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Odorant Receptors Govern the Formation of a Precise Topographic Map

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Summary

Olfactory neurons expressing a given odorant receptor project with precision to 2 of the 1800 glomeruli within the olfactory bulb to create a topographic map of odor quality. We demonstrate that deletions or nonsense mutations in the P2 odorant receptor gene cause the axons of these cells to wander rather than converge on a specific glomerulus. Receptor substitution experiments that replace the P2 gene with the coding region of the P3 gene result in the projection of P3→P2 axons to a glomerulus touching the wild-type P3 glomerulus. These data, along with additional receptor substitutions, indicate that the odorant receptor plays an instructive role in the establishment of the topographic map.

Introduction

In vertebrate sensory systems, peripheral neurons receive information from the environment and transmit this information to the brain where it is processed to provide an internal representation of the external world. The representation of the sensory world in the brain then translates stimulus features into a neural code to allow for the discrimination of complex sensory information. Most sensory systems spatially segregate afferent input from primary sensory neurons to construct a topographic map that defines the location of a sensory stimulus within the environment, as well as the quality of the stimulus itself (Udin and Fawcett, 1988). Olfactory sensory processing does not extract spatial features of the odorant stimulus. Relieved of the requirement to map the position of an olfactory stimulus in space, the olfactory system employs spatial segregation of sensory input to encode the quality of an odorant.

What features of the vertebrate olfactory apparatus might form the basis for a spatial map of olfactory information? Odorant stimuli are received from the environment by receptors on the cilia of olfactory sensory neurons in the olfactory epithelium. In mammals, the repertoire of olfactory receptors consists of about a thousand different genes, each encoding a putative seven-transmembrane domain receptor (Buck and Axel, 1991; Levy et al., 1991; Parmentier et al., 1992; Ben-Arie et al., 1994). Individual olfactory sensory neurons express only one of the thousand receptor genes, such that neurons are functionally distinct (Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994; C. Dulac and R. A., unpublished data). Discrimination among odors can therefore be reduced to a problem of distinguishing which neurons have been activated.

Cells expressing a given receptor in the olfactory epithelium are randomly dispersed within one of four broad but circumscribed zones (Strotmann et al., 1992, 1994; Ressler et al., 1993; Vassar et al., 1993). Sensory neurons extend a single unbranched axon to the olfactory bulb such that the projections from neurons expressing a specific receptor converge upon 2 of the 1800 glomeruli (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). The pattern of convergence is absolute and is invariant in all individuals in a species. The bulb therefore provides a spatial map that identifies which of the numerous receptors have been activated within the sensory epithelium such that the quality of an olfactory stimulus would be encoded by specific combinations of glomeruli activated by a given odorant. This model for olfactory discrimination is consistent with physiological studies suggesting that glomeruli represent functional units such that different odorants elicit spatially defined patterns of glomerular activity in the olfactory bulb (Stewart et al., 1979; Lancet et al., 1982; Kauer et al., 1987; Imamura et al., 1992; Mori et al. 1992; Katoh et al., 1993; Friedrich and Korsching, 1997; Joerges et al., 1997).

The observation that each of a thousand different subpopulations of sensory neurons project with precision to a small number of topographically fixed glomeruli poses an interesting but complex problem in axon guidance. How do neurons expressing a given receptor know which target to project to in the olfactory bulb? The topographic map elucidated in the olfactory system differs in character from the orderly representation inherent in the retinotopic, tonotopic, or somatotopic sensory maps. In these sensory systems, the peripheral receptor sheet is represented in the central nervous system (CNS) such that neighbor relations in the periphery are preserved in the CNS (for review, see Udin and Fawcett, 1988). In this manner, sensory neurons can both determine the position of a sensory stimulus in space and translate stimulus features into neural information. The orderly array of neurons in the periphery provides insight into how the topographic map of projections may be established. In these sensory systems, peripheral receptor cells may acquire a distinct identity that is determined by their relative position in a receptor sheet. Spatial patterning in the periphery can therefore endow individual neurons with positional information that directs their orderly representation in the brain (Udin and Fawcett, 1988; Holt and Harris, 1993).

The olfactory system, however, does not maintain an orderly representation of receptor cells in the periphery. Olfactory neurons differ from one another, not by virtue of their position in a receptor sheet, but rather by the nature of the receptor that they express. Order is restored, however, in the bulb where neurons expressing a given receptor converge on discrete loci to create a

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topographic map of odor quality (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). This tight linkage between the choice of an odorant receptor and the site of axonal convergence suggests a model in which the odorant receptor recognizes odorants in the periphery and also governs target selection in the bulb. In this manner, an olfactory neuron would be afforded a distinct identity that dictates the nature of the odorant to which it responds as well as the glomerular target to which its axon projects.

We have developed a genetic approach to visualize axons from neurons expressing a given receptor (the P2 receptor) as they project to the olfactory bulb. Substitution of the P2 coding sequence with that of a second odorant receptor (M12) alters the pattern of projections, suggesting that the odorant receptor indeed plays an instructive role in the guidance process (Mombaerts et al., 1996). In this study, we initially demonstrate that deletions or nonsense mutations in the P2 receptor gene cause the axons of these cells to wander broadly in the bulb without ever converging on a specific glomerulus. Receptor substitution experiments that replace the P2 gene with the coding region of the P3 gene, two linked genes expressed in the same epithelial zone, result in the projection of P3→P2 axons to a glomerulus touching the wild-type P3 glomerulus. These data, along with additional receptor substitutions, indicate that the odorant receptor plays an instructive role in the establishment of the topographic map as one of a complement of other guidance molecules that may reflect the zone of receptor expression in the epithelium.

Results

Odorant Receptor Is Required for Convergence of Axons

In initial experiments, we examined the consequence of receptor deletions on axon targeting. Previously, we used gene targeting to modify the P2 receptor gene by insertion of a cassette immediately 3′ of the P2 stop codon. This cassette consists of two parts: an internal ribosomal entry site (*IRES*) driving translation of *tau-lacZ* sequences followed by *LTNL* (a 5′ *loxP* site, the Herpes simplex thymidine kinase gene [*tk*], the neomycin resistance gene [*neo*], and the 3′ *loxP* site) (see Mombaerts et al., 1996, and references therein). Homologous recombination in the ES cells, followed by Cre recombinase-mediated deletion of the *tk* and *neo* genes, results in an insertion of *IRES-tau-lacZ* at the P2 locus. Cells that express this modified P2 allele now express the P2 receptor along with tau-lacZ. The association of tau-lacZ with microtubules in neural processes permits the direct visualization of the pattern of projections in the olfactory bulb. In these genetically altered strains of mice, we observe that neurons expressing the P2 receptor converge on only 2 topographically fixed glomeruli of the 1800 glomeruli in the mouse olfactory bulb (Figures 1F and 1G).

A second gene targeting strategy was chosen in which the endogenous P2 receptor coding sequences were substituted with *IRES-tau-lacZ* (Figure 1A). We have previously shown that neurons transcribe a given odorant receptor gene from only one of the two alleles (Chess et al., 1994). Therefore, cells that transcribe this modified P2 allele will express tau-lacZ but will not express the P2 receptor, allowing the direct visualization of the consequences of receptor deletion on the pattern of axonal projections in heterozygous animals. A targeting vector was constructed in which the P2 coding sequences spanning the start and stop codons of the P2 gene were replaced by a cassette containing *IRES-tau-lacZ-LTNL* (Figure 1A). This targeting vector was electroporated into ES cells, and a single G418-resistant clone resulting from homologous recombination at the P2 locus was identified by Southern blot hybridization. The targeted ES clone was then transiently transfected with a vector directing the expression of Cre recombinase (pBS185) (Sauer and Henderson, 1990), resulting in the deletion of the *tk* and *neo* sequences. In these ES cells, the P2 coding region is replaced by *IRES-tau-lacZ*, followed by a single *loxP* site. This clone was injected into blastocyst embryos and germline transmission was ultimately obtained (Figure 1B).

Whole-mount analysis of mice heterozygous or homozygous for the P2 deletion–IRES-tau-lacZ mutation reveals subpopulations of sensory neurons whose dendrites, cell bodies, and axons exhibit blue color after staining with X-gal (Figure 1C). The pattern of expression of the P2 deletion in the epithelium is indistinguishable from that of the wild-type P2 allele. Neurons expressing the wild-type P2 gene or the P2 deletion allele are restricted to zone III in the epithelium (Figures 1C and 1F). The blue axons in the P2 deletion–IRES-tau-lacZ mice are readily visualized as they emerge from the epithelium and pass through the cribriform plate into the olfactory bulb. Once in the bulb, however, the P2 deletion neurons do not converge. Rather, they appear to wander through a broad region in the outer nerve layer of the olfactory bulb (Figures 1D, 2A, and 2C). This is in sharp contrast to the precise convergence to one medial and one lateral glomerulus observed in P2-IRES-tau-lacZ mice (Figures 1G, 2B, and 2E). Moreover, the axon termini in P2 deletion mice appear thickened (Figure 1D), a feature not apparent in blue neurons expressing a wild-type receptor. Sections through the entire olfactory bulb reveal that the diffuse representation of axons is restricted to the outer nerve layer of the bulb with only a very small percentage of the fibers actually entering the glomerular layer (Figure 2A). The small subset of fibers that do enter the glomerular layer do not appear within the glomeruli themselves. Rather, they course around glomeruli and are therefore unlikely to synapse with the output neurons of the olfactory bulb. This is readily observed using immunofluorescence with antibodies directed against lacZ that permits the detection of single axons (Figures 2C and 2E). Similar results have been obtained for a second receptor, M12, in which blue neurons expressing the M12 deletion–IRES-tau-lacZ allele also project diffusely to the olfactory bulb (Mombaerts, F. W., and R. A., unpublished data).

The divergent character of the projections from neurons expressing the P2 deletion–IRES-tau-lacZ is also apparent during embryogenesis. At embryonic day 17 (E17), for example, wild-type P2 axons have already converged to form a protoglomerulus. In contrast, axons

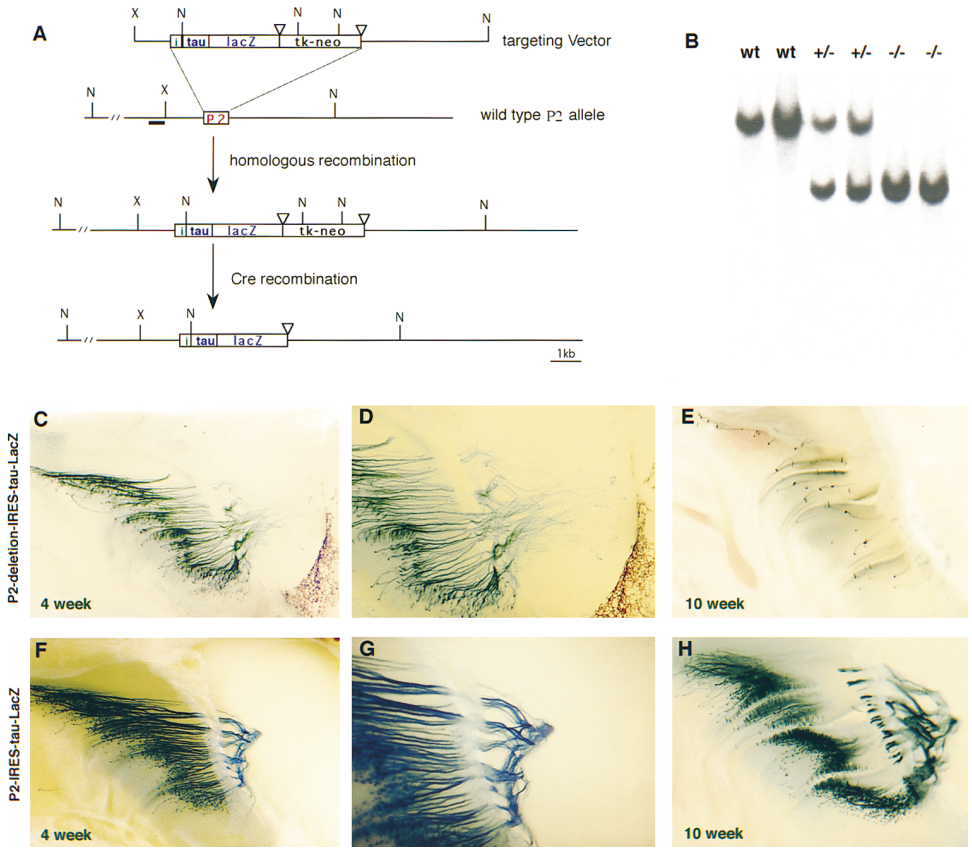

Figure 1. P2 Receptor Expression Is Required for Axon Convergence

(A) Schematic representation of the gene targeting strategy to delete the P2 receptor coding sequences. Homologous recombination between the targeting vector and the wild-type P2 gene results in the replacement of the P2 coding sequences with the *IRES-tau-lacZ-loxP-tk-neo-loxP* cassette. Transient transfection with a plasmid expressing the Cre recombinase results in the excision of the *tk-neo* cassette, leaving a single *loxP* site behind. Inverted triangles indicate loxP sites. X, XhoI; N, NcoI; i, *IRES*.

(B) Southern blot analysis of representative tail DNAs from wild type, heterozygous, and homozygous mice. DNA was digested with NcoI and hybridized with the 5′ P2 probe (denoted by the horizontal bar in [A]).

(C and D) Whole-mount view of nasal cavity and the medial aspect of the bulb of a heterozygous P2 deletion–IRES-tau-lacZ mouse (4-week-old) stained with X-gal. The axons project diffusely to the olfactory bulb and do not converge on a glomerulus.

(E) Whole-mount view of a 10-week-old homozygous P2 deletion–IRES-tau-lacZ mouse stained with X-gal. Very few blue cells are stained when compared with turbinates of younger mutant animals or with turbinates from homozygous P2-IRES-tau-lacZ mice of the same age (H).

(F and G) Whole-mount view of a heterozygous P2-IRES-tau-lacZ mouse (4-week-old) stained with X-gal. Convergence of axons at a single glomerulus is apparent.

(H) X-gal staining of a 10-week-old homozygous P2-IRES-tau-lacZ mouse.

from neurons expressing the P2 deletion–IRES-tau-lacZ allele at E17 project diffusely (data not shown). Thus, from the earliest times that sensory axons enter the bulb, a striking difference is observed in the character of the P2 projections in wild-type and P2 deletion mice. These data indicate that the divergence observed in the absence of a functional receptor results from an early defect in target selection and is not a reflection of early convergence and subsequent divergence as a consequence of the absence of a functional receptor.

Neurons expressing the P2 deletion allele do not project axons to a specific glomerular target despite the fact that the intact P2 glomeruli exist in heterozygous organisms. A given neuron will express the P2 receptor from only one of the two alleles. Therefore, in heterozygous animals, neurons that express the wild-type P2 allele will be interspersed among blue neurons expressing the modified P2 deletion allele. In situ hybridization with P2 RNA probes in mice heterozygous for the P2 deletion reveals one medial and one lateral P2 glomerulus reflecting the convergence of fibers from neurons expressing the unmodified allele (Figures 2G–2J). In contrast, blue fibers from cells expressing the P2 deletion do not converge on the P2 glomeruli (Figures 2G–2J). A similar conclusion emerges from the examination of compound heterozygotes that contain one allele encoding P2-IRES-tau-lacZ and a second allele containing the P2 deletion–IRES-tau-lacZ. Whole-mount analysis of these mice reveals a subset of blue fibers that converge on 2 of the 1800 glomeruli in the olfactory bulb and a second subset of fibers (presumably reflecting neurons that transcribe the deleted P2 allele) that are dispersed throughout a broad zone in the outer nerve layer of the olfactory bulb (data not shown).

These experiments suggest that receptor expression is required for the appropriate targeting of axons to

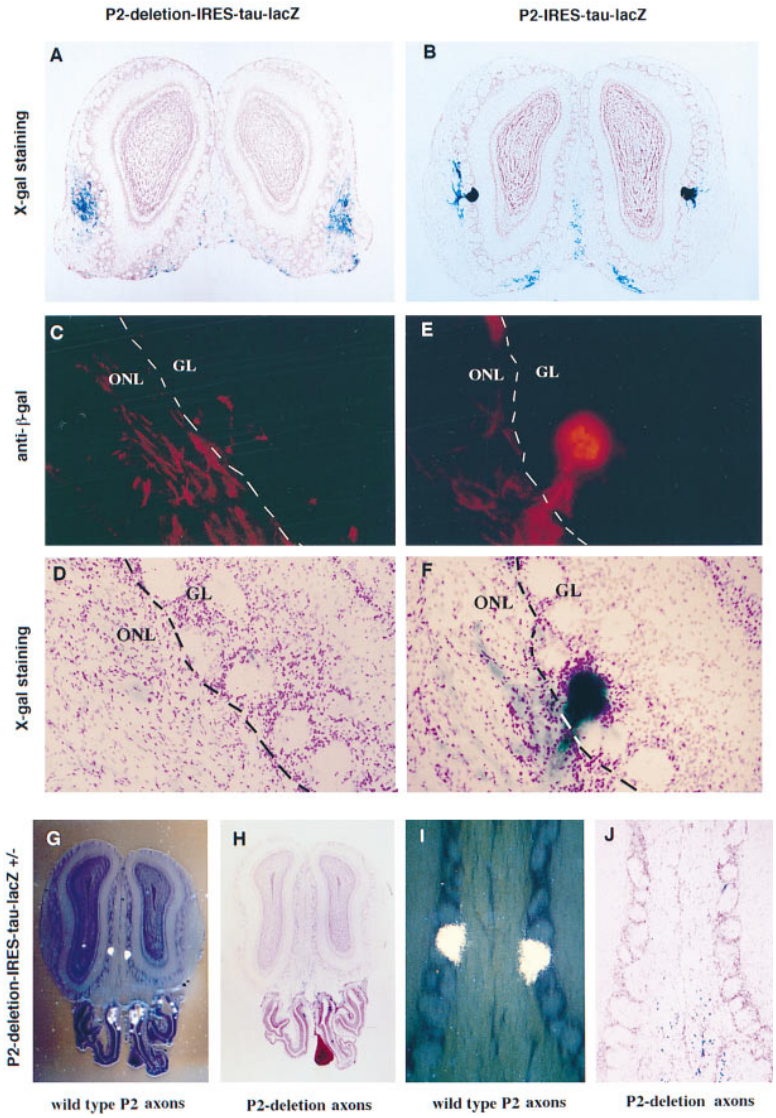

Figure 2. X-gal Staining and In Situ Hybridization Analysis on the Olfactory Bulb of P2-Deletion-IRES-tau-lacZ Mice

(A, C, and D) Coronal sections of olfactory bulbs from 2-week-old homozygous P2 deletion–IRES-tau-lacZ mice.

(B, E, and F) Coronal sections of olfactory bulbs from 2-week-old homozygous P2-IRES-tau-lacZ mice.

(A and B) X-gal staining. Blue axons in the P2 deletion–IRES-tau-lacZ mice remain largely in the outer nerve layer of the bulb (A). In contrast, blue axons in P2-IRES-tau-lacZ mouse converge on one glomerulus (B).

(C–F) Alternate sections through the olfactory bulb of P2 deletion–IRES-tau-lacZ mice (C and D) or P2-IRES-tau-lacZ mice (E and F) are subjected to either anti-β-gal immunohistochemistry (C and E) or X-gal staining (D and F). Note the persistence of majority of the labeled axons in the outer nerve layer of the P2 deletion–IRES-tau-lacZ mouse and the convergence of P2 axons on a single glomerulus in the P2-IRES-tau-lacZ mouse. The dashed lines indicate the boundary between outer nerve layer (ONL) and the glomerular layer (GL).

(G–J) A P2 glomerulus exists in the heterozygous P2 deletion–IRES-tau-lacZ mouse. Alternate coronal sections were either hybridized in situ with ^{33}P-labeled P2 RNA probe (G and I) or stained with X-gal (H and J). (I) and (J) are higher magnification views of the glomerular layer of the olfactory bulbs shown in (G) and (H), respectively. In the heterozygous mouse, neurons expressing the wild-type P2 allele still project to a single the P2 glomerulus, whereas axons from neurons expressing the P2 deletion–IRES-tau-lacZ allele do not converge on a glomerulus.

specific glomeruli in the olfactory bulb. It is formally possible, however, that a gene encoding a molecule governing axon guidance is linked to the P2 receptor and its regulatory sequences are disrupted as a consequence of the deletion. We have therefore generated mice with only three base changes in the P2 receptor gene to create nonsense mutations within both the second and sixth transmembrane domains. After the natural stop codon, we introduced *IRES-tau-lacZ*. In these mice, therefore, transcription of the modified allele will not result in the expression of a functional receptor, but these neurons will express tau-lacZ. The blue axons in these mice fail to converge on discrete glomeruli and exhibit a diffuse projection of axons to the olfactory bulb. This pattern is similar to distribution of blue fiber in the P2 deletion mice (data not shown). However, the level of expression of tau-lacZ is significantly reduced when compared to P2 deletion-IRES-tau-lacZ mice, perhaps as a consequence of the instability or reduced level of the mRNA due to the presence of the two premature nonsense codons (Kadowaki et al., 1990; van Hoof and Green, 1996). Thus, deletion of the P2 receptor, or nonsense mutations in the P2 receptor, results in a phenotype in which axons from P2 neurons fail to converge on their glomerular targets and project diffusely within the olfactory bulb. These data suggest that the expression of a functional odorant receptor is required for neurons to find their appropriate glomerular targets in the olfactory bulb.

An alternative explanation for these observations is that neurons that fail to express a functional P2 receptor transcribe a second receptor from the large repertoire of olfactory receptor genes. If the choice of a second receptor is random, then blue neurons could express a vast array of different receptors. Since there is a tight linkage between the choice of receptor and axon targeting, blue neurons could therefore project to multiple glomeruli. This would give the appearance of axon wandering when, in fact, the individual blue axons project to multiple different loci in the olfactory bulb. Two observations argue against this alternative interpretation. First, we observe that the axons of neurons that express

the P2 deletion allele largely remain in the outer nerve layer and only rarely enter the glomerular layer (Figures 2A and 2C). If cells expressing the P2 deletion allele had randomly chosen a second receptor, we would expect that the axons would enter multiple glomeruli, but this is not observed. In addition, we observe that cells expressing the P2 deletion allele are present at normal frequencies at birth, but over the ensuing weeks, the frequency of blue neurons falls such that at 10 weeks the numbers are less than 1% of wild-type P2 neurons (Figure 1E). These observations suggest that the half-life of neurons not expressing a functional P2 receptor is dramatically reduced, implying that these neurons are defective and are not transformed into normal olfactory neurons expressing a second functional receptor. However, definitive evidence that neurons that express the P2 deletion allele do not express a second receptor will require analysis of receptor RNA in isolated P2 deletion blue neurons.

Receptor Substitutions Alter the Patterns of Axon Projections

The P2 deletion experiments indicate that expression of the P2 receptor is required for the convergence of P2 axons on precisely defined loci within the olfactory bulb. A functional odorant receptor independent of the nature of the receptor sequences may be necessary for appropriate target selection. Alternatively, the pattern of projections may be a sensitive function of the nature of the receptor sequences such that the receptor plays an instructive role in the guidance process. In a previous experiment, we performed a single receptor substitution experiment in which the replacement of P2 coding sequences by M12 receptor DNA resulted in the convergence of axons to topographically fixed glomeruli distinct from either the wild-type M12 or P2 glomeruli (Mombaerts et al., 1996). These data suggested that the odorant receptor may play an instructive role in target selection as one of a complement of guidance receptors involved in the generation of the topographic map. However, this conclusion derived from only a single receptor substitution. Moreover, the M12 gene is expressed from a different chromosomal locus than is P2, and M12-expressing cells reside in a distinct zone within the epithelium. It is possible that the additional guidance molecules might reflect either the zone of receptor expression in the epithelium or the locus from which the receptor is expressed in the chromosome. We have therefore performed several additional receptor substitution experiments in which the P2 receptor coding sequences are replaced with receptors expressed either from the same chromosomal locus or in the same epithelial zone as the P2 gene.

The M71→P2 Receptor Substitution

In initial experiments, we performed a "radical" substitution analogous to the M12→P2 substitution in which the P2 coding sequences are replaced with receptor sequences that reside at different chromosomal loci and that are expressed in different zones in the epithelium. The M71 receptor gene resides on mouse chromosome 9 and is expressed in zone IV (Sullivan et al., 1996), the most dorsal zone in the sensory epithelium, whereas the P2 gene is located on chromosome 7 and is expressed in zone III of the epithelium (J. Edmondson, F. W., and R. A., unpublished data). Moreover, neurons expressing the wild-type M71 receptor project to glomeruli in the most dorsal posterior aspect of the bulb very distant from the ventral site of projection of P2 neurons (Ressler et al., 1994). The P2 coding sequences between the start and stop codons in a P2 targeting vector were replaced with the corresponding sequences from the M71 receptor gene. Immediately downstream of the stop codon of M71, we introduced the *IRES-tau-lacZ-LTNL* cassette (Figure 3A). This targeting vector was electroporated into ES cells, and two independent G418-resistant clones resulting from homologous recombination at the P2 locus were identified. These clones were injected into blastocysts and chimeric animals were generated with both targeted clones. Neurons that transcribe the modified P2 allele will express a bicistronic RNA encoding an M71 receptor along with tau-lacZ.

Whole-mount analysis of chimeric mice expressing the M71→P2-IRES-tau-lacZ allele demonstrates the expression of lacZ in neurons in zone III of the epithelium, the P2 zone (Figure 3B). The blue M71→P2 axons extend dorsally and posteriorly in the olfactory bulb and converge on one medial (Figure 3B) and one lateral glomerulus. The whole-mount analysis clearly distinguishes the M71→P2 glomerulus from the more ventral P2 glomerulus (Figure 3C) and the more dorsal M71 glomerulus (see Ressler et al., 1994). Thus, the substitution of P2 coding sequences by those of M71 results in the convergence of axons to topographically fixed glomeruli distant from the wild-type P2 glomerulus and somewhat closer to the wild-type M71 glomerulus. The analysis of M71→P2-IRES-tau-lacZ mice is thus far restricted to chimeric animals, but the relative position of the M71→P2 glomerulus is invariant in four independent chimeric mice. In a previous study, we performed a conceptually similar substitution of M12 coding sequences into the P2 gene. M12, like M71, resides on a different chromosome than P2 and is expressed in zone IV of the epithelium. The M12→P2 glomerulus, however, resides at a position close to the P2 glomerulus and distant from either the wild-type M12 or the M71→P2 glomerulus (Figure 6A). These data indicate that alterations in the nature of the P2 receptor sequences alter the patterns of projections of P2 neurons.

The M50→P2 Receptor Substitution

Our results suggest that the olfactory receptor plays an instructive role in axon targeting, but that the receptor may be one determinant in a more complex guidance process involving additional guidance molecules. We therefore performed more substitution experiments to ask whether these additional guidance molecules might reflect the nature of the chromosomal locus and the zone of receptor expression. The P2 gene resides within a linked array of receptor genes on mouse chromosome 7. We have isolated a yeast artificial chromosome (YAC) that encodes at least seven receptor genes in the relative order, P1, P2, P4, P3, M50, I7, and P5 (J. Edmondson, F. W., and R. A., unpublished data). The P genes (P1–P5)

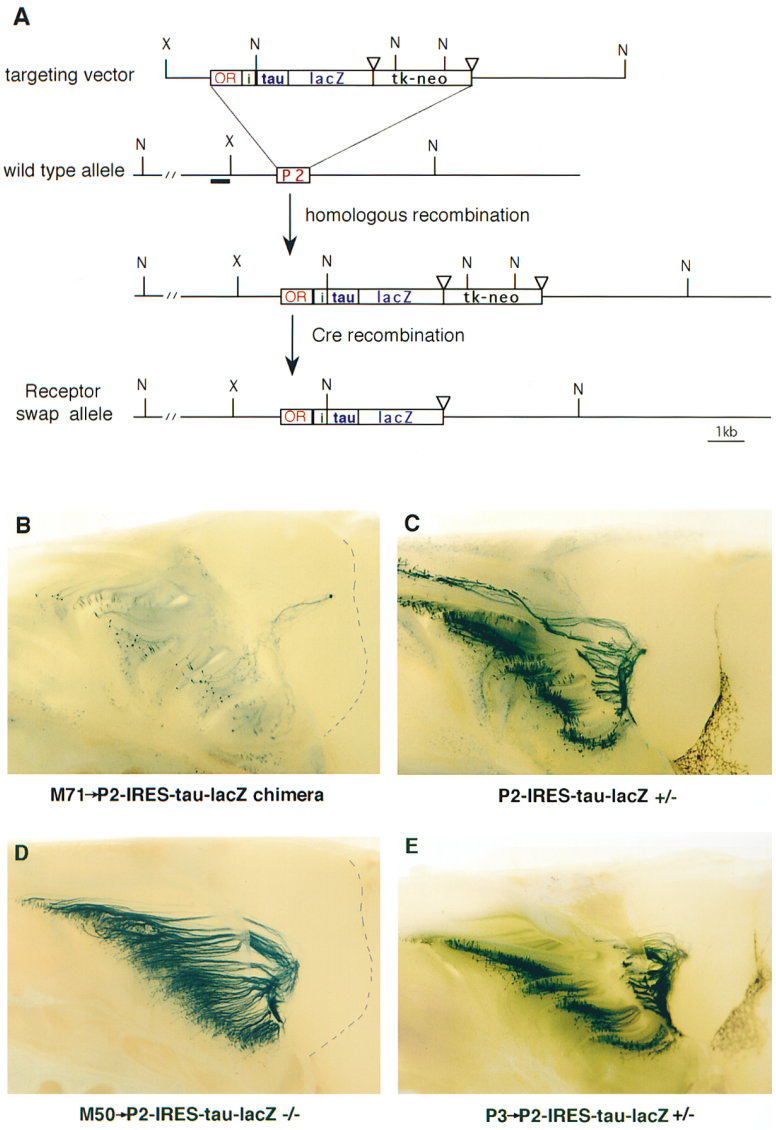

Figure 3. Receptor Substitutions Alter the Pattern of Axon Projections

(A) Schematic representation of the genetic strategy to substitute the P2 coding region with different odorant receptor (OR) sequences. Homologous recombination between the targeting vector and the wild-type P2 gene results in the replacement of the P2 coding sequences with the *OR-IRES-tau-lacZ-loxP-tk-neo-loxP* cassette. Transient transfection with a plasmid expressing the Cre recombinase results in the excision of the *tk-neo* cassette, leaving a single *loxP* site. This genetic manipulation results in the substitution of the P2 coding sequence with either the M71, M50, or P3 odorant receptor (OR), and introduces an *IRES-tau-lacZ* cassette. Inverted triangles indicate loxP sites. X, XhoI; N, NcoI; i, *IRES*.

(B) Whole-mount view of the nasal cavity and the medial aspect the olfactory bulb of an M71→P2-IRES-tau-lacZ chimera (12-day-old) stained with X-gal. Neurons expressing the M71→P2-IRES-tau-lacZ allele project to a posterior position in the bulb, distant from the wild-type P2 (C) or M71 glomerulus (data not shown).

(C) Whole-mount view of the medial aspect of the olfactory bulb of a heterozygous P2-IRES-tau-lacZ mouse (12-day-old) stained with X-gal. P2 neurons project to a medioventral position in the bulb.

(D) Whole-mount view of the nasal septum and the medial aspect of the olfactory bulb of a homozygous M50→P2-IRES-tau-lacZ mouse (5-week-old) stained with X-gal. Neurons expressing the M50→P2-IRES-tau-lacZ allele project to a posterior-ventral position in the olfactory bulb.

(E) Whole-mount view of a heterozygous P3→P2-IRES-tau-lacZ mouse (3-week-old) stained with X-gal. Neurons expressing the P3→P2-IRES-tau-lacZ allele project to a medioventral position in the olfactory bulb.

The dashed lines in (B) and (D) define the posterior boundary of the olfactory bulb.

share significant homology and are all expressed in zone III of the olfactory epithelium. M50 and I7 represent members of different subfamilies and are expressed in zone I in the epithelium (Buck and Axel, 1991; Vassar et al., 1993). We next examined the consequences of a receptor substitution in which the P2 coding region is replaced by the corresponding M50 sequences. A gene targeting strategy, similar to that described for the M71→P2-IRES-tau-lacZ substitution, was employed to obtain germline transmission of M50→P2-IRES-tau-lacZ mice (Figure 3A). Whole-mount analysis of mice either heterozygous or homozygous for the M50→P2-IRES-tau-lacZ mutation reveals the expression of tau-lacZ in zone III (the P2 zone) of the epithelium (Figure 3D). The expression of the M50→P2-IRES-tau-lacZ allele in zone III (the P2 zone) is also apparent in sections through the epithelium annealed either with P2 or M50 probes or stained for lacZ (Figures 4A–4C).

The blue axons identifying the projections of neurons expressing the M50→P2 allele converge on one medial and one lateral glomerulus, which occupy a medioventral position (Figure 3D). Serial sections were prepared from the entire olfactory bulb of four heterozygous M50→P2-IRES-tau-lacZ mice to determine more precisely the site of convergence of blue axons. Adjacent serial sections were either stained with X-gal to identify the neurons expressing the M50→P2-IRES-tau-lacZ allele, hybridized in situ with an M50 probe to identify the neurons expressing M50 receptor RNA from both the wild-type and modified allele, or hybridized in situ with a P2 probe to identify the neurons expressing the wild-type P2 allele (Figures 4D–4F).

Sections through the bulb reveal that the axons from neurons expressing the M50→P2-IRES-tau-lacZ allele project to glomeruli distinct from either the wild-type P2 or M50 glomeruli. The blue axons converge on a single glomerulus that resides 250 μm (about 4 glomeruli) posterior to the wild-type P2 glomerulus and 450 μm (about 6 glomeruli) dorsal to the wild-type M50 glomerulus (Figures 4D–4F). These relative positions are maintained on

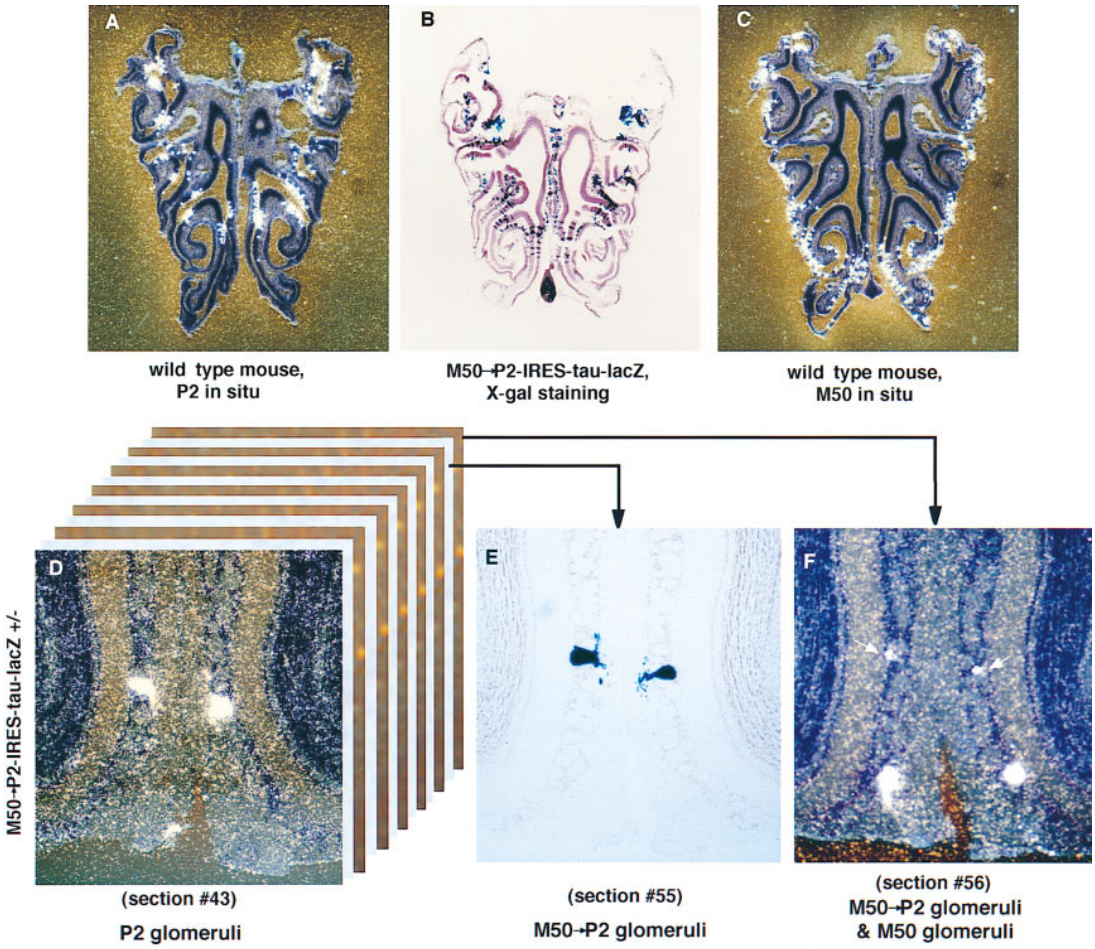

Figure 4. In Situ Hybridization and X-gal Staining Analysis of M50→P2-IRES-tau-lacZ Mice

(A) In situ hybridization with ^{33}P-labeled P2 RNA probe on coronal sections through the olfactory epithelium of wild-type mice. The P2 neurons are scattered in zone III.

(B) X-gal staining of the epithelium from a homozygous M50→P2-IRES-tau-lacZ mouse. Neurons expressing the M50→P2-IRES-tau-lacZ allele are distributed in zone III of the epithelium. Note that expression of the M50→P2 allele is restricted to zone III (the P2 zone) rather than zone I (the M50 zone, see [C]).

(C) In situ hybridization with ^{33}P-labeled M50 RNA probe to the epithelium of wild-type mice. M50 neurons are present in the most ventral zone (zone I).

(D–F) Analysis of the olfactory bulb of heterozygous M50→P2-IRES-tau-lacZ mice. Alternate 20 μm sections were either hybridized with a ^{33}P-P2 RNA probe, a ^{33}P-M50 RNA probe, or stained with X-gal to determine the relative positions of the P2, M50, and M50→P2 glomeruli. The M50→P2 glomerulus, identified by X-gal staining (E), is approximately 250 μm posterior to the wild-type P2 glomerulus (D), identified by in situ hybridization with the P2 probe, and is about 450 μm dorsal to the wild-type M50 glomerulus (F), identified by in situ hybridization with the M50 probe. Weak hybridization signals with the M50 RNA probe also identifies the M50→P2 glomerulus (arrows in [F]). Section numbers are indicated in the figure.

both the lateral and medial aspect of the bulb and are consistently observed in each of the four heterozygous animals examined. Moreover, in homozygous M50→P2 mice, which contain two copies of the modified allele and no P2 receptor sequences, the blue axons converge on a glomerulus in the same relative position as the M50→P2 axons in heterozygous animals (Figure 3D and data not shown). Thus, the substitution of P2 coding regions by those of M50 results in the convergence of axons to topographically fixed glomeruli distinct from either P2 or M50. These results suggest that substitutions between receptor genes expressed from the same chromosomal locus but in different epithelial zones still do not restore targeting to the wild-type glomeruli.

The P3→P2 Receptor Substitution

We next performed a more "conservative" substitution experiment in which we have replaced the P2 coding region with corresponding sequences from the P3 gene. P2 and P3 share 75% amino acid identity. Moreover, these two genes reside at the same chromosomal locus and are expressed in the same zone in the epithelium (data not shown). A gene targeting strategy similar to that described for the M50→P2 substitution (Figure 3A) was employed to generate germline transmission with two independent P3→P2 clones.

In wild-type mice, P3 neurons project axons to two glomeruli, one on the medial and one on the lateral aspect of the bulb. The P3 glomerulus resides about

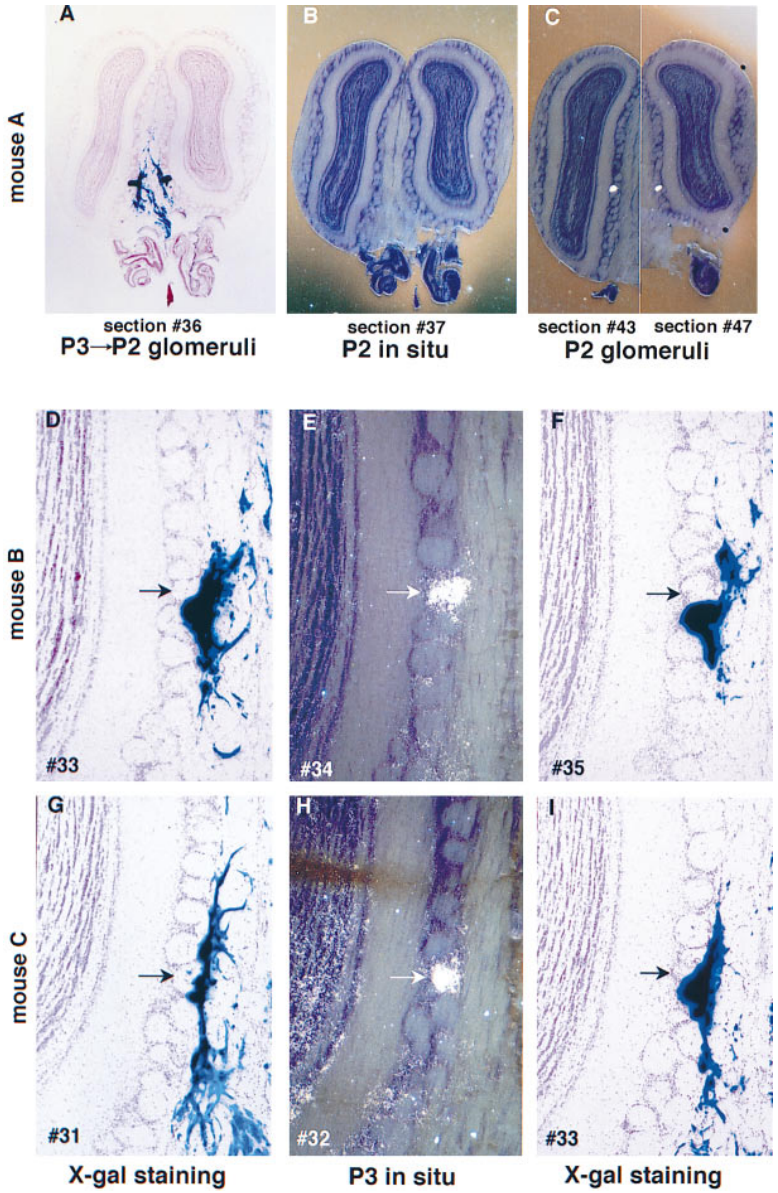

Figure 5. The P3→P2 Glomerulus Is Immediately Adjacent to the Wild-Type P3 Glomerulus

(A–C) The P3→P2 glomerulus is anterior to the P2 glomerulus. Alternate 20 μm sections from the olfactory bulb of a heterozygous P3→P2-IRES-tau-lacZ mouse were either stained with X-gal (A) or hybridized in situ with the ^{33}P-P2 RNA probe (B and C). The center of the medial P3→P2 glomerulus as identified by X-gal staining ([A], section #36) resides about 200 μm anterior to the wild-type P2 glomerulus as identified by in situ hybridization with the ^{33}P-P2 RNA probe ([C], section #43 and #47). In accord with this, sections adjacent to the P3→P2 glomerulus are negative for in situ hybridization with the P2 probe (B), and sections adjacent to the wild-type P2 glomeruli are negative for X-gal staining (data not shown).

(D–I) The P3→P2 glomerulus touches the wild-type P3 glomerulus. Adjacent 20 μm sections from the olfactory bulb of two heterozygous P3→P2-IRES-tau-lacZ mice were either stained with X-gal (D, F, G, and I) or hybridized in situ with ^{33}P-labeled P3 RNA probe (E and H). The P3→P2 glomeruli as revealed by X-gal staining ([D] and [F] for mouse B; [G] and [I] for mouse C) touch the wild-type P3 glomeruli as identified by in situ hybridization with the P3 probe ([E] for mouse B; [H] for mouse C). The arrows indicate the wild-type P3 glomerulus in these serial sections. Note that in situ hybridization with P3 probe does not detect the P3→P2 glomeruli (see text). Section numbers are indicated in the lower left corner. These relative positions are the same on both medial and lateral aspects of the bulb and in all four heterozygous mice examined.

200 μm (about 3 glomeruli) anterior to the P2 glomerulus (data not shown). Whole-mount analysis of mice heterozygous for the P3→P2-IRES-tau-lacZ mutation reveals the presence of blue neurons in zone III of the epithelium and the convergence of blue axons on one medial and one lateral glomerulus, which occupy a medial ventral position of the bulb (Figure 3E). It is not possible, however, by whole-mount analysis to determine whether the blue P3→P2 glomeruli are coincident with either the wild-type P2 or P3 glomeruli. Serial cryostat sections were therefore prepared through the entire olfactory bulb from heterozygous P3→P2-IRES-tau-lacZ mice. Adjacent 20 μm sections were either stained with X-gal or subjected to in situ hybridization with ^{33}P-labeled antisense P2 or P3 RNA probes. X-gal staining identifies the axonal projections from neurons expressing the P3→P2-IRES-tau-lacZ allele (Figures 5A, 5F, and 5I). In situ hybridization with the P2 probe identifies the projections from neurons expressing the wild-type P2 allele (Figure 5C), whereas in situ hybridization with the P3 probe identifies the site of convergence of neurons expressing the wild-type P3 allele (Figures 5E and 5H). We have observed previously that in situ hybridization cannot be reliably employed to detect glomeruli from neurons expressing modified alleles also expressing tau-lacZ, perhaps because the longer mRNAs are not present in axon termini.

Analysis of these sections reveals that neurons expressing the P3→P2-IRES-tau-lacZ allele project to glomeruli immediately adjacent to the wild-type P3 glomeruli. X-gal staining is restricted to one medial and one lateral glomerulus that abuts the glomeruli detected by in situ hybridization with P3 probes (Figures 5D–5I). These glomeruli are distinct from the wild-type P2 glomeruli that reside at more posterior positions as determined by in situ hybridization (Figures 5A–5C). The relative positions of the P2, P3, and P3→P2 glomeruli are maintained in both the medial and lateral aspect of the

bulb and are consistently observed in the right and left bulb in each of four mice studied. It should be emphasized that the position of the P3→P2 glomeruli, immediately adjacent to the P3 glomeruli, does not reflect animal-to-animal variance. In all P2-IRES-tau-lacZ mice studied, the neurons expressing the modified P2 allele project to the same glomeruli as do neurons expressing the wild-type P2 allele. In contrast, in all four P3→P2-IRES-tau-lacZ heterozygous mice, neurons expressing the modified P3→P2 allele project to a glomerulus adjacent to the wild-type P3 glomerulus and anterior to the P2 glomerulus (Figures 5D–5I). This analysis of heterozygous animals provides an internal control for slight variations in the map between different animals. Thus, the substitution of P2 coding sequences by those of P3 results in the convergence of axons to glomeruli immediately adjacent to the wild-type P3 glomeruli and distinct from the P2 glomeruli. These results contrast with all other substitutions that replace the P2 coding sequences with receptor sequences expressed either in different zones or from different chromosomal loci. Unlike the P3→P2 substitution, these substitutions result in the convergence of axons at glomeruli distinct and more distant from their wild-type counterparts (summarized in Figure 6).

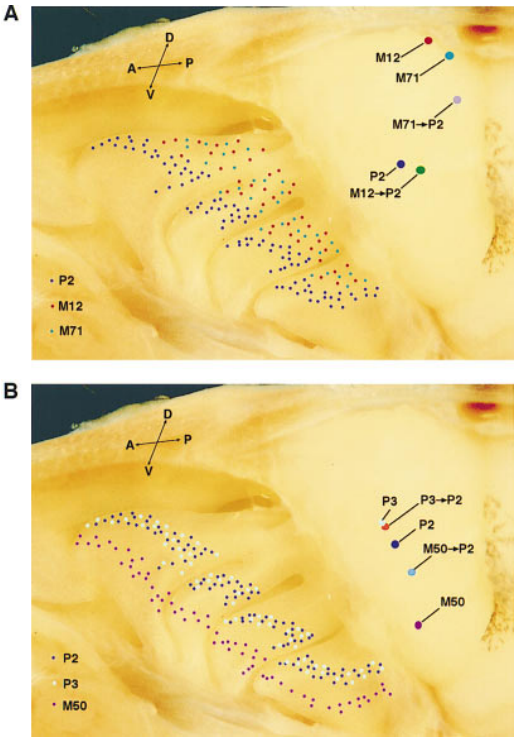

Figure 6. Schematic Representation of the Results of the Receptor Substitution Experiments

(A) Schematic representation of the pattern of expression of the wild-type P2, M12, and M71 neurons in the epithelium and the relative positions of the wild-type P2, M12, M71, and the M12→P2, M71→P2 glomeruli in the olfactory bulb.

(B) Schematic representation of the pattern of expression of the wild-type P2, P3, and M50 neurons in the epithelium and the relative positions of the wild-type P2, P3, M50, and the P3→P2, M50→P2 glomeruli in the olfactory bulb.

The P2, P3, and M50 genes are linked at one locus on mouse chromosome 7 whereas the M12 and M71 gene reside at two distinct chromosomal loci. M50 is expressed in zone I, P2 and P3 are expressed in zone III, and M12 and M71 are expressed in zone IV. Neurons expressing all substituted alleles are restricted to the P2 zone (zone III). Note that in each of the receptor substitutions, the targeted glomerulus maintains an A/P position aligned with the wild-type glomerulus of the donor sequence, rather than with the P2 glomerulus itself.

Discussion

Neurons connect to one another with remarkable precision. The specificity of connections in the nervous system is essential for the translation of neuronal activity into meaningful neural codes. In most sensory systems, peripheral neurons in the receptor sheet project axons to precise loci in the CNS to create an internal representation of the sensory world that translates stimulus features into neural information. In the olfactory system, neurons expressing a given odorant receptor, and therefore responsive to given odorants, project with precision to 2 of the 1800 glomeruli within the olfactory bulb. Since the positions of individual glomeruli are topographically defined, the bulb provides a spatial map that identifies which of the numerous receptors have been activated within the sensory epithelium. The quality of the olfactory stimulus would therefore be encoded by the specific combination of glomeruli activated by a given odorant.

Establishing a Topographic Map: Odorant Receptor as Guidance Molecule

The observation that each of 1000 different olfactory sensory neurons project with precision to a small number of topographically fixed glomeruli immediately poses the question as to how this precise topographic map is established. What is the nature of the molecules guiding the projections of sensory axons? The most parsimonious model linking receptor choice with target selection in the brain would argue that the odorant receptor is expressed on dendrites where it recognizes odors in the environment and is also expressed on axon termini where it would recognize a distinct set of spatial cues elaborated by the bulb. The consequences of receptor mutations and receptor substitutions on target selection strongly suggest that the odorant receptor indeed plays an instructive role in the guidance process. In initial experiments, we demonstrate that deletion or nonsense mutations of the P2 receptor gene cause axons of cells expressing these mutant alleles to “wander” broadly within the outer nerve layer of the bulb without converging on the P2 glomerulus. The projection of axons to the olfactory bulb is therefore not dependent on the expression of a functional receptor. Once in the bulb, however, receptor expression is essential for the convergence of P2 axons on discrete glomeruli. These data do not distinguish whether the receptor is playing a permissive or an instructive role in the guidance process. A functional receptor (independent of the nature of the receptor sequence) may be necessary to permit the expression of a distinct set of guidance molecules, which then serve to direct axonal projections to their appropriate glomerular targets. Alternatively, the odorant receptor may be expressed on axon termini such

that different receptors provide axons with different instructions to assure appropriate target selection in the bulb.

The results of the receptor substitution experiments strongly argue that odorant receptors play an instructive role in target selection but cannot be the sole determinant in the guidance process. We have replaced the coding region of the P2 gene with the corresponding coding regions of several other receptors and examined the consequences on the formation of the topographic map. In the most conservative experiment, substitution of P2 coding sequences with those of the P3 gene, two linked homologous genes expressed in the same epithelial zone, result in the projection of P3→P2 axons to a glomerulus immediately adjacent to the wild-type P3 glomerulus. All other substitutions that replace the P2 coding sequences with receptor sequences expressed either in different zones or from different chromosomal loci result in the convergence of fibers to glomeruli distinct and more distant from their wild-type counterparts (summarized in Figure 6). Convergence to aberrant glomeruli is observed even if the substitution is performed with a receptor gene (M50) expressed from the same chromosomal locus as the P2 gene, but in a distinct epithelial zone. These data indicate that selection of the precise glomerular targets requires both the odorant receptor and additional guidance receptors that may reflect the epithelial zone in which the receptor is expressed.

Our data suggest that the odorant receptor may dictate anterior–posterior (A/P) positioning of glomerular targets, whereas the zone of expression may contribute to dorsoventral (D-V) patterning of sensory projections. In each of the four substitutions of the P2 receptor gene, the targeted glomerulus maintains an A/P position aligned with the wild-type glomerulus of the donor sequence rather than with that of the P2 glomerulus itself. For example, the M50→P2 glomerulus is distinct from either the wild-type P2 or M50 glomeruli but maintains an A/P position aligned with wild-type M50 (Figures 4 and 6).

The four expression zones in the epithelium each occupy a discrete dorsoventral position such that the D-V pattern of sensory neurons in the epithelium is conserved in the projections to the olfactory bulb (Yoshihara et al., 1997; Saucier and Astic, 1986; Schoenfeld et al., 1994). This is also apparent upon examination of the glomerular targets of neurons expressing specific odorant receptors. Thus, the M50 neurons that reside in the most ventral zone project ventrally in the bulb, whereas M71 and M12 neurons that are restricted to dorsal regions of the epithelium project most dorsally in the bulb (Ressler et al., 1993) (Figure 6). In this manner, the odorant receptor along with guidance receptors reflecting the epithelial position of the sensory neuron may contribute information in two dimensions that govern the precision of target selection in the bulb. At present, we cannot discern whether additional guidance receptors are also expressed by sensory neurons that reflect the chromosomal locus from which the odorant receptor is expressed since we have not substituted P2 with receptor genes expressed in the same zone as P2 but from different chromosomal locations.

If precise targeting to the P3 glomerulus requires the P3 odorant receptor and additional guidance receptors that represent the zone and perhaps the chromosomal locus, why do P3→P2 fibers not converge precisely on the P3 glomerulus? If the odorant receptor indeed recognizes guidance cues, it is reasonable to assume that the nature of the odorant receptor sequence on axons and the number of receptors will alter the pattern of axonal projections. If graded guidance cues are read by receptors on the axon termini, then even slight variations in the level of P3 when expressed from the P2 chromosomal locus, rather than from the wild-type P3 locus, might cause subtle alterations in the position of projections. Alterations in the level of P3 receptor could also result from subtle sequence changes required to generate the targeted P3→P2-tau-lacZ allele (e.g., polylinkers, loxP sites, and sequence changes surrounding the translational start and stop sites). We might anticipate that conservative substitutions that introduce fewer sequence changes in the P2 locus might result in even greater precision of targeting. Finally, we cannot exclude the possibility that the convergence of P3→P2 fibers to a glomerulus touching the P3 glomerulus might result from other guidance mechanisms that bring the P3→P2 glomerulus and the P3 glomerulus together by chance. Conservative substitutions with other odorant receptors will ultimately allow us to distinguish among these alternatives. Whatever the interpretation of the P3→P2 substitution, the results of these experiments strongly suggest that the olfactory receptor plays an instructive role in axon targeting, but that the receptor is only one determinant in a more complex guidance process.

How may the odorant receptor play an instructive role in the guidance process? In one model that we favor, the odorant receptor is expressed on the axon termini along with other guidance receptors where it recognizes positional cues elaborated by the bulb. Each of the thousand distinct olfactory sensory neurons will therefore bear a unique combination of guidance receptors that define a code dictating the selection of a unique glomerular target in the olfactory bulb. Such a model does not necessarily imply that there are a thousand distinct cues, each spatially localized within the olfactory bulb. Rather, a small number of graded cues may cause the differential activation of the different odorant receptors on the axon termini. In this manner, the different affinities of individual receptors for one or a small number of cues, perhaps taken together with different levels of receptor, might govern target selection. Such a model is formally equivalent to models of retinotopy in which a gradient of guidance receptor on retinal axons is matched by a positional gradient guidance cue in the tectum (Holt and Harris, 1993; Drescher et al., 1997).

A second model argues that in the substitution experiments the odorant receptors govern target selection, not by virtue of their presence within the axon termini, but rather as a consequence of their activation within the dendrites. It is possible, for example, that the activation of different receptors on the dendrites results in the expression of different levels of distinct guidance receptors on the axon termini. In such a model, the nature of the receptor would indeed play an instructive role in the guidance process, but only indirectly by virtue of the induction of a second set of guidance molecules.

Activity-Dependent Processes

In other sensory systems, it is thought that guidance molecules during development generate a coarse topographic map that is subsequently refined by coordinate neural activity to achieve a precision of connections between the periphery and the brain (Constantine-Patton et al., 1990; Woolsey, 1990; King and Moore, 1991; Goodman and Shatz, 1993). The most prominent feature of the olfactory map is the segregation of like axons at invariant convergent loci within the bulb. What is the role of activity-dependent processes in the establishment of an olfactory topographic map? In the visual system, for example, eye-specific retinotopic maps exist first in the thalamus and the superior colliculus and then are multiply represented in the visual cortex. The orderly projections of retinal axons to the brain are thought to result from a graded set of guidance cues elaborated by their targets, which are recognized by receptors on axons to establish a coarse retinotopic map (Sperry, 1963; Holt and Harris, 1993; Drescher et al., 1997). The precision of this map is then afforded by the correlated activity of neighboring axons (Constantine-Patton et al., 1990) that results from both spontaneous synchronous waves of neuronal activity in the retina (Galli and Maffei, 1988; Shatz, 1990), as well as visual experience (Hubel et al., 1977; LeVay et al., 1980; Stryker and Harris, 1986). In this manner, genetically determined cues initially guide synapse formation, but subsequent refinement ultimately requires spontaneous neural activity as well as visual experience (Katz and Shatz, 1996).

In contrast, in the olfactory system, a topographic map encoding odor quality in the bulb develops without apparent contribution from activity-dependent processes. Mice lacking the olfactory cyclic nucleotide-gated ion channel fail to exhibit odor-evoked electrophysiologic responses in the sensory epithelium (Brunet et al., 1996). The pattern of convergence of like axons in the bulb, however, is unaltered in these mutant mice (L. Brunet, F. W., R. A., and J. Ngai, unpublished data), arguing strongly that olfactory experience is not required for the establishment or refinement of the topographic map. Similarly, dramatic reductions in odor-evoked responses are observed in mice with homozygous deficiency in G_{olf}, the major G protein in olfactory sensory neurons, yet precise convergence of olfactory sensory projections is maintained in these mutant animals (Belluscio et al., 1998). Whereas spontaneous, correlated activity of like axons may elicit spatial segregation even in the absence of odor-evoked activation of sensory neurons, it is difficult to envisage a mechanism for the generation of a correlated activity pattern that would result in the spontaneous activation of a random distribution of like neurons in the absence of odor stimulation.

It remains possible, however, that segregation of the axons of neurons bearing substitutions at the P2 locus from the wild-type P2 axons is the result of activity-dependent sorting as a consequence of competition under these artificial experimental circumstances. For example, neurons that choose to express the P2 allele may all initially project to the P2 target within the bulb independent of the nature of the receptor sequences introduced into the P2 coding region. In this model in a heterozygous animal, neurons expressing an M50→P2 allele, for example, and neurons expressing the wild-type P2 allele may both initially project to a single glomerular target. Activity-dependent processes could then result in the segregation of like axons such that the M50→P2 fibers are displaced from the wild-type P2 glomerulus. Several lines of experimental evidence argue against such a model. First, axons from neurons bearing four different substitutions at the P2 locus project to four different invariant positions in the olfactory bulb. Whereas activity-dependent guidance mechanisms can be envisaged that affect the segregation of axons from neurons bearing the same receptor, it is difficult to invoke pure activity-dependent mechanisms that precisely determine the positions of convergence in a stereotyped and invariant fashion. Second, in homozygous mice bearing two copies of the M50→P2 allele, no P2-expressing axons remain, yet the axons from neurons expressing the modified allele project to the same position as the displaced glomerulus observed in heterozygous animals. These data argue strongly against activity-dependent sorting as a mechanism for the displacement of axons from neurons bearing substitutions at the P2 receptor.

These observations do not, however, exclude a role for activity-dependent processes in the maintenance or potential plasticity of the map after it is established. We observe, for example, that neurons that do not express functional odorant receptor appear to have a significantly shorter half-life. Activity-dependent processes may therefore be essential for the survival of olfactory neurons postnatally and in this manner may alter the input to individual glomeruli, altering the sensitivity to individual odors. Taken together, these data argue that activity-dependent processes do not play a significant role in the formation of the olfactory map. Rather, they strongly suggest that the precision of the topographic map is a consequence of the elaboration of spatial cues by the bulb that guide axons to the 1800 invariant loci.

Comparison with Other Sensory Systems

The topographic map in the olfactory bulb differs in character from the representation of sensory cells in the auditory, somatosensory, and visual systems. In these sensory systems, neurons within the peripheral receptor sheet project to the central nervous system in a highly ordered manner such that neighbor relations in the periphery are maintained in the brain. The orderly representation of cells in the peripheral receptor sheet may therefore transmit information to the brain concerning both the quality of a sensory stimulus and the position of a stimulus in space. The visual world, for example, is projected onto the retina, which in turn projects its representation of visual space first to the thalamus and ultimately to higher sensory centers in the visual cortex. Similarly, the somatosensory neurons provide a neural representation of the body surface in the somatosensory cortex. Finally, in the auditory system, a tonotopic map exists both in the cochlea and is represented in the auditory cortex, such that hair cells tuned to respond to diminishing frequencies of sound are linearly arrayed along one axis of the cochlea. This orderly array in the periphery is maintained in the projections of cochlear ganglion cells to higher auditory centers.

These observations immediately suggest that the relative position of a sensory neuron in the periphery will determine its pattern of projection to the brain. Individual sensory neurons would therefore be endowed with a positional identity that can direct their ordered representation in the brain. In accord with this model, if retinal axons are transplanted to inappropriate targets in the optic tectum (the first relay in the brain), they will reorient and ultimately synapse at their appropriate target location in the tectum (Harris, 1986, 1989). The positional information on the retinal afferents and on their brain targets is thought in part to depend upon an anterior–posterior gradient of an Eph receptor (Mek-4/EphA3) on retinal axons and overlapping gradients of two Eph ligands (RAGS/ephrinA5 and E1F-1/ephrinA2) in the tectum (Cheng et al., 1995; Drescher et al., 1995; Nakamoto et al., 1996). In this manner, topographically ordered sensory cells can exploit inherent spatial information in the periphery to direct their orderly projections to the brain.

In the olfactory system, however, neurons expressing a given receptor, and therefore responsive to a given odorant, are randomly dispersed within zones in the sensory epithelium. Spatial order is restored more centrally in the olfactory bulb where like axons converge on two positionally invariant glomeruli to generate a topographic map encoding odor quality. In contrast to other sensory systems, olfactory neurons are therefore unlikely to acquire a positional identity that defines its projection targets in the brain. Rather, our data suggest that an olfactory neuron is afforded a distinct identity by virtue of the nature of the receptor it expresses, and this receptor choice also dictates the pattern of projection in the olfactory bulb.

Experimental Procedures

Construction of P2-Deletion (C57Bl/6) and P2-Deletion (129/sv) Plasmids

A genomic clone deleted in P2 coding sequences was constructed to allow the substitution of different receptor coding regions into the P2 gene. A genomic clone (XhoI-NcoI), encoding the P2 gene (Mombaerts et al., 1996), was modified to delete the P2 coding sequence from the start to stop codon. In brief, a 5′ PacI restriction site was created via recombinant PCR immediately 5′ to the presumptive P2 start codon, and a 3′ PacI site was created three nucleotides 3′ to the P2 stop codon. Cleavage with PacI and religation generates a plasmid deleted in the P2 coding sequence that allows for the ready insertion of other genes. This P2 deletion plasmid was constructed both with a P2 clone of C57Bl/6 and 129/sv origin.

Generation of Targeted Mutations

P2-Deletion-IRES-tau-lacZ

The ETLpA$^-$/LTNL fragment (see Mombaerts et al., 1996, for details) containing *IRES-tau-lacZ*, followed by *loxP-tk-neo-loxP*, was cloned into the P2 deletion (C57Bl/6) plasmid as a PacI restriction fragment. This construct was linearized at the 5′ XhoI site (35 μg) and electroporated into 30×10^6 E14 ES cells (Hooper et al., 1987) at 800 V and 3 μF with a Bio-Rad Gene Pulser. Homologous recombinants were identified by Southern blotting. Genomic DNA from *neo*^{r} ES cell clones was digested with NcoI and hybridized with a 5′ external probe. One targeted clone was identified and was subsequently transiently transfected with the plasmid pBS185 (GIBCO-Life Technologies), which contains Cre recombinase under the control of the *CMV* promoter. After transfection, *tk*$^-$ clones were isolated, and genomic DNA was digested with BamHI and hybridized with a 3′ P2 probe to identify subclones that underwent Cre-mediated excision of the *tk-neo* sequences. Two such subclones were injected into C57Bl/6 blastocysts to produce chimeras that transmitted the P2 deletion allele through the germline. Mice are in a mixed (129 × C57Bl/6) background.

P3→P2-IRES-tau-lacZ

A genomic clone containing the P3 receptor gene was modified by recombinant PCR to introduce a PacI site three nucleotides 5′ to the presumptive P3 start codon, and AscI and PacI sites were inserted in tandem three nucleotides 3′ to the P3 stop codon. The P3 coding sequences were removed by PacI digestion and cloned into the PacI site of the P2 deletion (129/sv) plasmid substituting P2 coding sequences with those of P3. ETLpA$^-$/LTNL was subsequently ligated into this plasmid as an AscI restriction fragment. Gene targeting and Cre-mediated excision were performed as described for P2 deletion–IRES-tau-lacZ, except that W9.5 ES cells (Stewart, 1993) were used as a recipient. Germline transmission was obtained with two independently targeted clones. The resultant mutant mice are in mixed (129 × C57Bl/6) background.

M50→P2-IRES-tau-lacZ

The M50 coding sequence was modified by recombinant PCR to introduce 30 nucleotides encoding the human c-myc epitope in-frame between the second and the third M50 codon. A PacI site was then created via recombinant PCR three nucleotides 5′ to the M50 start codon. AscI and PacI sites were introduced in tandem three nucleotides 3′ to the M50 stop codon. The M50 coding sequence was excised by PacI digestion and cloned into the P2 deletion (C57Bl//6) plasmid to replace the P2 coding region with that of M50. ETLpA$^-$/LTNL was then introduced into the AscI site of this plasmid. Gene targeting, Cre recombination, and the generation of germline chimeras were performed as described for P2 deletion–IRES-tau-lacZ. One targeted M50→P2 clone was isolated and used to generate mutant mice that are in mixed (129 × C57Bl/6) background. It should be noted that the M50→P2 gene contains a c-myc epitope, two amino acids from the NH_2 terminus. This epitope is unlikely to affect the pattern of projections since control experiments reveal that epitope-tagged P2 and unmodified P2-expressing cells project axons to the same glomerulus (data not shown).

M71→P2-IRES-tau-lacZ

The M71 gene was modified in a manner similar to P3 and M50 to generate a 5′ PacI site immediately 5′ to the presumptive start codon, and tandem AscI-PacI sites three nucleotides 3′ to the stop codon. The M71 coding region was ligated into P2 deletion (129/sv) plasmid as a PacI restriction fragment. ETLpA$^-$/LTNL was introduced as an AscI fragment. Gene targeting and Cre-mediated recombination were performed as described for P3→P2-IRES-tau-lacZ. Chimeras were generated by injecting targeted ES cells into C57Bl/6 blastocysts. Four chimeras obtained from two independently targeted clones were analyzed.

Immunohistochemistry

Immunohistochemistry to detect β-galactosidase was performed on 2-week-old mice. The olfactory turbinate and bulb were dissected and frozen in OCT (Miles). Twenty-eight micrometer coronal sections were collected on Superfrost slides (Fisher). Slides were then fixed in 4% paraformaldehyde at 4°C and washed three times in PT (**P**BS + 0.1% **T**riton X-100) at room temperature. Tissue sections were then reacted with rabbit anti-β-galactosidase antibody (Capel) at 1:1000 dilution in PTS (**P**BS + 0.1% **T**X-100+ 1% goat **s**erum, GIBCO). The bound primary antibody was then visualized using Cy3-conjugated anti-rabbit IgG (Jackson Laboratories).

X-gal staining and in situ hybridization were performed as described previously in detail (Vassar et al., 1993, 1994; Mombaerts et al., 1996).

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