An Olfactory Sensory Map Develops in the Absence of Normal Projection Neurons or GABAergic Interneurons

Alessandro Bulfone,† Fan Wang,* Robert Hevner,* Stewart Anderson,* Tyler Cutforth,† Sandy Chen,* Juanito Meneses,* Roger Pedersen,* Richard Axel,* and John L. R. Rubenstein‡

* Nina Ireland Laboratory of Developmental Neurobiology
  Center for Neurobiology and Psychiatry
  Department of Psychiatry and Programs in Neuroscience
  Developmental Biology and Biomedical Sciences
  University of California
  San Francisco, California 94143-0984
† Department of Biochemistry and Molecular Biophysics and
Howard Hughes Medical Institute
College of Physicians and Surgeons
Columbia University
New York, New York 10032

Summary

Olfactory sensory neurons expressing a given odorant receptor project to two topographically fixed glomeruli in the olfactory bulb. We have examined the contribution of different cell types in the olfactory bulb to the establishment of this topographic map. Mice with a homozygous deficiency in Tbr-1 lack most projection neurons, whereas mice with a homozygous deficiency in Dlx-1 and Dlx-2 lack most GABAergic interneurons. Mice bearing a P2-ires-tau-lacZ allele and deficient in either Tbr-1 or Dlx-1/Dlx-2 reveal the convergence of axons to one medial and one lateral site at positions analogous to those observed in wild-type mice. These observations suggest that the establishment of a topographic map is not dependent upon cues provided by, or synapse formation with, the major neuronal cell types in the olfactory bulb.

Introduction

Most sensory systems segregate input from peripheral receptor neurons to provide a spatial map that defines the quality of a stimulus. What features of the vertebrate olfactory apparatus might form the basis for a map of olfactory information? Odorant stimuli are detected by receptors on the cilia of sensory neurons in the olfactory epithelium. Each olfactory neuron projects a single unbranched axon to the brain, where it synapses with the dendrites of mitral/tufted and periglomerular cells of the olfactory bulb. These synapses are confined to ~1800 discrete loci or glomeruli within the mammalian olfactory bulb (Royet et al., 1989). 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 receptor gene, such that neurons are functionally distinct (Ngai et al., 1993; Chess et al., 1994). Analysis of the spatial pattern of receptor expression in the olfactory epithelium by in situ hybridization reveals that cells expressing a given receptor are randomly dispersed within one of four epithelial zones (Ressler et al., 1993; Vassar et al., 1993). In contrast, the axonal projections from neurons expressing a specific receptor converge upon two glomeruli within each mouse olfactory bulb (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). The bulb therefore provides a spatial map that identifies which neurons have been activated within the sensory epithelium. By this means, the quality of an olfactory stimulus is encoded by a distinct spatial pattern of activity defined by the specific combination of glomeruli activated by a given odorant.

The observation that the thousand different sensory neurons project with precision to two topographically fixed glomeruli in the olfactory bulb poses a complex problem in axon guidance. In other sensory systems, it is thought that spatial cues in the target afford a coarse topographic map that is subsequently refined by activity-dependent processes (reviewed by Constantine-Paton et al., 1990; Goodman and Shatz, 1993). In the olfactory system, a precise topographic map can be established in the absence of odorant-elicted activity (L. J. Brunet et al., personal communication). It is therefore likely that spatial cues in the olfactory bulb are recognized by sensory axons and direct olfactory projections to precise glomerular targets. Three major cell types reside within the olfactory bulb: projection neurons (the mitral and tufted cells), local inhibitory interneurons (periglomerular and granule cells), and glia (Shipley and Ennis, 1996). In this study, we examine the contribution of the different neuronal cell types in the olfactory bulb to the establishment of a topographic map.

Recent experiments have identified several transcription factors that are expressed in subpopulations of neurons in the developing forebrain and olfactory bulb. Two mammalian homeobox genes, Dlx-1 and Dlx-2, are expressed in the interneurons of the bulb (Salinas and Nusse, 1992; Porteus et al., 1994). Gene targeting experiments have demonstrated that mice with a deletion of both Dlx-1 and Dlx-2 lack mature GABAergic olfactory bulb interneurons, namely the periglomerular and granule cells. These mutant mice also exhibit defects in the differentiation of most striatal projection neurons and neocortical interneurons (Anderson et al., 1997a, 1997b). Projection neurons of the olfactory bulb express a distinct set of transcription factors including T-brain-1 (Tbr-1), a mammalian brachyury homolog (Bulfone et al., 1995). In this study, we demonstrate that mice with a homozygous deficiency in the Tbr-1 gene fail to form normal mitral and tufted cells. Tbr-1 and Dlx-1/Dlx-2 mutant mice each exhibit defects in specific subsets of...
neurons in the olfactory bulb, allowing us to examine independently the role of projection neurons and local interneurons in the establishment of a topographic map.

In previous experiments, we have used targeted mutagenesis to generate strains of mice in which neurons that express the P2 olfactory receptor also express tau-lacZ, a fusion of the microtubule-associated protein tau and β-galactosidase. In mice bearing this modified gene (P2-RES-tau-lacZ), it is possible to follow the pattern of projections of P2 neurons to two distinct glomeruli in each olfactory bulb (Mombaerts et al., 1996). Genetic crosses between P2-RES-tau-lacZ mice and mice deficient in Tbr-1 reveal a convergence of P2 axons to one medial and one lateral site at positions analogous to those observed in wild-type mice. A similar pattern of convergence of P2 axons is observed in crosses with mice mutant for Dlx-1 and Dlx-2. These observations suggest that the establishment of a topographic map is not dependent upon cues provided by the major neuronal cell types in the olfactory bulb—the mitral/tufted cells or the GABAAergic interneurons. Moreover, the establishment of the map does not appear to be dependent upon synapse formation with the output neurons of the olfactory bulb.

Results

Tbr-1 Mutant Mice Lack Projection Neurons

Tbr-1, a member of the brachyury/T box gene family, is expressed in a regionally restricted pattern in postmitotic cells of the developing and adult telencephalon of the mouse (Bulfone et al., 1995). In the developing olfactory bulb, Tbr-1 is expressed in postmitotic projection neurons, suggesting that it may function in the control of differentiation of the mitral and tufted cells (Bulfone et al., 1995). We have therefore used gene targeting to generate mice with a homozygous deficiency in the Tbr-1 gene. The mouse Tbr-1 gene is encoded by six exons (boxes); the initiation codon is in exon 1, and the termination codon is in exon 6. The empty boxes indicate the 5’ and 3’ untranslated regions, the dashed boxes the coding region, and the black boxes the T box domain. Introns are indicated by thin lines connecting the boxes. Arrows indicate initiation codons in Tbr-1 and neo. PGK neo was inserted between NotI and BamHI sites; the direction of transcription of these genes is antiparallel. The locations of the Southern probe (A) and the in situ probe (B) are indicated. The black arrowheads indicate PCR primers for Tbr-1; the white arrowheads indicate PCR primers for neo. Abbreviations: B, BamHII; N, NotI; X, XhoI; PGK, phosphoglycerate kinase promoter; neo, neo resistance gene; and tk, thymidine kinase gene.

(B) Examples of the Southern and PCR genotyping assays are shown. On the left is a photograph of an autoradiograph of a Southern blot showing the sizes of the wild-type (6.0 kb) and mutant (7.5 kb) alleles following digestion with BamHI and probing with probe A. The middle panel shows a photograph of an agarose gel that displays the reaction products following a PCR assay for the wild-type allele. The template was mouse tail DNA. Abbreviations: P. C., positive control (wild-type genomic DNA); N. C., negative control (dH2O). Size markers are on the flanking lanes. The panel on the right side shows the results of a PCR analysis for the Neo gene. Lane 1, molecular size markers; lane 2, +/+ genomic DNA; lane 3, +/− genomic DNA; and lane 4, −/− genomic DNA.

Figure 1. Organization of the Wild-Type and Mutant Tbr-1 Alleles

(A) Schematic representations of the Tbr-1 cDNA, Tbr-1 wild-type allele, Tbr-1 targeting vector, and mutant Tbr-1 allele. The wild-type Tbr-1 allele has six known exons (boxes); the initiation codon is in exon 1, and the termination codon is in exon 6. The empty boxes indicate the 5’ and 3’ untranslated regions, the dashed boxes the coding region, and the black boxes the T box domain. Introns are indicated by thin lines connecting the boxes. Arrows indicate initiation codons in Tbr-1 and neo. PGK neo was inserted between NotI and BamHI sites; the direction of transcription of these genes is antiparallel. The locations of the Southern probe (A) and the in situ probe (B) are indicated. The black arrowheads indicate PCR primers for Tbr-1; the white arrowheads indicate PCR primers for neo. Abbreviations: B, BamHII; N, NotI; X, XhoI; PGK, phosphoglycerate kinase promoter; neo, neo resistance gene; and tk, thymidine kinase gene.

(B) Schematic representations of the Tbr-1 cDNA, Tbr-1 wild-type allele, Tbr-1 targeting vector, and mutant Tbr-1 allele. The wild-type Tbr-1 allele has six known exons (boxes); the initiation codon is in exon 1, and the termination codon is in exon 6. The empty boxes indicate the 5’ and 3’ untranslated regions, the dashed boxes the coding region, and the black boxes the T box domain. Introns are indicated by thin lines connecting the boxes. Arrows indicate initiation codons in Tbr-1 and neo. PGK neo was inserted between NotI and BamHI sites; the direction of transcription of these genes is antiparallel. The locations of the Southern probe (A) and the in situ probe (B) are indicated. The black arrowheads indicate PCR primers for Tbr-1; the white arrowheads indicate PCR primers for neo. Abbreviations: B, BamHII; N, NotI; X, XhoI; PGK, phosphoglycerate kinase promoter; neo, neo resistance gene; and tk, thymidine kinase gene.
the Tbr-1 mutation do not nurse and die on the second postnatal day. The brains of Tbr-1 mutant mice are smaller than those of heterozygous or wild-type littermates (data not shown). Preliminary analysis of the forebrain in these mice indicates that cortical neurons are present, but there is a severe disruption of their laminar organization. Preplate cells are few in number, and there is a marked deficiency in their derivatives, the subplate and Cajal-Retzius cells (data not shown). The olfactory cortex is hypoplastic (see below).

The olfactory bulb in Tbr-1 mutant mice is small, owing in part to a marked reduction in the number of cells (Figure 2). The glomerular layer in the mutants contains 3-4 smaller glomeruli in its radial dimension rather than the 1-2 glomeruli observed in wild-type mice (Figures 2E and 2F). The mitral cell layer is absent, and there are no histologically identifiable ectopic mitral or tufted cells in Nissl-stained sections (Figure 2), whereas in wild-type mice, the mitral cell bodies reside within a distinct lamina, and each mitral cell sends its apical dendritic projections to a single glomerulus, where it synapses with olfactory sensory axons (Figure 4C). In situ hybridization to sections of wild-type olfactory bulb with RNA probes for three mRNAs (Id-2, Reelin, and Tbr-1) reveals hybridization to cells in the mitral cell layer and to other scattered cells that are likely tufted cells (Figures 3K, 3M, and 3O). In contrast, hybridization to olfactory bulb sections from Tbr-1 mutant mice reveals no hybridization to cells within a distinct mitral cell layer and only weak, sparse hybridization to cells that are centrally located in proximal regions of the olfactory bulb (Figures 3L, 3N, and 3P).

The loss of cells expressing genes characteristic of projection neurons is apparent early in the development of the olfactory bulb. Mitral and tufted cells are present in the bulb primordium at around embryonic day 13 (E13). By E14.5, the mitral cell markers Id-2 and Tbr-1 are apparent in cells in the developing olfactory bulb (Figures 3A and 3C). Id-2 and Tbr-1 expression are greatly reduced in the E14.5 olfactory bulb of Tbr-1-deficient mice (Figures 3B and 3D), whereas their expression continues in the cerebral cortex. At this stage, synapses between sensory axons and mitral cells are first present in the olfactory bulb (Hinds and Hinds, 1976a, 1976b). At E16.5, the mitral cell layer is clearly identified by in situ hybridization using neurotensin (NT), a mitral cell marker (Figure 3E; Kiyama et al., 1991). In Tbr-1 mutant mice at this stage, only weak NT hybridization signals are observed in cells restricted to the more central region of the olfactory bulb (Figure 3F). Sections through the entire bulb reveal that this ectopic expression of mitral cell markers is restricted to the regions of the bulb proximal to the telencephalon; more distal regions lack detectable expression (data not shown). Finally, in situ hybridization with a probe to tyrosine hydroxylase (TH), a marker for a subpopulation of the periglomerular and granule cells (Figure 3G), demonstrates the presence of these interneurons in mutant animals (Figure 3H). The organization of the interneuron cell bodies, however, is distorted as a consequence of the absence of an intact mitral cell layer (compare Figures 3G and 3H).

The axons of the mitral cells fasciculate as they leave the bulb to form the lateral olfactory tract (LOT), which projects to the piriform or primary olfactory cortex. If crystals of Dil are placed either in the olfactory bulb or the primary olfactory cortex of wild-type animals, axon fibers are observed connecting the primary olfactory cortex with the olfactory bulb (Figures 4A and 4D). In contrast in mutant animals, the placement of Dil crystals either in the olfactory bulb or in the position of the primary olfactory cortex does not reveal a fiber tract connecting the olfactory bulb to the cortex (Figures 4B and 4E). In addition, the LOT was absent, based on the lack of calretinin immunoreactive on the surface of the ventrolateral cortex in Tbr-1 mutants at E15.5 (Figures 4F and 4G) and P0 (data not shown). These data are consistent with the absence of functional projection neurons in Tbr-1 mutants. Mice with a homozygous deficiency in the Tbr-1 gene therefore exhibit a striking reduction in mature olfactory bulb projection neurons that is apparent when target selection and synapse formation are occurring in the olfactory bulb.

Sensory Axon Targeting in Tbr-1 Mice

The observation that Tbr-1 mutant mice have a profound reduction in mitral and tufted cells allows us to examine...
the role of these cell types in the generation of a precise topographic map. In previous experiments, we used gene targeting to generate a strain of mice in which neurons that transcribe the P2 receptor gene also express tau-lacZ in their axons, permitting the direct visualization of the pattern of projections of sensory axons to the brain (Mombaerts et al., 1996). We have therefore crossed homozygous P2-IRES-tau-lacZ mice with mice heterozygous for the Tbr-1 deficiency to ultimately obtain homozygous Tbr-1 mutants that also carry a P2-IRES-tau-lacZ allele. Analysis of the patterns of projections of P2 neurons reveals the convergence of blue fibers to one medial and one lateral glomerulus in both control and Tbr-1 mutant mice. The convergence of P2 fibers is apparent both on whole mount analysis and on sections through the olfactory bulb, despite the absence of normal projection neurons in Tbr-1 mutant mice (Figure 5). The approximate positions of the P2 glomeruli are conserved in each of the four Tbr-1 mutants examined. The hypoplastic and deformed character of the bulb in Tbr-1 mutants renders it difficult to directly compare the precise position of the P2 glomerulus in mutant and wild-type animals. For example, the P2 glomerulus in the mutant bulb is deeper than is observed in wild type (Figures 5D and 5F). Nonetheless, the presence of a single ventromedial and ventrolateral glomerulus approximates the pattern of P2 glomeruli in the wild-type olfactory bulb. Convergence to these glomeruli is observed in each of the seven Tbr-1 mutant mice examined. These observations suggest that projection neurons...
within the olfactory bulb are not essential for the establishment of a precise topographic map of sensory projections.

**Axon Targeting in the Absence of GABAergic Interneurons in Dlx-1 and Dlx-2 Mutant Mice**

Mice with a homozygous deficiency in Dlx-1 and Dlx-2 show a marked reduction in GABAergic interneurons within the bulb (Anderson et al., 1997b), affording the opportunity to examine the role of this neuronal cell type in the generation of a topographic map. Dlx-1 and Dlx-2 are murine homeobox genes that are expressed in ventral thalamus and basal telencephalic regions from about day 9.5 of mouse development (Bulfone et al., 1993a, 1993b; Liu et al., 1997). In the olfactory bulb, Dlx-1 and Dlx-2 are both expressed in the periglomerular and granule cells but is not detectable in mitral cells (Figure 6C). Dlx-1/Dlx-2 mutants die within a few hours of birth without milk in their stomachs (Qiu et al., 1997). Mutant animals reveal abnormalities in the development of the striatal subventricular zone and in the differentiation of striatal matrix neurons (Anderson et al., 1997a). The olfactory bulbs of Dlx-1/Dlx-2 mutant mice are hypoplastic; the diameter is \(~50\%~\) that of wild-type littermates, and the ventricle is consistently enlarged (Figure 6). Nissl staining reveals the absence of interneurons surrounding individual glomeruli and a thinning of the granule cell layer (Figure 6B). However, the laminar organization of the bulb is maintained, and the projection neurons appear unaffected by the Dlx-1/Dlx-2 deletion (Figures 6F and 6G). Dlx-1/Dlx-2 mutant animals exhibit no detectable glutamic acid decarboxylase-67 (GAD-67) expression in the olfactory bulb, a marker for the GABAergic interneurons. In situ hybridization and immunohistochemical analyses demonstrate that additional markers characteristic of the interneuronal population within the bulb, including TH and calbindin, are either absent or markedly reduced in mutant animals (Figures 6K, 6M, and 6O). In contrast, staining for the mitral cell marker Reelin reveals roughly normal numbers of mitral cells in Dlx-1/Dlx-2 mice (Figures 6F and 6G).

The absence of GABAergic interneurons in Dlx-1/Dlx-2 mutants with an apparently normal population of mitral cells allowed us to ask whether these cells are essential for the establishment of a precise topographic map. Homozygous P2-ires-tau-lacZ mice were therefore crossed with mice bearing a heterozygous deficiency in the Dlx-1 and Dlx-2 genes to ultimately generate Dlx-1/Dlx-2 mutant mice bearing the P2-ires-tau-lacZ allele. Examination of the patterns of projections of P2 neurons in either mutant mice or wild-type littermates reveals a normal pattern of P2 expression in the epithelium and the convergence of P2 fibers on a single glomerulus on both the medial and lateral aspect of the olfactory bulb (Figure 7). Both the size of the glomerulus and its relative position within the olfactory bulb appear unaltered in each of seven Dlx-1/Dlx-2 mutant mice examined, suggesting that interneurons are not required for the generation of an olfactory topographic map.

**Discussion**

Distinct Genetic Pathways Control the Development of Olfactory Projection Neurons and Interneurons

The development of the two major neuronal cell types in the olfactory bulb, the projection neurons and the
Figure 6. Dlx-1/Dlx-2 Mutant Mice Lack Olfactory Bulb GABAergic Interneurons

Histochemical analysis of molecules expressed in subtypes of olfactory bulb cells. A superior sector of each olfactory bulb is shown. Pairs of heterozygous (+/−) and homozygous mutant (−/−) specimens that are analyzed with the same cRNA probe or antibody are adjacent to each other (except for DLX-1, for which only the heterozygote is shown).

(A and B) Nissl-stained sections.
(C) Anti-DLX-1 protein immunohistochemistry stains cells in the glomerular, granule cell, and subventricular layers.
(D and E) Olfactory marker protein (OMP) immunohistochemistry stains the olfactory nerve layer.
(F and G) Reelin in situ hybridization stains the mitral cell layer.
(H and I) GABA.
(J and K) GAD-67 immunohistochemistry stains interneurons in the glomerular and granule cell layers.
(L and M) TH immunohistochemistry stains interneurons in the glomerular layer.
(N and O) Calbindin immunohistochemistry stains interneurons in the olfactory and granular cell layers.

Abbreviations: EPL, external plexiform layer; GL, glomerular layer; GCL, granule cell layer; MCL, mitral cell layer; ONL, olfactory nerve layer; OV, olfactory ventricle; and SVZ, subventricular zone. White bar in (A) is 0.2 mm.

inhibitory interneurons, are under distinct genetic control. We have demonstrated that mutants deficient in the Tbr-1 gene exhibit a striking reduction in the number of projection neurons, whereas mutants in Dlx-1 and Dlx-2 reveal a severe loss of GABAergic interneurons. In addition to the distinct genetic pathways that regulate the formation of these two neuronal lineages, there is evidence indicating that the precursors of these two lineages are anatomically distinct. Tbr-1 expression is largely restricted to the cortical telencephalon, whereas early expression of Dlx genes is observed in the subcortical telencephalon (Bulfone et al., 1995). In the cortex, the expression of Tbr-1 appears to respect a strict boundary at the pallial/subpallial limit (roughly the cortical/subcortical boundary; J. L. R. R., unpublished data). In the olfactory bulb primordium, Tbr-1 expression is apparent by E12.5 (Bulfone et al., 1995) and is later restricted to the mitral and tufted cells. In Tbr-1-deficient mice, the olfactory bulb is hypoplastic, the mitral cell layer is histologically absent, projection neurons are dramatically reduced, and axon tracing experiments fail to reveal the LOT connecting the olfactory bulb with the piriform cortex. Moreover, olfactory bulb cells expressing markers for projection neurons (Tbr-1, Id-2, Reelin, NT [Figure 3], and neuropilin [data not shown]) are greatly reduced early in development and in neonatal animals. Although these data do not exclude the possibility that mitral and tufted cell precursors are indeed present within the bulb, the anatomic and molecular characteristics of olfactory bulb projection neurons are absent in Tbr-1 mutants.

Expression of the Dlx-1 and Dlx-2 genes is restricted to GABAergic interneurons in the bulb. Around E12.5, cells expressing the Dlx genes in the subcortical telencephalon migrate across the pallial/subpallial limit and enter the mantle and subventricular zones of the cerebral cortex and olfactory bulb (Porteus et al., 1994; Anderson et al., 1997a). Mice deficient in Dlx-1 and Dlx-2 reveal a block in the migration and differentiation of subventricular zone-derived cells from the basal telencephalon (Bulfone et al., 1995). In the cortex, the expression of Tbr-1 appears to respect a strict boundary at the pallial/subpallial limit (roughly the cortical/subcortical boundary; J. L. R. R., unpublished data). In the olfactory bulb primordium, Tbr-1 expression is apparent by E12.5 (Bulfone et al., 1995) and is later restricted to the mitral and tufted cells. In Tbr-1-deficient mice, the olfactory bulb is hypoplastic, the mitral cell layer is histologically absent, projection neurons are dramatically reduced, and axon tracing experiments fail to reveal the LOT connecting the olfactory bulb with the piriform cortex. Moreover, olfactory bulb cells expressing markers for projection neurons (Tbr-1, Id-2, Reelin, NT [Figure 3], and neuropilin [data not shown]) are greatly reduced early in development and in neonatal animals. Although these data do not exclude the possibility that mitral and tufted cell precursors are indeed present within the bulb, the anatomic and molecular characteristics of olfactory bulb projection neurons are absent in Tbr-1 mutants.

An Olfactory Sensory Map Develops in the Absence of Projection Neurons or GABAergic Interneurons

In the olfactory system, neurons expressing a given odorant receptor project with precision to 2 of the 1800 glomeruli within the olfactory bulb. Since the positions of these glomeruli are topographically invariant, the bulb...
provides a spatial map that identifies which receptors have been activated within the olfactory epithelium. The generation of mutant mice deficient in either projection neurons or interneurons has allowed us to determine the contribution of these two neuronal populations to the generation of a spatial map. In other sensory systems, it is thought that a coarse topographic map is initially generated during development by spatially patterned guidance cues in the target that are recognized by specific receptors on the growth cones of sensory axons. This pattern is then subsequently refined by coordinated neural activity to achieve a precision of connections between the periphery and the brain.

The projection neurons and interneurons in the bulb could contribute to the patterning of sensory projections either by elaborating guidance cues or by participating in activity-dependent refinement of synaptic connections. When mice deficient in either Tbr-1 or Dlx-1/Dlx-2 are crossed with mice bearing the P2-IRES-tau-lacZ allele, lacZ expression is observed in the expected subset of sensory neurons within the appropriate zone of the olfactory epithelium. Moreover, the axons from these P2 neurons project to the olfactory bulb, where they converge upon one glomerulus on both the medial and lateral aspects of the bulb. Although the olfactory bulb is hypoplastic, and the histological organization is abnormal in both the Tbr-1 and Dlx-1/Dlx-2-deficient mice, the relative positions of both the medial and lateral P2 glomeruli roughly approximate their position in wild-type mice.

It is possible to obtain a more precise representation of the topographic map by performing in situ hybridization to the olfactory bulb with probes for different odorant receptor mRNAs, a procedure which demonstrates that relative positions of different glomeruli are invariant (Ressler et al., 1994; Vassar et al., 1994). This procedure is not sufficiently sensitive to be performed on neonatal animals, precluding a more precise definition of the relative position of the P2 glomeruli in the Tbr-1 and Dlx-1/Dlx-2 mutants. Nonetheless, the convergence of P2 axons to positions that approximate their wild-type location strongly suggests that the appropriate guidance cues necessary to establish this complex topographic map do not require the presence of either the projection neurons or interneurons within the olfactory bulb.

It remains possible that both the projection neurons and the interneurons express redundant spatial cues, such that the presence of either neuronal population is adequate to guide sensory axons to their appropriate glomerular target. We have attempted to address this possibility by generating triple mutant mice (Tbr-1, Dlx-1/Dlx-2) that express the P2-IRES-tau-lacZ allele. We have recently obtained these recombinant strains (Tbr-1 and Dlx-1/Dlx-2 each reside on chromosome 2), and preliminary analysis reveals that the olfactory bulb is deficient in both neuronal cell types. As a consequence, the bulb is markedly hypoplastic and anatomically deformed, rendering it difficult if not impossible to interpret the projection patterns of incoming sensory axons. Thus, we cannot exclude the unlikely possibility that the presence of either projection neurons or GABAergic interneurons alone is adequate to establish the olfactory sensory map.

What other cell types may be the source of putative guidance cues in the olfactory bulb? One attractive candidate is the radial glia, a transient population of cells detectable as early as E14 in the developing olfactory bulb (Valverde et al., 1992). Radial glia extend processes...
Activity-Dependent Processes and the Formation of an Olfactory Map

Our data provide additional support for the suggestion that the topographic map in the olfactory system develops without apparent contribution from activity-dependent processes (Belluscio et al., 1998; Wang et al., 1998). In other sensory systems, genetically determined cues initially guide synapse formation, but subsequent refinement may require spontaneous neural activity as well as sensory experience (Katz and Shatz, 1996). In the visual system, for example, a retinotopic map exists first in the thalamus and the superior colliculus and is then multiply represented in the visual cortex (Goodman and Shatz, 1993). The precision of this map is then refined by the correlated activity of neighboring neurons that results from both spontaneous synchronous waves or neural activity in the retina as well as visual experience. A different picture emerges from our analysis of the development of the olfactory map. For example, mice lacking the olfactory cyclic nucleotide-gated ion channel exhibit no odor-evoked electrophysiological activity in the sensory epithelium (Brunet et al., 1996). The pattern of axonal convergence to the olfactory bulb, however, is unaltered in these mutant mice (Brunet et al., personal communication). Similarly, severe reductions in odor-evoked responses are observed in mice lacking G(o)α, the major G protein α subunit in olfactory sensory neurons, yet convergence of olfactory sensory projections is maintained in these mutant animals (Belluscio et al., 1998).

In this study, we demonstrate that the convergence of like axons at a topographically fixed position approximating that observed in wild-type animals occurs in the absence of synapse formation with either the projection neurons or the inhibitory interneurons of the olfactory bulb. In other neural systems, activity-dependent plasticity is thought to require the participation of the post-synaptic cell, such that correlated activity in presynaptic neurons results in the release of factors that subsequently induce or enhance the presynaptic arbors, thereby refining the topographic map (reviewed by Cramer and Sur, 1995; Fitzsimonds and Poo, 1998). In contrast, a precise topographic map of olfactory sensory projections develops in the absence of an apparent contribution from either of the two synaptic targets, the inhibitory neurons or mitral cells. Taken together, these data argue strongly that odor-evoked activity is not required for the establishment or refinement of the topographic map during development. Rather, they suggest that the precision of the topographic map is a consequence of spatial cues elaborated by the bulb that are recognized by receptors on sensory axons. Recent data demonstrate that the odorant receptor plays an instructive role in axon targeting as one determinant in the complex guidance process that directs axons to 1 of 1800 invariant glomerular targets. Our data indicate that the spatial information recognized by these guidance receptors must be provided by cells other than the major synaptic targets of the sensory axons in the bulb and further argue that synapse formation between sensory neurons and their bulb targets may not be required for the establishment of the olfactory topographic map.

Experimental Procedures

Isolation of Tbr-1 Genomic Clones and Generation of Targeting Vectors, Mutant ES Cells, and Mutant Mice

Tbr-1 genomic DNA was isolated from 129 strain mice and the exon-intron organization was mapped (Figure 1). This information was used to generate a targeting vector (Figure 1; see Qiu et al. [1995] for a description of the components).

The targeting vector was electroporated into J-M-1 ES cells, and mutant ES clones were selected and characterized by the methods described in Qiu et al. (1995). Genotyping of ES cells and mouse tails was performed by either Southern or PCR assays (Figure 1). The PCR assay for the Tbr-1 mutation used oligonucleotide primers (primer 1, 5′-ACATTCTGTCGCTGATCC-3′; primer 2, 5′-TGCACATTGTGTCGCCG-3′) that amplify a 130 bp fragment of Tbr-1 encoded by exon 2 (nucleotides 814-944, referring to the cDNA sequence in GenBank, accession number U49251). The PCR conditions for these primers are 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s; and 1 cycle at 72°C for 7 min. The other set of primers are located in the neo gene; their sequences are 5′-CCAGATTGGTCGACCGCAG-3′ and 5′-CATCTGTATCGCACAAGAC-3′. The PCR conditions for these primers are 1 cycle at 94°C for 2 min; 35 cycles at 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min; and 1 cycle at 72°C for 7 min. Genotyping by the Southern blot method was performed as follows. The genomic DNA was digested with BamHI and analyzed with probe A (Figure 1). The wild-type allele is ~6.0 kb, and the mutant allele is ~7.5 kb.

RNA Expression Analysis

Radiolabeling and nonradioactive in situ RNA hybridization was performed as described in Qiu et al. (1995) and Shimamura et al. (1995), respectively. Riboprobes were made from the following cDNA templates: Id-2 (Zhu et al., 1995), Tbr-1 (Bulfone et al., 1995), Reelin (D’Arcangelo et al., 1995), rat TH (1.1 kb, from D. Chikarashii), and rat NT (~580 bp, PCR-generated by T. Cutforth).

Immunohistochemistry

Immunohistochemistry was performed by standard methods as described in Porteus et al. (1994). The following antibodies were used to study neuronal cell types in 10 μm thick cryostat-sectioned paraffin-embedded tissues: rabbit anti-GABA from Sigma used at a 1:2000 dilution; rabbit anti-GAD-67 from Chemicon, used at a 1:2000 dilution; rabbit anti-calretinin from Chemicon, used at a 1:2000 dilution; rabbit anti-calbindin from SLI5 Antibodies, used at 1:5000; rabbit anti-TH from Chemicon, used at 1:1000; and goat anti-OMP from Frank Marquart, used at a 1:1000 dilution. The Vectastain ABC kit was used for antibody detection.

Analysis of Olfactory Projections Using X-Gal Histochemistry

Whole mount preparations were fixed for 30 min with ice-cold 4% paraformaldehyde, 100 mM phosphate buffer (pH 7.4), and 2 mM MgSO4, washed three times in PBS and 2 mM MgCl2 for 5 min each at room temperature; and then stained in the dark in 100 mM...
phosphate buffer (pH 7.4), 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM potassium-ferrocyanide, 5 mM potassium-ferrocyanide, and 0.5 mg/ml X-gal (USB) at 37°C. For sections, the tissues were frozen in OCT (Miles), and 20 µm sections were collected, stained with X-gal, counter stained with neutral red (Sigma), and dehydrated and mounted with Cytoseal 60 mounting medium (Stephens Scientific).

Dil Tract Tracing
Four mice were studied at P0.5 (two wild-type or heterozygote, two homozygous mutant), and six mice were studied at P1.5 (three wild-type or heterozygote, three homozygous mutant). Animals were anesthetized by cooling on ice and then perfused transcardially with fixative (4% paraformaldehyde, 4% sucrose in PBS). Their brains were removed and postfixed for a minimum of 12 hr. Single crystals (100-200 µm) of Dil (Molecular Probes, "Large Crystalline Dil") were inserted with an insect pin into the main olfactory bulb (n = 6) or piriform cortex region (n = 4). About three separate crystals were radially inserted into each olfactory bulb. The tissue was stored at room temperature for 3-6 weeks in fixative solution. The tissue was then rinsed with PBS, embedded in 4% low-melt agarose in PBS, and sectioned coronally on a Vibratome at 80 µm. Sections were counterstained with DAPI, coverslipped in 30% sucrose in PBS, and examined with an epifluorescence microscope. Axon fluorescence with Dil indicated anterograde or retrograde transport. Retrograde labeling was conclusively recognized by the presence of labeled cell bodies. Anterograde transport was presumptively recognized when axon fluorescence occurred in the absence of labeled cell bodies.

Acknowledgments
This work was supported by research grants to J. L. R. R. from Nina Ireland, the National Alliance for Research on Schizophrenia and Depression, the National Institute of Neurological Disorders and Stroke (NS34663-01A1), and the National Institute of Mental Health (K02 MH01046-01); to S. A. from the National Alliance for Research on Schizophrenia and Depression and the Veterans Administration; to R. H. from the National Institute of Neurological Disorders and Stroke (NS01973-02) to R. A. P. from NICHD-1 (HD26732) and the United States Department of Energy/0HER (contract number DE-AC03-76SF001012); and to R. A. from the Howard Hughes Medical Institute. Care of experimental animals was in accordance with institutional guidelines.

Received June 22, 1998; revised October 14, 1998.

References


