two of the eight ephrins at high levels: ephrin-A3 and -A5 (Figures 1A–1F). Expression of these two ephrins persists beyond the first postnatal week, when all other Eph and ephrin proteins are no longer found on sensory axons. The levels of both ephrin-A3 and ephrin-A5 mRNA appear uniform in the sensory epithelium with no spatial pattern evident. Moreover, ephrin-A3 and ephrin-A5 mRNA appear equivalent in the four dorsoventral zones of the sensory epithelium.

We have also examined the expression of Eph and ephrin genes in the olfactory bulb. As in the sensory neurons, previous studies have demonstrated the transient expression of several Ephs and ephrins in the multiple cell types of the olfactory bulb during embryonic and early postnatal stages (Knöll et al., 2001; St. John and Key, 2001; St. John et al., 2000, 2002; Zhang et al., 1996, 1997). EphA3 and EphA5 mRNA are detected in

mitral and tufted cells and periglomerular cells at an early postnatal age by in situ hybridization (Figures 1G–1J). No spatial pattern of EphA expression can be discerned either by in situ hybridization or with an ephrin-A5 alkaline phosphatase fusion reagent that detects the sum of EphA protein levels (data not shown). Thus ephrin-A expression is observed on incoming olfactory sensory axons, and the cognate receptors, EphA3 and EphA5, are present in the olfactory bulb.

If ephrin expression by olfactory sensory neurons is involved in the formation of a topographic map in the olfactory bulb, we expect that ephrin-A proteins will be present on axon termini. We employed an EphA3 receptor ectodomain fused to alkaline phosphatase (EphA3-AP) to detect ephrin-A protein. This receptor binds to all ephrin-A family members (Cheng and Flanagan, 1994) and therefore allows us to identify sites of ephrin-A protein expression in the olfactory system. EphA3-AP binding is not observed on either the dendrites or cell bodies of the sensory neurons in the epithelium, but high levels of ephrin protein can be detected on axons innervating the olfactory bulb (Figures 2A and 2B). Sections through the olfactory bulb reveal intense staining in the outer nerve layer and within the glomeruli (Figures 2C–2G). The staining in glomeruli is likely to reflect ephrin-A expression in presynaptic sensory neurons rather than postsynaptic bulbar neurons since intense staining is also observed in the outer nerve layer, and levels of ephrin-A3 and ephrin-A5 mRNA are very low within the olfactory bulb (data not shown).

As described previously (St. John et al., 2000, 2002; Zhang et al., 1996), a mosaic pattern of ephrin-A protein expression is observed among individual glomeruli, with some glomeruli exhibiting intense expression and others containing far lower protein levels (Figures 2E–2G). The levels of ephrin-A differ among glomeruli but appear uniform within a given glomerulus. This mosaic pattern is apparent throughout the olfactory bulb, and glomeruli expressing high levels of ephrin-A are interspersed with those exhibiting low expression, with no discernible spatial regularity. Mosaic expression is observed for both ephrin-A3 and ephrin-A5 proteins individually since similar staining patterns with EphA3-AP are observed in mice mutant for either ephrin-A3 or ephrin-A5 alone (see below).

These experiments demonstrate that olfactory sensory neurons expressing different ORs and converging on different glomeruli express different levels of ephrins on their axon termini. We have employed the EphA3-AP fusion protein to detect ephrin-A levels in the olfactory bulb, and it is conceivable that this reagent binds only to free ephrin-A. Similar patterns of mosaicism, however, have been reported in the rat olfactory bulb using polyclonal antisera directed against ephrin-A5 (St. John et al., 2002). Thus EphA3-AP is an accurate reporter of ephrin-A levels, and the mosaic pattern is due to differential expression by OSNs rather than variations in free ephrin protein caused by differential EphA expression in the olfactory bulb. Similarly, we have used an ephrin-A5-AP fusion protein (Feldheim et al., 1998) to detect EphA protein in the olfactory bulb and observe similar staining in tissue from wild-type mice or mutants lacking both ephrin-A3 and ephrin-A5 (data not shown).

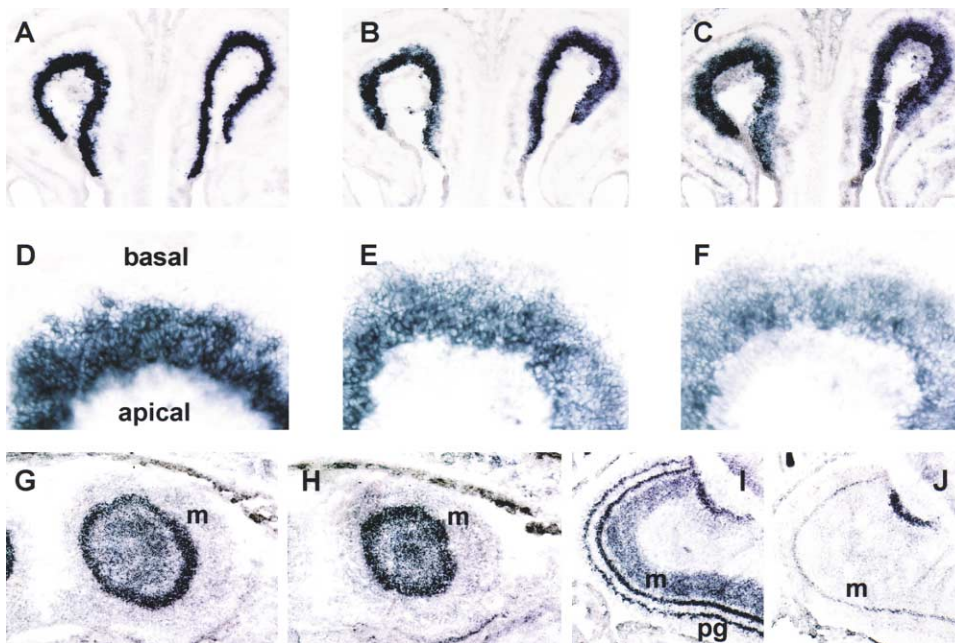If distinct ephrin-A levels are meaningful for the pre-

Figure 1. Expression of Ephrin-A and EphA Genes in the Olfactory System

Coronal sections through the mouse olfactory epithelium at postnatal day 3 reveal expression of mRNA for olfactory marker protein (A and D), ephrin-A3 (B and E), and ephrin-A5 (C and F). Ephrin expression extends more basally than OMP, indicating that immature olfactory sensory neurons contain ephrins. In coronal sections of the olfactory bulb at postnatal day 1, both EphA3 (G) and EphA5 (H) are present in the ring of mitral cell nuclei. Sagittal sections at age P5 reveal expression of EphA3 in the outer layer of periglomerular cells and inner layer of mitral cells (I), as well as weaker expression in the central granule cell layer. The mitral cell layer at this age is identified by expression of the $GABA_A\beta1$ receptor subunit (J) (Zhang et al., 1990). There is no large-scale gradient of either ephrin mRNA in the olfactory epithelium nor of EphA mRNA in the olfactory bulb. m, mitral cell layer; pg, periglomerular cell layer.

cise targeting of olfactory sensory axons, we would expect that neurons expressing a given OR consistently exhibit the same amount of ephrin-A. We therefore asked whether levels of ephrin expression in subpopulations of neurons expressing a given OR are conserved in different animals. Previously, we have used gene targeting to generate strains of mice in which neurons expressing a given OR also express the fusion protein tauLacZ (Mombaerts et al., 1996). In this manner, we can identify the individual glomeruli innervated by neurons expressing a given receptor. In this study, we have employed two different modified OR alleles: SR1 and P2 (also designated MOR256-3 and MOR263-5, respectively) (Zhang and Firestein, 2002). Alternating sections from mice containing labeled SR1- or P2-expressing neurons were stained with either X-gal or EphA3-AP to determine the relative levels of ephrin expression in defined glomeruli. Visual inspection reveals intermediate levels of ephrin in both the SR1 (Figures 3A–3D) and P2 glomeruli.

We have quantitated the ephrin levels more precisely (Figures 3E and 3F) by determining the intensity of ephrin staining for every glomerulus in a coronal section containing either an SR1 (eight sections, n = 230) or P2 (four sections, n = 103) glomerulus. This experiment demonstrates that there is a 5-fold difference in ephrin levels between the weakest- and strongest-staining glomeruli in the olfactory bulb. If the lowest ephrin-A level is assigned a value of 1 and the highest a value of 5, then the P2 glomeruli fall within a range of 1.6–1.9 rela-

tive units. The SR1 glomeruli occupy a larger but nevertheless restricted range of ephrin levels between 2.0 and 3.6 relative units. Thus these two different sets of glomeruli sort within a restricted range of ephrin-A levels. Moreover, all SR1 glomeruli exhibit more ephrin-A protein than P2 glomeruli. These experiments indicate that neurons expressing different receptors express different levels of ephrins on their axons within glomeruli, and these levels fall within a characteristic, restricted range in different animals. Thus, there is a correlation between the nature of the OR expressed by a given neuron and its level of ephrin-A expression.

Generation of Mice Lacking Ephrin-A in Olfactory Sensory Neurons

We have examined the role of ephrin-As in the development of the olfactory sensory map by generating mice deficient for both ephrin-A3 and ephrin-A5. We constructed a strain lacking ephrin-A3 by replacement of exons 2–4 in the mouse ephrin-A3 gene with a PGK-neo cassette, by means of homologous recombination in embryonic stem cells (Figures 4A–4C). Presumed splicing of exon 1 to exon 5 in mutant animals would lead to a frameshift encoding an ephrin-A3 protein truncated after 45 amino acids, and thereby lacking 90% of the ectodomain required to bind Eph receptors (Himanen et al., 2001; Toth et al., 2001). We are therefore confident that this mutation represents a null allele. Homozygous *ephrin-A3* mutant mice are viable, fertile, and display no gross morphological or behavioral abnormalities, ei-
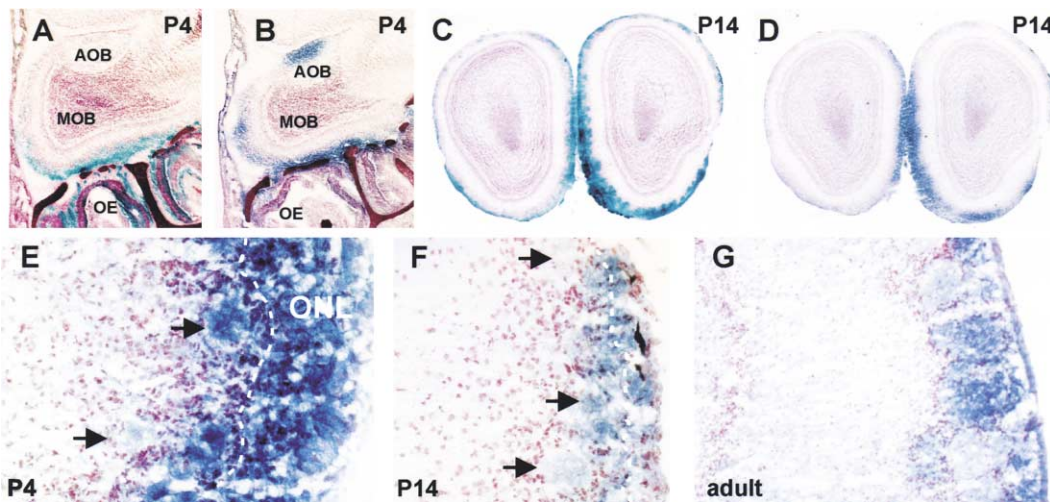
Figure 2. Mosaic Pattern of Ephrin-A Protein Levels in Olfactory Bulb Glomeruli

Adjacent sections from mice homozygous for the OMP-tauLacZ allele, which labels all olfactory sensory neurons with β-galactosidase (Mombaerts et al., 1996), were analyzed for either β-galactosidase activity using X-gal (A and C) or ephrin-A protein using an EphA3-alkaline phosphatase soluble fusion protein (B, D, E, F, and G). Panels A and B show sagittal sections at postnatal day 4, with anterior to the left and dorsal toward the top, and panels C and D are coronal sections through the olfactory bulb at postnatal day 14 with dorsal toward the top. The boundary between the outer nerve layer and glomerular layer is indicated with a dotted white line (E and F). While X-gal stains the olfactory dendrites, cell bodies, and axons innervating the bulb, ephrin protein is concentrated in the axon termini. At P4, there are clear differences in ephrin-A levels in the newly forming glomeruli (arrows in [E]). At P14 (C, D, and F), the spherical glomeruli are apparent by X-gal staining, immediately below the outer nerve layer (C). While ephrin-A protein is present throughout the circumference of the outer nerve layer (D), under higher magnification there are different levels of ephrin-A protein present in individual glomeruli (arrows in [F]). (G) shows the complex mosaic pattern of ephrin-A protein in the glomeruli of the adult olfactory bulb. AOB, accessory olfactory bulb; MOB, main olfactory bulb; OE, olfactory epithelium; ONL, outer nerve layer.

ther in a mixed 129/SvEvTac-C57BL/6 or congenic C57BL/6 background.

Mice mutant for ephrin-A5 have been generated previously by gene targeting (Frisén et al., 1998). Examination of ephrin-A3 protein expression in olfactory bulbs from *ephrin-A5* mutant animals, or conversely ephrin-A5 protein in *ephrin-A3* mutant mice, reveals that each protein is independently expressed in a mosaic pattern (Figures 4D and 4E compared with Figure 2G). We interbred *ephrin-A3* and *ephrin-A5* mutant strains to eliminate all ephrin expression from olfactory sensory neurons. The specificity of EphA3-AP staining is demonstrated by the absence of detectable ephrin-A in doubly mutant mice lacking both ephrin-A3 and ephrin-A5 (Figure 4F). Doubly mutant mice are often noticeably smaller than littermates and they exhibit no striking behavioral or morphological phenotypes except for the failure of females to nurse their young. Although the double mutants as well as *ephrin-A5* single mutants have a diminished survival rate up to weaning (Frisén et al., 1998), sufficient numbers of progeny can be recovered to permit an analysis of the olfactory sensory map in the absence of ephrin-A expression.

Targeting Defects in *Ephrin-A3/A5* Mutant Mice

We examined the projections of olfactory sensory neurons expressing a labeled OR allele in *ephrin-A3/A5* mutant mice. Mice bearing a genetically modified P2 allele were crossed into the *ephrin-A3/A5* mutant background. A similar cross was performed with mice bearing the SR1-IRES-tauLacZ allele. Both P2 and SR1 glomeruli are located centrally along the A/P dimension in the

ventral region of the bulb, with SR1 lying slightly posterior to P2. The relative positions of the P2 and SR1 glomeruli were determined in olfactory bulbs from each of the compound mutants and compared to the locations of the wild-type P2 and SR1 glomeruli. Each glomerulus was assigned a coordinate on the anteroposterior and dorsoventral axes relative to internal landmarks within the bulb. Examination of 122 SR1 glomeruli reveals a posterior shift in the location of both the medial and lateral glomerulus (Figure 5A). This shift is statistically significant ($p < 0.05$) and results in a posterior displacement of the SR1 glomeruli over a distance approximately 20% of the length of the the olfactory bulb (Table 1).

Examination of 94 P2 glomeruli reveals a similar posterior shift (Table 1). On the medial aspect of the olfactory bulb only, a smaller ventral shift is observed for the SR1 glomerulus whereas the P2 glomerulus is shifted dorsally (Figure 5B). Thus mice deficient in ephrin expression exhibit alterations in glomerular targeting that result in significant posteriorization of the two specific glomeruli examined. Moreover, the larger shift exhibited by SR1 glomeruli correlates with the higher levels of ephrin-A protein present on SR1-expressing axons. No other morphological changes in glomerular number or character are readily apparent, and there is no statistically significant difference in the size of the olfactory bulb between wild-type and *ephrin-A3/A5* mutant mice. Since glomeruli are still present at the anterior region of olfactory bulbs from the doubly mutant mice, it is unlikely that the several hundred unlabeled glomeruli are all similarly shifted in mice lacking ephrin-A3 and
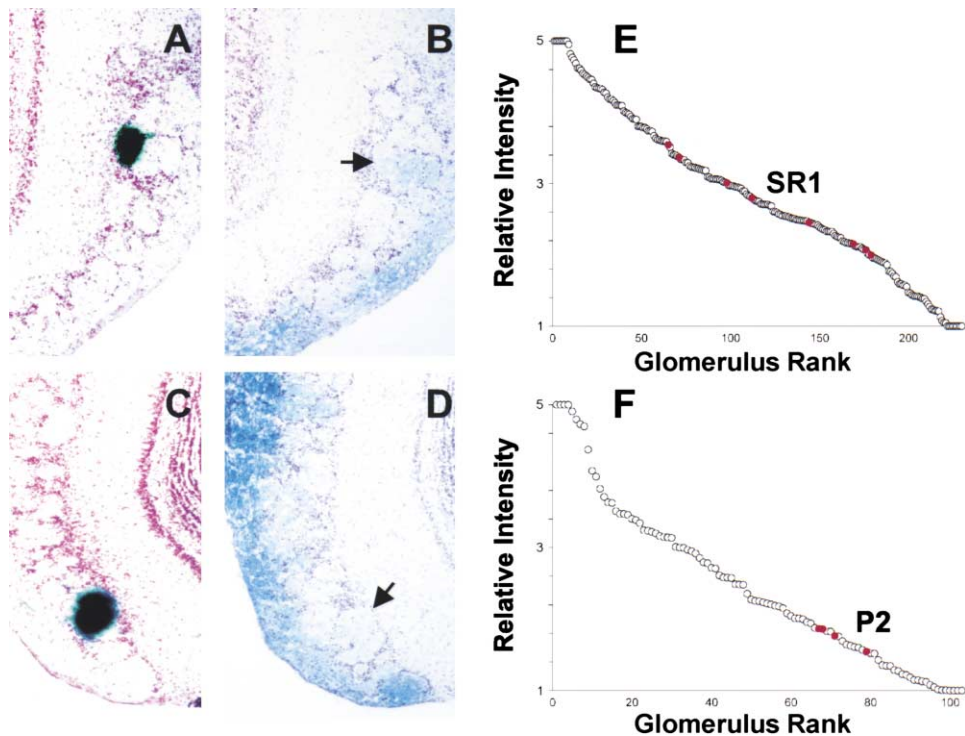
Figure 3. Neurons Expressing a Given Olfactory Receptor Exhibit Similar Levels of Ephrin in their Target Glomerulus

Coronal sections through the olfactory bulbs from mice containing an SR1-IRES-tauLacZ allele were analyzed for ephrin-A levels in glomeruli innervated by tauLacZ-expressing axons. Sections adjacent to defined SR1 glomeruli (A and C) were stained with EphA3-AP (B and D), demonstrating that ephrin-A levels in the SR1 glomerulus were intermediate compared to the range of glomerular staining in their coronal plane. The SR1 glomeruli are indicated with an arrow in the EphA3-AP-stained sections (B and D). Due to variations in the plane of section from animal to animal, the configuration of glomeruli, and therefore the pattern of ephrin-A protein, in the vicinity of the labeled glomerulus are not identical in different individuals. The range of ephrin-A levels observed in glomeruli from sections containing either an SR1 or P2 glomerulus was quantitated and plotted in descending order of intensity, with the defined glomeruli shown as filled red circles (E and F). Values were normalized between 1 and 5 for each section, then combined for either SR1 (230 glomeruli) or P2 (103 glomeruli). Note that the intensities for the SR1 glomeruli lie within the range of 2.0–3.6 (25%–64%), which does not overlap with the narrower range of 1.6–1.9 (14%–22%) for the P2 glomeruli.

ephrin-A5. The magnitude and direction of miswiring of mutant axons is likely to depend on their endogenous ephrin-A levels, such that neurons with high levels of ephrin-A will show the maximal posterior shift and neurons with low ephrin-A levels may exhibit a minimal shift and by necessity be displaced anteriorly.

Ephrin-A Overexpression Alters the Sensory Map

The loss-of-function experiments indicate that the ephrins may serve as guidance molecules that participate in the formation of a precise topographic map. We have extended these observations with gain-of-function experiments in which neurons expressing the P2 receptor overexpress ephrin-A5 in a genetic background that is either wild-type or mutant for ephrin-A expression. Gene targeting was employed to construct a strain of mice containing the tricistronic allele P2-IRES-ephrin-A5-IRES-tauLacZ (Figure 6A), such that olfactory sensory neurons that express this P2 allele will overexpress the ephrin-A5 protein. These mice were then crossed with homozygous animals that contain the P2-IRES-GFP allele (Gogos et al., 2000). Monoallelic expression of the OR genes ensures that, in heterozygotes resulting from this cross, P2 neurons with wild-type ephrin levels are marked by GFP, whereas P2 neurons that overexpress ephrin-A5 will contain tauLacZ. As a control, mice bearing the P2-IRES-GFP allele were crossed with mice bearing a P2-IRES-tauLacZ allele, such that the two differently labeled P2 alleles in this case will contain equal ephrin-A levels.

In control heterozygotes bearing a P2-IRES-GFP and a P2-IRES-tauLacZ allele, neurons expressing P2 invariably co-converge on the same glomerulus (Figure 6B). The positions of GFP-labeled and tauLacZ-labeled P2 glomeruli were then examined on sections of the olfactory bulb from six heterozygous mice containing the ephrin-A5 overexpressing allele in trans to the GFP-labeled allele. In the 12 olfactory bulbs, the glomeruli formed by neurons expressing the P2-IRES-GFP allele and neurons expressing the P2-IRES-ephrin-A5-IRES-tauLacZ allele never overlap (Figure 6C). In 60% of 21 GFP- and tauLacZ-labeled P2 glomerular pairs, there is an anterior shift in the neurons overexpressing ephrin-A5. This anteriorization of P2 glomeruli by ephrin-A5 overexpression occurs over a range of 1–2 glomeruli. Moreover, on the lateral face of the bulb, 76% of lacZ-labeled glomeruli are displaced ventrally compared to their control glomerulus, which is opposite to the shift observed for P2 glomeruli lacking ephrin-A.
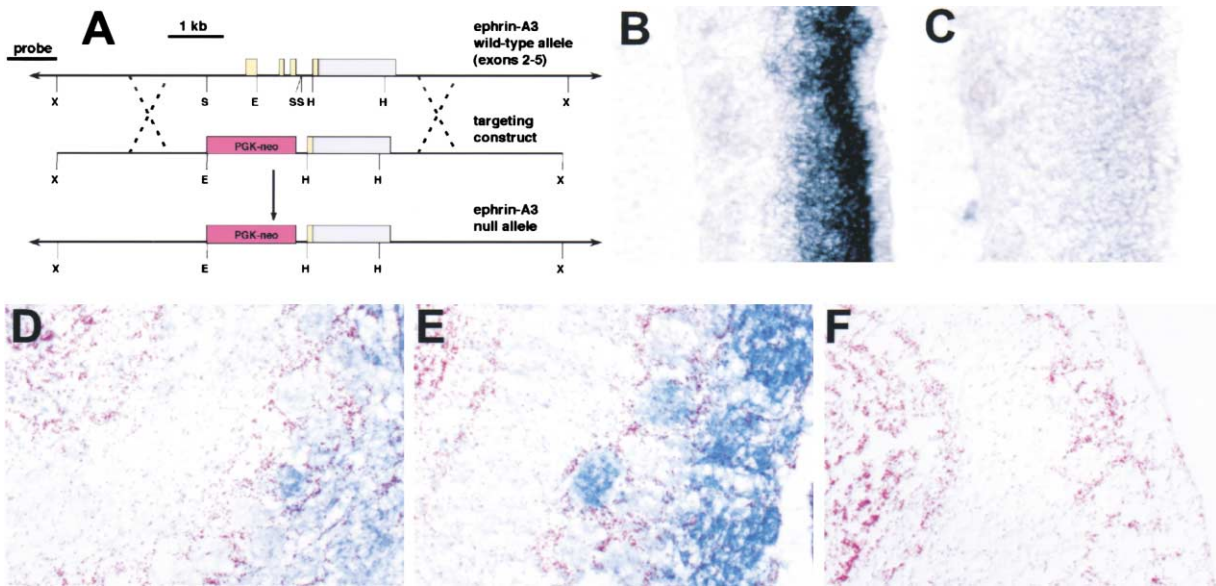
Figure 4. An *Ephrin-A3* Null Allele Reveals the Independent Contributions of Ephrin-A3 and Ephrin-A5 to Mosaic Protein Levels in Glomeruli

(A) The genomic organization of mouse *ephrin-A3* is indicated for exons 2–5 (E = EcoRI, H = HindIII, S = SphI, X = XbaI) (Cerretti and Nelson, 1998). Yellow boxes indicate coding regions, and the 3′ UTR is depicted in gray. The SphI fragment containing exons 2–4 was replaced by the PGK-neo selectable marker within a 9.1 kb XbaI fragment to produce the targeting construct.

(B and C) Expression of ephrin-A3 mRNA in the olfactory epithelium was analyzed on coronal sections at postnatal day 3 from either wild-type (B) or *ephrin-A3* homozygous mutant (C) animals. The antisense RNA probe corresponds to the three deleted exons. No expression is apparent in the mutant animal.

(D–F) Coronal sections of the olfactory bulb from mice at 1 month were analyzed for ephrin-A protein expression using the EphA3-AP fusion reagent. Tissue from *ephrin-A3* homozygous mutant (D) and *ephrin-A5* homozygous mutant (E) animals exhibits the characteristic mosaic expression pattern (see Figure 2G). Ephrin-A5 is present at lower levels, in accordance with lower ephrin-A5 mRNA levels in the olfactory epithelium (compare panels E and F in Figure 1). In the *ephrin-A3/A5* doubly mutant mice (F), there is no apparent ephrin-A protein in the olfactory bulb.

If olfactory sensory neurons employ differences in ephrin-A levels to discriminate precisely among glomeruli, then we expect that axons from neurons expressing the P2-IRES-ephrin-A5-IRES-tauLacZ allele would display a greater separation from GFP-labeled axons in an *ephrin-A3/A5* doubly mutant background as compared to the wild-type situation. In accord with this prediction, the axons from neurons expressing this allele in doubly mutant mice converge to a location 3–4 glomeruli displaced from the P2 glomerulus innervated by GFP-expressing axons (Figure 6D). This displacement is always greater than we observe for this heterozygous combination in a wild-type background and occurred in all 16 pairs of P2 glomeruli from four mice doubly mutant for *ephrin-A3/A5*.

P2 neurons overexpressing ephrin-A5 exhibit only a relatively small displacement in glomerular position; however, this shift is consistent in multiple animals. The extent of displacement is likely to reflect the level of ephrin-A5 produced from the P2-IRES-ephrin-A5-IRES-tauLacZ tricistronic allele. The efficiency of translation from an IRES can vary by a factor of 100 or more (Hennecke et al., 2001). We have therefore obtained an estimate of the amount of ephrin-A5 protein produced by this allele using EphA3-AP staining of olfactory bulbs from P2-IRES-ephrin-A5-IRES-tauLacZ homozygotes mutant for endogenous ephrin-A3/A5. In these mice, the only ephrin-A protein present in olfactory sensory neurons is derived from two copies of the tricistronic

P2 allele. In each of three mice, the amount of ephrin-A protein detected by EphA3-AP staining is less than 20% of the levels of endogenous ephrin-A protein present in the wild-type P2 glomerulus (data not shown). This relatively low level of ephrin-A expressed under translational control of the IRES may explain the small but consistent displacement in glomerular position that we observe. Thus, deletion of ephrin genes results in a characteristic posterior and dorsal shift in glomerular targeting for P2 axons, whereas overexpression of ephrin-A5 leads to a reciprocal anterior and ventral displacement of glomeruli, providing evidence that the ephrin-As instruct the formation of a precise olfactory sensory map.

Discussion

Ephrin-As Participate in the Formation of an Olfactory Sensory Map

Most sensory systems spatially segregate afferent input from primary sensory neurons to provide a representation of the sensory world in the brain. The topographic map in the olfactory bulb differs from the orderly representation inherent in retinotopic, tonotopic, or somatotopic sensory maps. In those systems, the spatial arrangement of neurons in the peripheral receptor sheet (the retina, cochlea, or body surface) is maintained in the projections to the CNS. In the olfactory system, however, spatial order of receptor cells in the periphery is not maintained in the projections to the brain. Rather,

A

SR1 LATERAL   SR1 MEDIAL

● wild-type
○ mutant

B

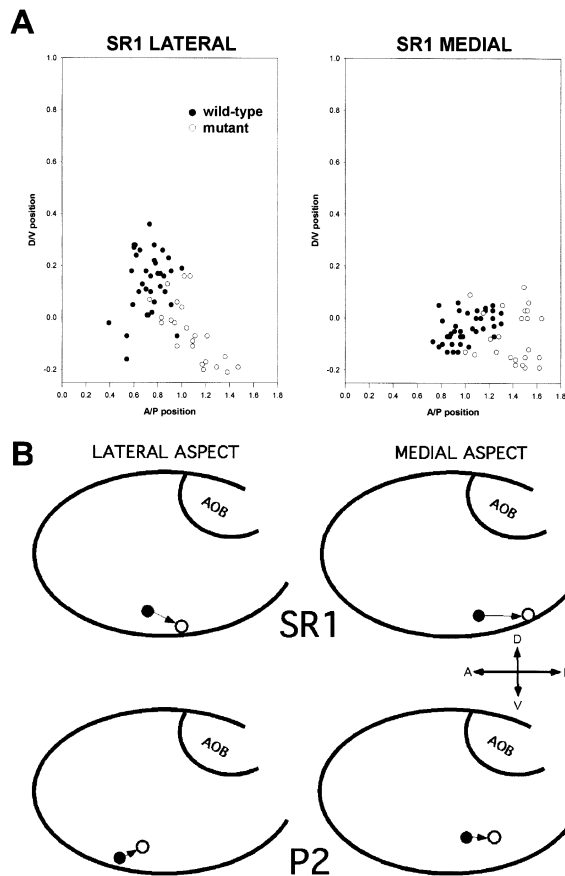LATERAL ASPECT   MEDIAL ASPECT

SR1

P2

Figure 5. Mice Lacking Ephrin-A3 and Ephrin-A5 Display a Shift in the Glomerular Map

(A) Positions of the glomeruli innervated by SR1-expressing neurons in wild-type (n = 19 bulbs) and *ephrin-A3/A5* doubly mutant mice (n = 16 bulbs) were scored on either the lateral (left panel) or medial face (right panel) of the olfactory bulb and presented as a scatter plot. Mice were approximately one month old when sacrificed for analysis to allow unambiguous identification of the glomeruli. As has been noted previously, the mediolateral plane of symmetry is not parallel to the rostrocaudal axis, and therefore glomeruli on the lateral face are located more anterior to their medial counterparts. On each face, the SR1 glomerulus is shifted in the mutant mice.
(B) The mean glomerular position and direction of shift are diagrammed for either SR1- or P2-expressing sensory neurons on each face of the olfactory bulb. Note that on the lateral face only, SR1 glomeruli are displaced ventrally whereas P2 glomeruli move dorsally.

neurons expressing a given receptor appear to be randomly dispersed within one of four zones in the epithelium. Spatial order is imposed in the bulb when neurons expressing a given receptor converge on discrete loci to create a precise topographic map capable of encoding odor quality.

The generation of a precise spatial map poses a complex problem in axon guidance and the specificity of neural connectivity. How do apparently randomly positioned neurons acquire the requisite guidance information to assure their convergence at spatially discrete glomeruli? Each of the 1000 distinct neuronal subpopulations must express either a qualitatively or quantitatively unique complement of guidance receptors. One

simple solution exploits the only known molecular distinction between the 1000 distinct neurons, namely the ORs, and posits that the ORs also serve as guidance receptors. Genetic experiments involving either deletions or substitutions of OR coding sequences lead to alterations in the positions of specific glomeruli, demonstrating that the OR indeed plays an instructive role in the formation of the olfactory sensory map. For example, substitution experiments that replace the P2 coding sequence with those of several different ORs result in convergence at novel glomeruli distinct from P2. These data suggest that ORs act in concert with other guidance receptors to direct axons to their appropriate glomerular target.

Several additional candidate guidance factors have been identified in the developing and mature olfactory system, but functional evidence for their involvement in the precise connectivity of olfactory sensory neurons is limited. Expression of a dominant-negative neuropilin-1 protein, which serves as a receptor for several secreted semaphorins, causes chick olfactory neurons to enter the telencephalon prematurely (Renzi et al., 2000). Mutations in galectin-1, Sema3A, neuropilin-2, p75NTR, CHL1, or N-CAM-180 (Puche et al., 1996; Schwarting et al., 2000; Walz et al., 2002; Tisay et al., 2000; Montag-Sallaz et al., 2002; Treloar et al., 1997) result in subtle perturbations in the overall pattern of olfactory axon projections; however, the role of these molecules in the precision of glomerular targeting has not been examined.

In this study, we demonstrate that ephrin-As govern the formation of a topographic map in the bulb. Neurons expressing different odorant receptors express different levels of ephrin-A protein on their axons. As a consequence, a mosaic pattern of ephrin-A expression is observed among different glomeruli but the relative levels of ephrin-A in the SR1 and P2 glomeruli are conserved in different animals. Thus, there is an intimate linkage between the nature of the odorant receptor and the level of ephrin-A expression in a given sensory neuron. Gene targeting experiments reveal that alterations in the levels of ephrin alter the glomerular map. Deletion of ephrin-A genes results in a posterior displacement of the glomeruli innervated by P2 and SR1 neurons, whereas overexpression of ephrin-A5 in P2 neurons results in an anterior shift in the P2 glomerulus. There are also opposing dorsal and ventral shifts for the medial P2 glomeruli in the loss- and gain-of-function ephrin-A experiments. Thus the ephrin-As are differentially expressed in distinct subpopulations of neurons and are likely to participate in the targeting of like axons to precise locations in the bulb.

The conclusions from gain-of-function experiments, however, must be tempered by the possibility that genetic manipulations of the P2 locus that introduce two IRES elements may lead to alterations in glomerular targeting quite independent of ephrin levels. For example, axons from neurons expressing a P2-IRES-rtTA-IRES-tauLacZ tricistronic mRNA, containing a tetracycline-dependent transcription factor, segregate from axons expressing P2-IRES-GFP (Gogos et al., 2000). However, axons from neurons expressing M72-IRES-tauGFP-IRES-OCNC1 (OCNC1 is a cyclic nucleotide-gated ion channel) and those expressing M72-IRES-tau-

Table 1. SR1 and P2 Glomerular Coordinates in Wild-Type and *Ephrin-A3/A5* Double Mutants

SR1 Receptor	Lateral Face		Medial Face	
	Wild-type (n = 35)	Mutant (n = 24)	Wild-type (n = 39)	Mutant (n = 24)
A/P axis	0.73 ± 0.04	1.06 ± 0.08*	1.02 ± 0.05	1.40 ± 0.08***
D/V axis	0.14 ± 0.04	−0.05 ± 0.05***	−0.04 ± 0.02	−0.07 ± 0.04

P2 Receptor	Lateral Face		Medial Face	
	Wild-type (n = 32)	Mutant (n = 18)	Wild-type (n = 25)	Mutant (n = 19)
A/P axis	0.52 ± 0.04	0.69 ± 0.10**	0.94 ± 0.08	1.13 ± 0.12*
D/V axis	0.03 ± 0.03	0.12 ± 0.04***	0.20 ± 0.03	0.18 ± 0.03

Glomeruli were assigned a dorsoventral and anteroposterior position based on internal olfactory bulb landmarks (see Experimental Procedures). Using this coordinate system, the posterior limit of the olfactory glomeruli is approximately 1.5, while the dorsoventral positions in a given coronal section range from −0.1 to 1.1. Values reported here are the mean ± SEM (95% confidence interval) for each coordinate on either the medial or lateral face of the bulb. For the eight sets of comparisons between mutant and wild-type positions, all differences are statistically significant except for the dorsoventral position of both SR1 and P2 glomeruli on the medial face only. Triple asterisk, $p < 0.0001$; double asterisk, $p < 0.005$; single asterisk, $p < 0.05$ (Student's t test, 2 tails, unequal variance).

LacZ innervate the same glomerulus (Zheng et al., 2000). Although the observed anterior shift in axons overexpressing ephrin-A5 is consistent with the predictions from loss-of-function mutants, we cannot at present exclude genetic modifications at the P2 locus as a source of targeting errors. We have examined the fate of axons expressing the P2-IRES-ephrin-A5-IRES-tauLacZ allele in both the wild-type and *ephrin-A3/A5* doubly mutant background. In accordance with the prediction that ephrin-A5 overexpression is responsible for the observed anterior shift, we consistently observe a greater displacement between the two glomeruli in the doubly mutant background. If ephrin-A levels are responsible for the shift, we expect that the greater the relative difference in ephrin-A levels between the two classes of P2 neurons, the greater the displacement of their glomeruli.

How do the ephrin-As cooperate with the odorant receptors to form a precise topographic map? In one model, the OR may be responsible for the initial formation of the map and the ephrins may participate in subsequent refinement to 1000 discrete glomeruli. Refinement may be a particularly important component in the formation of the olfactory sensory map. Different ORs can exhibit as much as 98% amino acid identity (Lane et al., 2001). This minimal sequence dissimilarity may alter the specificity of odorant recognition in the periphery without altering glomerular targeting in the bulb. The expression of different ephrin-A levels in different neurons could provide a mechanism for segregation of axons bearing extremely similar ORs. The ephrins, for example, might provide a mechanism to regulate growth cone migration in response to other attractive cues recognized by ORs in axons. In general, guidance cues that attract axons pose the problem as to how all axons are prevented from migrating en masse to the source of the cue, rather than forming a distributed neural map. One cue might therefore attract axons, affording a vector or direction to the migratory process, whereas a second set of guidance molecules, including the ephrin/Eph pairs, could dictate the magnitude of axon migration.

The intimate linkage between the nature of the OR and the level of ephrin-A in sensory axons, and the requirement for ephrin-As for the precise glomerular targeting, pose a series of interesting molecular problems. First, how do the GPI-linked ephrin-As transduce a signal across the membrane to guide axon migration? Second, how do the differences in ephrin-A levels contribute to glomerular targeting despite the fact that their presumed ligands, the Ephs, are uniformly expressed in the olfactory bulb? Finally, how is the level of ephrin-A expression coordinated with the choice of specific odorant receptors?

Ephrin-A as a Receptor

How might the GPI-linked ephrin-A proteins transduce a signal across the membrane to guide axon growth? Ephrin-A proteins are attached to the cell surface via a glycosylphosphatidylinositol (GPI) linkage, whereas their interacting partners, the EphAs, contain a tyrosine kinase domain. The distinction between ligand and receptor is often blurred when considering Eph/ephrin interactions since either Eph- or ephrin-bearing cells can receive a signal depending on their developmental context (reviewed in Kullander and Klein, 2002).

In the visual system, a retinotopic map in the brain employs Eph/ephrin signaling to ensure precise connectivity along two orthogonal axes. Complementary gradients of EphA proteins on retinal ganglion cells and ephrin-A2 and ephrin-A5 in their corresponding target regions, through a repulsive mechanism, results in the formation of a retinotopic map in the superior colliculus or tectum (reviewed in Wilkinson, 2001). Overexpression of EphA3 in a spatially random subpopulation of retinal neurons causes axons to form two non-overlapping topographic maps, demonstrating that neurons can distinguish quantitative differences in Eph signaling to guide their connectivity (Brown et al., 2000). An additional level of complexity is afforded by the coexpression of ephrins and Eph receptors within retinal ganglion cells themselves, which may desensitize axons to small differences in ephrin levels in the target (Hornberger et al., 1999). Recent data suggest that along the dorsoventral axis in the retina, gradients of EphB or ephrin-B proteins on retinal ganglion cells are matched by an opposing
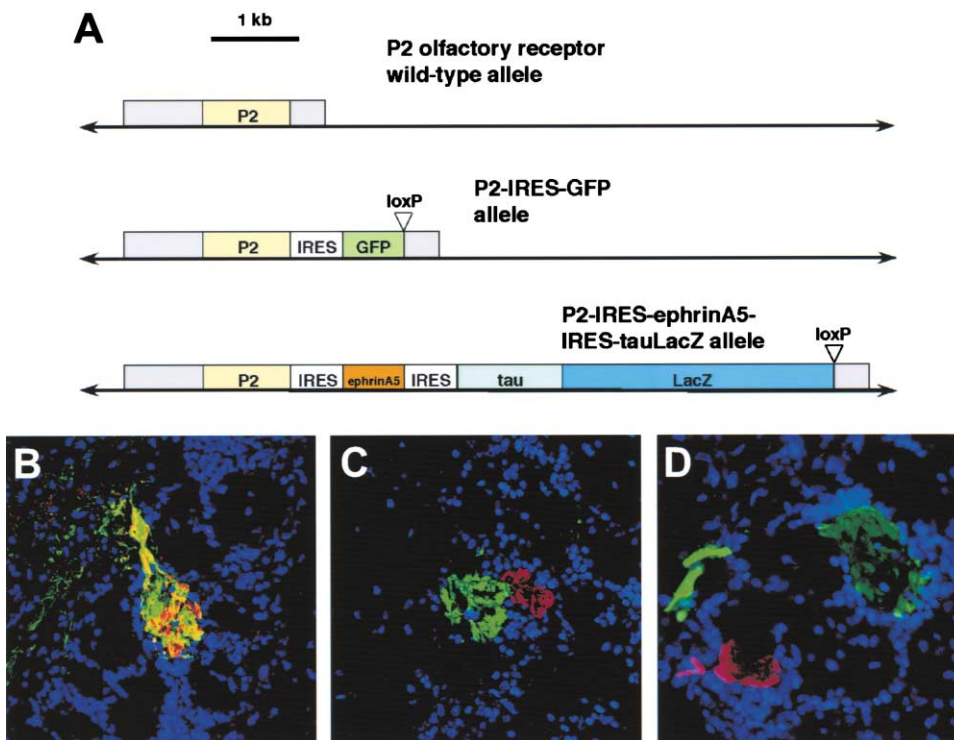
Figure 6. Ephrin-A5 Overexpression Segregates Projections of P2 Neurons

(A) A schematic diagram of wild-type and genetically modified P2 alleles, designed to coexpress GFP (Gogos et al., 2000) or tauLacZ plus ephrin-A5 (this study). The various coding sequences are color-coded, the IRES sequence is indicated as a white box, and the noncoding segments of the P2 exon are shaded gray. The two modified alleles contain a single loxP site immediately following the P2 stop codon, which remains after Cre-mediated removal of the neo^R selection cassette.

(B) A confocal micrograph of a coronal section through the olfactory bulb from a P2-IRES-tauLacZ/P2-IRES-GFP heterozygous mouse, stained with anti-lacZ (red) and anti-GFP (green) antibodies. The nuclei of surrounding periglomerular cells are visualized in blue using TOTO-3. Every glomerulus examined in such mice displays co-convergence of tauLacZ- and GFP-expressing axons.

(C) A confocal micrograph of a coronal bulb section from a P2-IRES-GFP/P2-IRES-ephrin-A5-IRES-tauLacZ heterozygous mouse, stained for the axonal markers as described above. In all cases, the two populations of P2 neurons segregate to separate glomeruli. The majority of glomeruli innervated by axons overexpressing ephrin-A5 are displaced in the opposite direction from their shift in *ephrin-A3/A5* double mutants.

(D) A confocal micrograph from a coronal bulb section of a P2-IRES-GFP/P2-IRES-ephrin-A5-IRES-tauLacZ heterozygous mouse that is also lacking both ephrin-A3 and ephrin-A5, stained for the axonal markers as described above. In the absence of endogenous ephrin-A in the sensory axons, the relative difference in ephrin-A levels between the two populations of P2-expressing neurons is greater, and their projections exhibit a greater separation than in the wild-type.

gradient in the superior colliculus or tectum, and this governs the appropriate placement of retinal axons along the M/L or D/V axis of the target (Hindges et al., 2002; Mann et al., 2002). Whereas appropriate termination along the anteroposterior axis mediated by EphA/ephrin-A is likely to involve repulsion, targeting by EphB/ephrin-B is thought to be a consequence of both attractive and repellent cues (McLaughlin et al., 2003).

In the olfactory system, however, ephrin-As rather than Eph receptors are prominently expressed on incoming sensory axons. The ephrin-A ligands are tethered to the membrane via a GPI linkage, immediately posing the question as to how guidance information is transmitted intracellularly. In cultured cells, activation of ephrin-As by Eph receptors results in an integrin-dependent adhesion to laminin (Huai and Drescher, 2001) and may involve intracellular kinases such as MAPK and Fyn (Davy et al., 1999; Davy and Robbins, 2000). Recently, ephrin-A5 has also been implicated in the guidance of vomeronasal sensory neurons to the accessory olfactory bulb (Knöll et al., 2001). Moreover,

VNO axons preferentially grow on an EphA-containing substrate when compared to a laminin control, suggesting that VNO neurons may exhibit an attractive response to ephrin-A signaling. The GPI-anchored ephrin-A proteins presumably transduce signals across the membrane by association with other transmembrane proteins capable of activating intracellular signaling pathways. We do not know the nature of the postulated ephrin-A co-receptors on olfactory sensory axons or on any ephrin-A responsive cell.

One unifying model for olfactory sensory axons postulates that ephrin-As and ORs do not act independently but rather form a multimeric complex that recognizes guidance information in the target. Alternatively, it is possible that the two guidance pathways may act independently of one another, perhaps in a temporal sequence, to assure the precision of axon targeting. It is now increasingly clear that the formation of even relatively simply neural pathways can involve multiple guidance systems. Dorsoventral migration of motor neurons in the worm *C. elegans*, for example, employs attractive

ventral cues, the netrins, and dorsal repulsion mediated by the slit/robo system (Hao et al., 2001). This push-pull mechanism is also apparent in the ventral migration of commissural axons in the mouse spinal cord, where the floor plate expresses the attractant netrin (Serafini et al., 1994) while the dorsally placed roof plate secretes BMP family members as repellents (Augsburger et al., 1999). Although we cannot at present discern the mechanistic contributions of either the ephrin-As or ORs, our genetic data indicate that these receptors provide information that governs the precise placement of like axons in the bulb, contributing to the formation of the complex olfactory sensory map.

Eph Expression in the Bulb and Ephrin Expression on Sensory Neurons

The expression of EphA molecules, the presumed ligands for the ephrin-As, is not discernibly graded in any dimension in the olfactory bulb. Rather, we observe a uniform level of EphA3 and A5 in both projection neurons and interneurons of the bulb. This contrasts with the opposing gradients of EphAs on retinal ganglion axons and ephrin-As on their targets in the brain. Ephrin-As are present in a mosaic pattern in the bulb, with neurons expressing different receptors also containing different ephrin levels. The absence of a spatial pattern of ephrin-As predicts that EphA levels may not exhibit a spatial pattern across the bulb since this may not contribute to the precision of the guidance process. Indeed, we observe a uniform distribution of Eph levels in the target. Whether ephrins elicit attraction or repulsion, neurons bearing different ephrin-A levels may travel different distances in a homogeneous target of Eph receptors.

Finally, how are the levels of odorant receptor and ephrin-As coordinately controlled such that neurons expressing different odorant receptors contain different ephrin levels? One model for the control of OR expression invokes the existence of 1,000 different cell types in the sensory epithelium, each expressing a unique combinatorial of transcription factors. These transcriptional codes could regulate both the nature of the OR and the level of ephrin-As expressed by a given neuron. A second model of odorant receptor gene expression posits that all odorant receptor genes are regulated by the same set of transcription factors, but a mechanism exists within the cell to assure that only one receptor is chosen for expression in each neuron. In this model, which invokes a stochastic choice of the odorant receptor gene, the nature of the OR expressed must be closely linked with the level of ephrin-A. How might the nature of the expressed OR govern ephrin-A levels? One unlikely possibility invokes signaling through OR mRNA or protein as a regulator of ephrin-A3 and ephrin-A5 transcription. Alternatively, the different ORs could determine ephrin-A protein levels in the axon. In the simplest form of this mechanism, OR and ephrin-A proteins might directly interact, thereby regulating ephrin-A levels at axon termini and concomitantly providing an intracellular signaling partner for the GPI-linked ephrin-A proteins. Whatever the mechanism, our data reveal an intimate linkage between odorant receptor gene expression and ephrin-A levels and provide evidence that these two sets of molecules serve to guide olfactory sensory axons to precise targets in the brain.

Experimental Procedures

In Situ Hybridization

RNA in situ hybridization using digoxigenin-labeled probes was essentially as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993) with slight modifications. The gene segments used as probes are: for mouse ephrin-A3, a segment corresponding to amino acids 47–207 (exons 2 through 4) that was amplified by PCR from mouse brain cDNA; for mouse ephrin-A5, the complete open reading frame; for mouse EphA3, a 780 bp fragment derived from the intracellular domain that was amplified by PCR; for mouse EphA5, a 700 bp fragment obtained from an EST that corresponds to the C terminus of the protein; and for mouse OMP, a 1.4 kb BamHI fragment corresponding to nucleotides 820–2891 in the published cDNA (Genbank accession #U01213) that is lacking the internal 680 bp NcoI fragment.

EphA3-AP Staining

Mouse tissue was fresh-frozen in OCT (Tissue-Tek), then sectioned on a cryostat and post-fixed for 10 min at 4°C in 4% p-formaldehyde/PBS. Sections were then washed for 5 min in PBS at room temperature and incubated briefly in HBAH buffer before continuing with the published staining protocol (Flanagan et al., 2000). Following NBT/BCIP development, sections were either mounted directly in Glycergel (Dako) or counterstained with 0.33% neutral red (Sigma) in PBS, dehydrated using a series of washes with increasing ethanol concentration, then washed in xylene and mounted in Cytoseal XYL (Stephens Scientific).

To quantitate EphA3-AP staining in individual glomeruli, digital images were captured with an RT Slider SPOT camera (Diagnostic Instruments, Inc.) and analyzed using the ImageJ software package (NIH). The mean pixel gray value was determined for an oval drawn completely within the periglomerular cell nuclei surrounding each glomerulus, and the range of intensities for each section was normalized from 0 to 1, then combined for all sections. For three of the SR1-containing sections, one intensely staining glomerulus, which may have been an erroneous inclusion of ephrin-A from the outer nerve layer, was discarded as an outlier since the next highest intensity in each case was <0.7. To determine the approximate range of ephrin-A levels in the wild-type olfactory bulb, a similar analysis was performed on images of sections from a wild-type and an ephrin-A3/A5 doubly mutant mouse that were processed in parallel for EphA3-AP activity. The mean pixel value from the doubly mutant glomeruli was subtracted from the wild-type values to yield an estimate of the range of ephrin-A levels in absolute rather than relative terms.

Generation of an *Ephrin-A3* Mutant Strain

A 9.1 kb genomic clone containing exons 2 through 5 of the ephrin-A3 transcript (Cerretti and Nelson, 1998) was isolated from a 129/SvJ lambda phage library (Stratagene). A 1.8 kb SphI fragment containing exons 2–4 was replaced with the PGK-neo selectable marker. Electroporation into a 129/SvEv mouse ES cell line followed by G-418 selection resulted in 4/96 homologous recombinant clones as determined using an external 0.9 kb probe on Southern blots of EcoRI-digested ES cell genomic DNA. The probe distinguishes between the 9.5 kb wild-type allele and the 8.5 kb targeted allele (Figure 4A). Chimeric C57B/6 males that transmitted the mutant allele were obtained for two independent clones, and one clone was propagated for further analysis following confirmation that both clones yielded an identical phenotype. The following primers and PCR conditions were used for genotyping *ephrin-A3* progeny: 5'-CCGAGAAGTTCCAGCGTTACAG and 5'-GCAGCAGACGAACACCTTCATC (0.7 kb product) for the wild-type allele and 5'-TCATGTAGGAGATACAGGGC and 5'-ACCAAAGAACGGAGCCGGTTGGCG (0.3 kb product) for the mutant allele. Thirty cycles of PCR (94°C for 30 s, 57°C for 30 s, 72°C for 1 min) were sufficient to amplify the product from 1/100 crude tail DNA.

Analysis of P2 and SR1 Projections

Brains were prepared for fixation, sectioning, and X-gal staining as described previously (Mombaerts et al., 1996). Wild-type and *ephrin-A3/A5* doubly mutant mice were approximately one month of age

(ranging from postnatal days 28–37) when sacrificed for analysis. Serial 30 μ sections on the coronal plane were collected from the anterior to the posterior of the olfactory bulb. The position of each glomerulus was assigned a fractional coordinate on both the A/P and D/V axes, relative to the following internal landmarks: first appearance of the granule cell layer (defined as 0) and the rostral limit of the accessory olfactory bulb (defined as 1) along the A/P axis, and the ventral (defined as 0) and dorsal (defined as 1) limits of mitral cell nuclei along the D/V axis. This essentially maps the hemiovoid surface of each face of the olfactory bulb onto a rectangular coordinate system and is comparable to other proposed bulbar coordinate schemes (Schaefer et al., 2001) with respect to the resulting distribution of wild-type glomerular positions. In addition, mapping the glomeruli relative to internal landmarks controls for factors such as age- or sex-dependent size variations, distortion of the bulb during dissection, and variations in the plane of sectioning.

Overexpression of Ephrin-A5 in P2 Neurons
The coding region of a mouse ephrin-A5 cDNA was modified by PCR to introduce NcoI (5′) and BamHI (3′) ends. This causes a conservative L→V substitution at the second amino acid position, which is within the putative signal sequence of ephrin-A5 and should not perturb its function. This modified ephrin coding sequence was cloned into the NcoI/BamHI sites of plasmid ETLpA-/LTNL (Mombaerts et al., 1996) to produce the IRES-ephrin-A5 cassette. This cassette and an IRES-tauLacZ-ACN cassette (Bunting et al., 1999) were cloned in tandem into a modified pNEB vector, and the entire IRES-ephrin-A5-IRES-tauLacZ moiety was inserted as an AscI fragment into an 8.6 kb mouse genomic clone containing the P2 OR gene with AscI and PacI sites engineered immediately following the stop codon. Electroporation into a 129/SvEv ES cell line yielded several homologous recombinant clones. Chimeric males transmitting the modified allele, from which the neo gene is excised by germline expression of Cre within the ACN cassette, were mated to homozygous P2-IRES-GFP females to produce heterozygous offspring for analysis.

For fluorescence immunohistochemistry of tauLacZ- and GFP-labeled projections, dissected brains were fixed for 30 min in 4% paraformaldehyde/PBS, then washed in PBS and cryoprotected in 30% sucrose/0.1 M sodium phosphate buffer (pH 7.4) overnight at 4°C. Brains were then frozen in OCT, and 20 μ sections collected on a cryostat. The primary antisera were goat α-lacZ (Biogenesis, 1:1000 dilution) and rabbit α-GFP (Molecular Probes, 1:1000 dilution). Fluorescent secondary antisera were Alexa Fluor 488 donkey α-goat (Molecular Probes, 1:1000) and Cy3-conjugated donkey α-rabbit (Jackson ImmunoResearch, 1:800), with nuclei visualized using the TOTO-3 stain (Molecular Probes, 1:1000). Images were collected on a BioRad MRC 1024ES confocal microscope.

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