

Sorting and Convergence of Primary Olfactory Axons Are Independent of the Olfactory Bulb

JAMES A. ST. JOHN,¹ HEIDI J. CLARRIS,¹ SONJA McKEOWN,²
STEPHANIE ROYAL,¹ AND BRIAN KEY^{1*}

¹Department of Anatomy and Developmental Biology, School of Biomedical Sciences,
University of Queensland, Brisbane, Queensland 4072, Australia

²The Murdoch Childrens Research Institute, Royal Children's Hospital,
Parkville, Victoria 3052, Australia

ABSTRACT

Primary olfactory axons expressing the same odorant receptor gene sort out and converge to fixed sites in the olfactory bulb. We examined the guidance of axons expressing the P2 odorant receptor when they were challenged with different cellular environments *in vivo*. In the mutant extratoes mouse, the olfactory bulb is lacking and is replaced by a fibrocellular mass. In these animals, primary olfactory axons form glomerular-like loci despite the absence of normal postsynaptic targets. P2 axons are able to sort out from other axons in this fibrocellular mass and converge to form loci of like axons. The sites of these loci along mediolateral and ventrodorsal axes were highly variable. Similar convergence was observed for larger subpopulations of axons expressing the same cell surface carbohydrates. The sorting out and convergence of like axons also occurred during regeneration following bulbectomy. Olfactory axon behaviour in these models demonstrates that sorting and convergence of axons are independent of the target, which instead provides distinct topographic cues for guidance. *J. Comp. Neurol.* 464:131–140, 2003. © 2003 Wiley-Liss, Inc.

Indexing terms: axon navigation; axon guidance; glomeruli; development

In the olfactory system, primary sensory neurons expressing the same odorant receptor are typically dispersed mosaically over one of at least four zones in the nasal cavity (Ressler et al., 1993; Vassar et al., 1993; Strotmann et al., 1994a,b). The axons of these neurons are intermixed with axons expressing other odorant receptors, yet, as they enter the outer nerve fiber layer of the olfactory bulb during embryogenesis, they begin to converge to their presumptive target site (Mombaerts et al., 1996; Royal and Key, 1999). Over the next several days, these axons condense and form topographically fixed glomeruli. In the mouse, there are approximately 1,800 glomeruli (Royet et al., 1988) and 1,000 different odorant receptors (Zhang and Firestein, 2002), and all primary sensory olfactory neurons expressing the same odorant receptor typically project to two topographic glomeruli, one each on the medial and lateral surfaces of the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994; Nagao et al., 2000).

The odorant receptor appears to have a dual role both in olfactory transduction and in axon guidance. Deletion of the P2 odorant receptor causes axons to fail to converge and form glomeruli in the olfactory bulb (Wang et al.,

1998). Instead, these axons form a loose aggregate in the nerve fiber layer. Moreover, when the coding region of the P2 gene is replaced by the coding region of the M12 odorant receptor, axons now expressing this swapped receptor continue to converge but do so at sites distinct from either the original P2 or original M12 glomeruli (Mombaerts et al., 1996). This receptor-swap approach has now been repeated for a number of different receptors, and the same

Grant sponsor: National Health and Medical Research Council (NHMRC) (J.A.S., B.K.); Grant number: 210146; Grant number: 142919; Grant sponsor: Garnett Passe (H.J.C., J.A.S.); Grant sponsor: Rodney Williams Memorial Foundation (H.J.C., J.A.S.); Grant sponsor: NHMRC Peter Doherty Fellowships (H.J.C., J.A.S.).

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

Received 26 December 2002; Revised 19 February 2003; Accepted 4 March 2003

DOI 10.1002/cne.10777

Published online the week of July 28, 2003 in Wiley InterScience (www.interscience.wiley.com).

conclusions have been reached (Wang et al., 1998). Consequently, odorant receptors are necessary but not sufficient for topographic targeting. Although several adhesion molecules and guidance receptors, including galectin-1 (Puche et al., 1996), N-CAM (Treloar et al., 1997; Aoki et al., 1999) and its signaling pathway (Morse et al., 1998), neuropilin-1 (Pasterkamp et al., 1998) and its ligand semaphorin 3A (Schwartz et al., 2000), and neuropilin-2 (Cloutier et al., 2002; Walz et al., 2002) are involved in olfactory axon guidance, the cues responsible for generating the topographic map remain elusive.

Where are these cues for convergence and targeting localised? Recent analyses of mice with a reduced complement of mitral cells have suggested that these postsynaptic neurons are not involved in the sorting and convergence of primary olfactory axons to their topographic targets (Bulfone et al., 1998). However, it was unclear whether cues present on the remaining mitral and tufted cells or on other olfactory bulb cells in these mutant mice were sufficient for the convergence and targeting of olfactory axons. We have also shown that the laminar organization of mitral cells is not critical for convergence and targeting of primary olfactory axons (Royal et al., 2002). However, a rigorous assessment of the role of bulbar neurons in the convergence of like axons requires the complete removal of the olfactory bulb. After removal of the olfactory bulb (bulbectomy) in neonatal mice, primary olfactory axons regenerate and form glomeruli-like structures in the forebrain (Graziadei et al., 1978). What remains unknown is whether these glomeruli represent the convergence of axons expressing the same odorant receptor. Do regenerating axons of the same type have the ability to sort out from all other axons and converge to form glomeruli? There is a naturally occurring mouse mutant called *extratoes* that appears to lack a histologically identifiable olfactory bulb (Johnson, 1967; Franz, 1994; Theil et al., 1999). Although odorant receptor genes are expressed by the primary olfactory neurons in these mice (Sullivan et al., 1995), it is unknown whether like axons expressing the same odorant receptor are able to sort out and converge to form glomeruli in the absence of a normal target.

In the present study, we have addressed the role of the olfactory bulb in providing cues for convergence by examining both the naturally occurring mutant mice *extratoes* that lack olfactory bulbs (Johnson, 1967) and mice that have been bulbectomized. We demonstrate that axon convergence of like axons occurs independently of the olfactory bulb, indicating that convergence and targeting are separate events in the olfactory pathway.

MATERIALS AND METHODS

Extratoes animals

Homozygous P2-IRES-tau:LacZ transgenic mice (Mombaerts et al., 1996) were mated with mice heterozygous for the mutated *Gli3* gene (*extratoes*, Xt). These mice were then crossed to produce mice homozygous (Xt/Xt) for the Xt mutation and either heterozygous or homozygous for the P2-IRES-tau:LacZ allele. Animals were analysed at E14.5, E16.5, and P0.5. Mice were anaesthetised with Nembutal (80 μ l/100 g body weight; Boehringer Ingelheim, Indianapolis, IN) and killed by decapitation. Heads were fixed in 4% paraformaldehyde for 30 minutes

at room temperature. After fixation, heads were cryoprotected in 30% sucrose with 0.1% sodium azide. Heads were placed in embedding matrix (O.C.T. compound; Miles Scientific, Naperville, IL) and snap frozen by immersion in isopentane that had been cooled by liquid nitrogen. Serial coronal sections (30 μ m) were cut on a cryostat microtome, collected on slides coated with 2% gelatin and 0.1% chromalum, air dried overnight, and stored at -25°C . All procedures were carried out with the approval of, and in accordance with, the University of Queensland Animal Ethics and Experimentation Committee.

Surgical ablation of olfactory bulbs

Unilateral bulbectomies were performed on day P4.5 homozygous P2-IRES-tau:LacZ transgenic mice (Mombaerts et al., 1996). Pups were anaesthetised by placing them on ice for 8 minutes (Danneman and Mandrell, 1997), and a midline incision was made to expose the cranial bones. The bone directly over the olfactory bulb was removed using a surgical drill with a 0.5-mm burr. The entire olfactory bulb was aspirated using a pulled-glass pipette attached to a vacuum pump. The skin covering the site was sutured, and the antiinflammatory drug Flunixin was administered via intramuscular injection (active ingredient flunixin, 1 mg/kg body weight; Parnell Laboratories, Australia, Pty. Ltd., Alexandria, New South Wales, Australia). Animals were allowed to recover for 3 or 6 weeks.

5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside histochemistry

X-gal staining was performed as previously described (Royal and Key, 1999). Sections of *extratoes* mice were counterstained in 0.1% eosin. Sections of olfactory-bulb-ectomy mice were counterstained with nuclear fast red.

Immunohistochemistry

Immunohistochemistry was performed using the protocol described by Key and Akeson (1993). Sections were reacted overnight at 4°C with one of the following primary antisera: rabbit against β -galactosidase (20 μ g/ml; Cortex Biochem, Inc., San Leandro, CA), rabbit anti- γ -aminobutyric acid (GABA; 1:1,000; Sigma, St. Louis, MO), goat antiolfactory marker protein (OMP; 1:6,000; Keller and Margolis, 1975), rabbit anti-Id2 (1:1,500; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human p75 (1:200; Promega Corp., Madison, WI), rabbit anti-neuropeptide Y (Jenkinson et al., 1999), RC-2 (kindly provided by Dr M. Yamamoto, Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki, Japan), and Pbx-1 (Santa Cruz Biotechnology). Secondary antibodies used were goat anti-rabbit or rabbit anti-goat immunoglobulins conjugated to biotin (Vector Laboratories, Burlingame, CA), and sections were then incubated in Vectastain Elite avidin-biotin horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories) and reacted with 3,3'-diaminobenzidine (0.5 mg/ml) in the presence of 0.02% H_2O_2 . Control sections incubated with 2% bovine serum albumin (BSA) with Triton X-100 in Tris-buffered saline (TBS), pH 7.4, in place of the primary antibodies produced negligible background staining.

For double-label immunofluorescence, sections were incubated in the primary antibodies rabbit anti- β -galactosidase (20 μ g/ml; Cortex Biochem, Inc.) and goat

anti-OMP (1:1,000). Sections were incubated with secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or TRITC as appropriate. Fluorescence images were collected using a Bio-Rad MRC 1024 confocal laser scanning microscope. Z-series images for TRITC and FITC were collected every 1.5 μm through the depth of the section and merged.

Lectin staining

Lectin staining with biotinylated *Dolichos bifloris* agglutinin (DBA) was performed as described elsewhere (Key and Akeson, 1993).

RNA expression analysis

Nonradioactive in situ hybridisation was performed as described by Ressler et al. (1993). Digoxigenin-labelled sense and antisense riboprobes were generated from reelin cDNA (D'Arcangelo et al., 1995).

Image analysis and photography

The distribution of P2-targeted glomeruli in Xt/Xt mice was depicted in camera lucida drawings obtained using a 10 \times objective lens. Images were traced and scanned into Adobe Photoshop 7 (Adobe Systems, San Jose, CA) and compiled in Corel Draw 9 (Corel Corporation Ltd., Dublin, Ireland).

RESULTS

Extratoes mouse lacks olfactory bulbs

To address the role of target tissue in convergence of primary olfactory axons, we took advantage of the naturally occurring mutant mouse extratoes (Xt/Xt; Johnson, 1967), which is deficient in the transcription factor Gli3 (Pohl et al., 1990; Schimmang et al., 1992) and appears to lack olfactory bulbs. In wild-type mice (Fig. 1A,B) and mice heterozygous for extratoes (Fig. 1C,D) primary olfactory axons arising from the olfactory neuroepithelium lining the nasal cavity terminate in the glomerular layer of the olfactory bulb. In the neonatal mouse, the olfactory bulb is laminated and consists of rudimentary glomerular and mitral cell layers and a large subventricular zone. In contrast, in extratoes mice, primary olfactory axons enter the cranial cavity and terminate in a small fibrocellular mass (FCM) rather than an olfactory bulb (Fig. 1E). This FCM lacks the laminar organisation displayed by wild-type olfactory bulb (Fig. 1F). This amorphous tissue does not fill the anterior cranial cavity and instead remains closely adherent to the cribriform plate (Fig. 1E).

Immunostaining for OMP, a marker of primary olfactory neurons (Keller and Margolis, 1975), labels the axons of these neurons in the nerve fiber and glomerular layers, which form the outer circumference of the olfactory bulb (Fig. 2A). In the extratoes mouse, these OMP-positive fibers were distributed throughout the depth of the FCM, without any evidence of a defined outer nerve fiber layer (Fig. 2D), which confirmed the absence of its cytoarchitectonic layers. Immunostaining for the low-affinity nerve growth factor receptor p75, a marker of olfactory ensheathing cells (Ramón-Cueto and Avila, 1998), revealed their presence in the olfactory nerve fiber layer of normal mice (Fig. 2B). In extratoes mice, p75 expression was present in cells principally located toward the periphery of the FCM (Fig. 2E). Neuropeptide Y, an independent

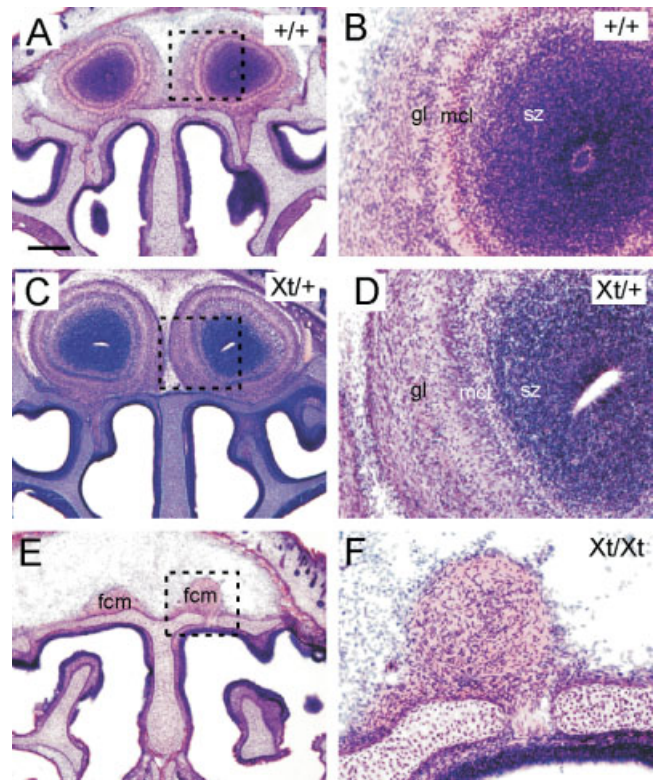


Fig. 1. Coronal cryostat sections through the nasal cavity of P0.5 wild-type (+/+; A,B), heterozygous extratoes (Xt/+; C,D), and homozygous extratoes (Xt/Xt; E,F) mice were stained using haematoxylin and eosin. Boxed regions in A, C, and E are shown enlarged in B, D, and F, respectively. The region usually occupied by olfactory bulbs in wild-type mice is instead filled with an amorphous fibrocellular mass (fcm) in mice homozygous for the extratoes mutation (E,F). This fibrocellular mass lacks the normal laminar organisation of wild-type and heterozygous olfactory bulbs. gl, Glomerular layer; mcl, mitral cell layer; sz, subventricular zone. Scale bar = 250 μm in A,C,E, 50 μm in B,D,F.

marker of olfactory ensheathing cells in the outer nerve fiber layer of normal mice (Ubink and Hokfelt, 2000; Fig. 2C), was present in cells restricted to the periphery of the FCM in extratoes mice. Immunostaining of 60- μm -thick cryostat sections clearly revealed the ensheathing cells that encapsulated the FCM (Fig. 2F). These results demonstrate that olfactory ensheathing cells migrate with the primary olfactory axons into the cranial cavity of the extratoes mouse, as they do during normal development in the wild-type mouse (Chuah and Au, 1991).

To confirm the absence of target neurons in the FCM, we screened for expression of molecules that are localised to distinct neuronal cell types in the olfactory bulb. The FCM lacked expression of Pbx-1 (Fig. 2J), a global marker of many different olfactory bulb neurons in wild-type mice (Redmond et al., 1996; Fig. 2G). Reelin (Fig. 2H) and Id2 (Fig. 2I) are markers of mitral cells (Bulfone et al., 1998), but both are absent from the FCM (Fig. 2K,L). In wild-type mice, GABAergic interneurons (Ribak et al., 1977) are clearly present within the glomerular and granule cell layers of the bulb (Fig. 2M). In contrast, only a few of these cells were dispersed in the peripheral margins of the FCM in each tissue section (arrowheads, Fig. 2O). Together, the

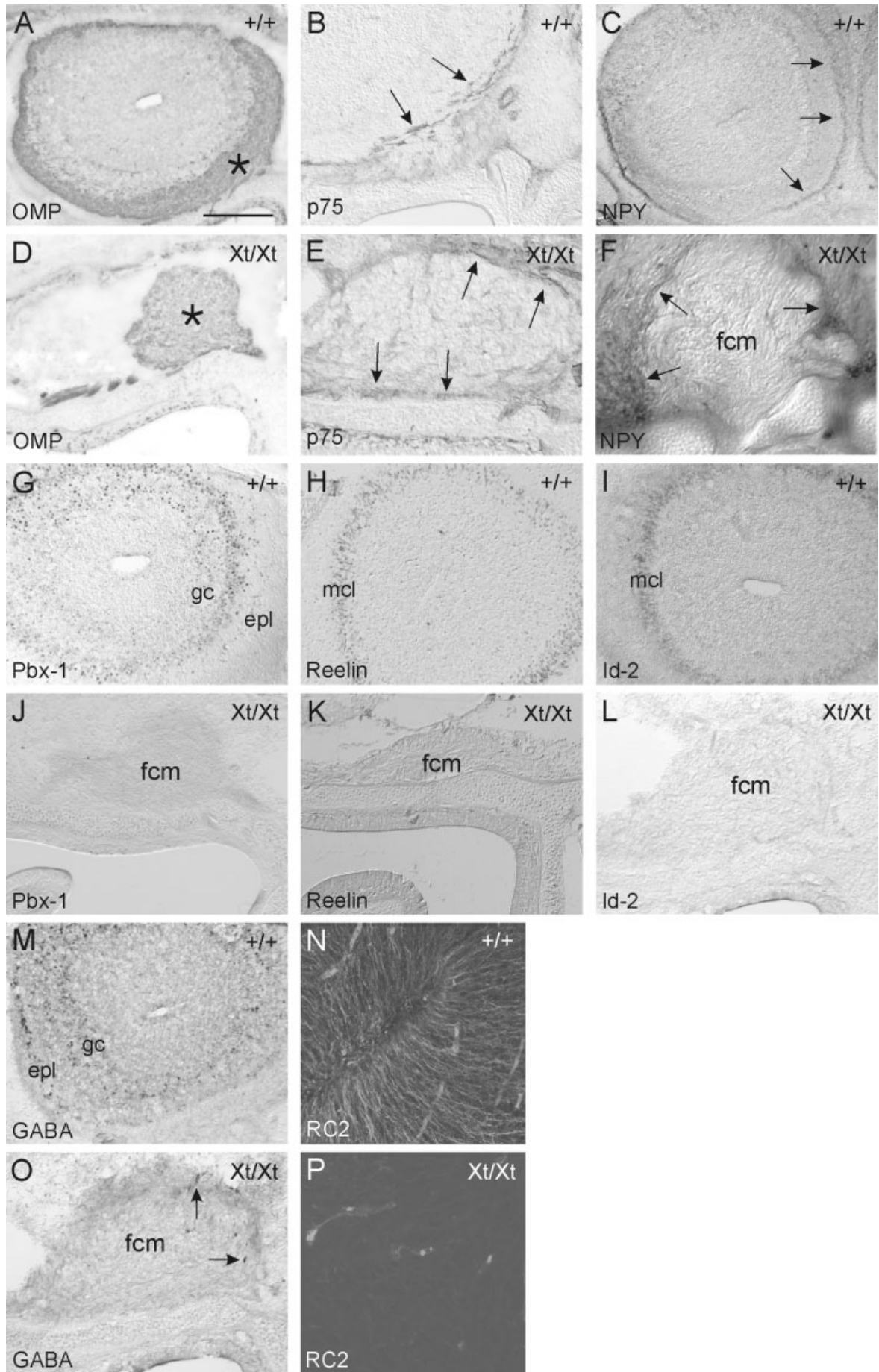


Figure 2

lack of expression of the olfactory bulb neuronal-specific markers revealed that the FCM is completely devoid of projection neurons and contains only a small number of GABAergic interneurons. Neonatal olfactory bulbs are typically immature and still contain radial glia (Treloar et al., 1997). We examined the extratoes mouse for the presence of these cells using the RC2 radial glia-specific antibody. Although these cells projected radially across the depth of the olfactory bulb in control mice (Fig. 2N), they were absent from the FCM of extratoes mice (Fig. 2P).

Primary olfactory axons sort out and converge in the absence of postsynaptic targets

To determine whether olfactory axons expressing the same odorant receptor sort out and converge in the FCM, we crossed mice homozygous for the P2-IRES-tau:LacZ allele (Mombaerts et al., 1996) and heterozygous for the extratoes mutation. In P2-IRES-tau:LacZ mice, all olfactory neurons expressing the P2 odorant receptor gene express a fusion protein between tau and LacZ that allows the trajectory of their axons to be visualised by histochemical staining with X-gal. P2 neurons are located in a discrete semiannular band or zone in the nasal cavity (Mombaerts et al., 1996; around the region of the dashed line in Fig. 3A) of control (P2-IRES-tau:LacZ) and extratoes mice (crossed with P2-IRES-tau:LacZ; around the region of the dashed line in Fig. 3C). In control mice, the P2 neurons projected to at least two topographically fixed glomeruli, one each on the medial and lateral surfaces of the olfactory bulb (Fig. 3B), as previously described (Royal and Key, 1999). In extratoes mice, P2 axons also displayed convergence to discrete sites in the FCM (Fig. 3D). We refer to these sites as *loci* rather than as *synaptic glomeruli* because of the absence of postsynaptic neurons in the FCM. Although P2-targeted loci were observed in 11 of 12 mice examined, their number, size, and shape varied both between animals and between the FCMs in the same animal. Whereas control animals typically have four topographically fixed P2 glomeruli, 9 of 12 extratoes mice examined had fewer than four P2 loci (average of three). Despite the absence of normal postsynaptic partners, P2 axons continued to sort out from other axons in the FCM and converge to discrete loci (Fig. 3D). This convergence was more clearly revealed in thick sections by scanning confocal laser microscopy (Fig. 3E) and appeared indistin-

guishable from that previously described in P2-IRES-tau:LacZ mice (Royal and Key, 1999). When sections were doubly labeled for P2 and OMP, it was clear that the axons from P2-expressing neurons sorted out from axons expressing other receptor types (Fig. 3F).

Next we examined the topographical positions of P2 loci about orthogonal axes in the FCM. These positions were determined from camera lucida drawings of serial 40- μ m coronal sections from 11 extratoes mice. The position of individual P2 loci about the dorsoventral and mediolateral axes in 11 animals was compiled and represented diagrammatically (Fig. 3G). There was no consistency in the mediolateral or dorsoventral positions of loci targeted by P2 axons either within the same animal or between animals. In contrast, loci were always restricted to two zones about 240 μ m in length along the rostrocaudal axis of the FCM (average length 740 μ m). The most rostral loci were restricted to a zone located between 160 and 400 μ m from the rostral pole of the FCM (as defined by OMP-stained axons), whereas the caudal loci were between 320 and 560 μ m from the rostral pole. Thus, the positions of P2 loci appear to be restricted along the rostrocaudal axis, whereas they were dispersed along the dorsoventral and mediolateral axes in extratoes mice.

Although olfactory glomeruli do not become morphologically distinct until early in postnatal development in wild-type mice, the convergence of P2 olfactory axons to loci begins embryonically (Royal and Key, 1999). P2 loci were absent from both control and extratoes mice at E14.5. However, several P2-positive axons could be observed entering the nerve fiber layer of control mice (arrow, Fig. 3H) and the FCM of extratoes mice (arrow, Fig. 3I). By E16.5, convergence of P2 axons was detected in both control (arrows, Fig. 3J) and extratoes (arrow, Fig. 3K) mice. These results indicate that the timing of convergence was not affected by the extratoes mutation.

We next determined whether other subsets of primary olfactory axons converged to loci in the FCM of extratoes mice. Histochemical staining with the plant lectin DBA has previously revealed a large subpopulation of primary olfactory axons that target glomeruli in the mouse olfactory bulb (Key and Akeson, 1993). This subset of olfactory axons is considerably larger than and mutually exclusive of the subpopulation of axons expressing the P2 receptor. These DBA-stained axons converged to glomeruli in control (Fig. 4A,B) and to loci in extratoes (Fig. 4C–F) mice.

Fig. 2. Analysis of the expression of different markers within the olfactory bulbs of wild-type mice and the fibrocellular mass (FCM) of mice homozygous for the extratoes mutation. Coronal cryostat (A–G, I–J, L–P) and paraffin (H, K) sections of P0.5 animals sections were stained with antisera raised against OMP (A,D), p75 (B,E), neuropeptide Y (C,F), Pbx-1 (G,J), Id2 (I,L), GABA (M,O), and RC2 (N,P). Sections shown in H and K were reacted with digoxigenin-labelled riboprobes corresponding to the reelin gene. OMP-positive fibers are observed within the nerve fiber layer and the glomerular layers (asterisk) of wild-type olfactory bulb (A), whereas, in extratoes homozygous mice (D), OMP-positive fibers fill the FCM (asterisk). p75 Immunostaining is confined to olfactory ensheathing cells (arrows) present within the outer layer of the nerve fiber layer of wild-type mice (B), but, in extratoes mice (E), p75-positive ensheathing cells are found mostly around the boundaries of the FCM (arrows), although a few p75-positive cells are dispersed within this structure. In wild-type mice, neuropeptide Y expression (C) is confined to the outer nerve fiber layer (arrows), whereas, in extratoes mice (F), neuropeptide Y positive cells are found principally at the edge of the FCM (arrows). G:

Pbx-1 was expressed within neurons of the glomerular, mitral, and granule cell layers of the wild-type olfactory bulb, whereas, in extratoes mice (J), no Pbx-1-immunoreactive neurons were observed within the FCM. (H) Reelin mRNA was observed within the mitral cell layer (mcl) of the wild-type olfactory bulb, but reelin signal was not observed within the FCM in mice homozygous for the extratoes mutation (K). I: Id-2 was expressed within the mitral cell layer of the wild-type olfactory bulb, but, in the FCM of extratoes mice (L), Id-2 immunoreactivity was not detected. M: GABA was expressed within the inhibitory interneurons of the glomerular and granule cell layers in the olfactory bulbs of wild-type mice. O: Only several dispersed cells (arrows) were GABA-immunoreactive neurons in the FCM of extratoes mice. N: In olfactory bulbs of wild-type mice, the radial glia-specific antibody RC2 labelled cells across the depth of the olfactory bulb. In extratoes mice (P), only background staining of blood vessels was apparent in the FCM. epl, External plexiform layer; fcm, fibrocellular mass; gc, granule cell layer. Scale bar = 200 μ m in A–N, 100 μ m in O,P.

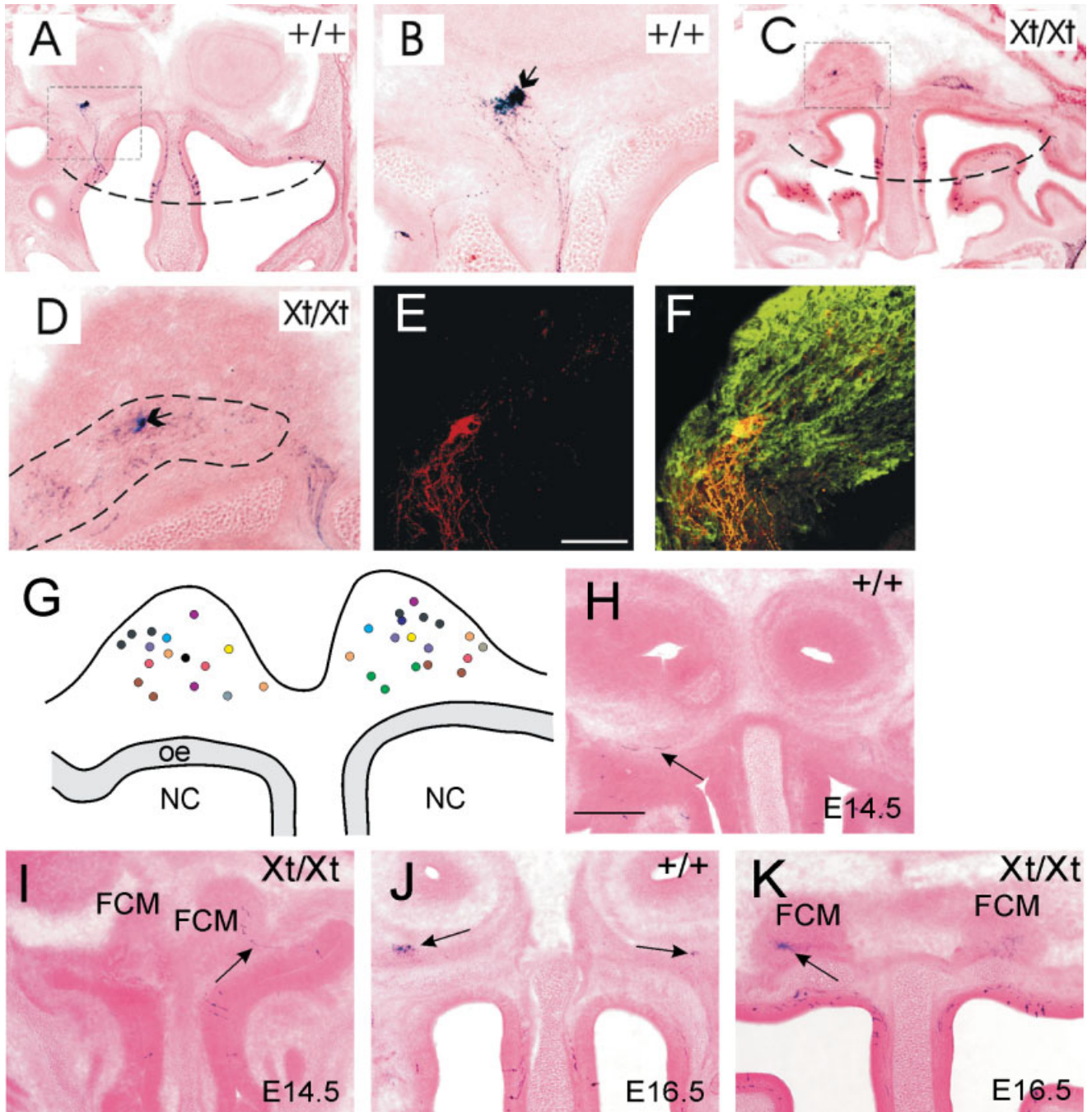


Fig. 3. Distribution of P2-expressing neurons and their axonal projections. Coronal cryostat sections were stained using X-gal histochemistry (A–D, H–K) or were stained with anti- β -gal (E) or anti- β -gal and OMP antibodies (F). A: In wild-type mice, P2 was expressed in neurons found in zone three (around the region of the dashed line) of the olfactory neuroepithelium. A single P2 glomerulus was present within the dorsolateral region of the left bulb. This glomerulus (arrow) is shown enlarged in B. C: In extratoes (Xt/Xt) mice, the P2 odorant receptor was also expressed within primary olfactory neurons found in zone three of the nasal cavity. P2 axons converged to a discrete locus in the left bulb in this animal, which is shown enlarged in D (arrow). P2-expressing axons appeared to be more diffusely dispersed in extratoes animals compared with wild-type (wt) animals. E: Immunohistochemistry using antibodies against LacZ revealed the con-

vergence of P2-lacZ axons. F: Double labeling of the same section as in E using antibodies against OMP (green) and β -gal (red). G: Camera lucida compilation of P2-targeted loci in 11 different extratoes mice. Different colors represent different animals. The figure reveals that, in the dorsoventral and mediolateral axes, there appears to be no consistency in the position of P2-targeted loci. Analysis of the P2-targeting behavior in wt and Xt/Xt mice during embryonic development revealed that, at E14.5, there was only an occasional P2 axon (arrow) in either wt (H) or extratoes (I) mice. However, at E16.5, small but discrete loci (arrow) were observed in the olfactory bulbs of wt (J) and the fibrocellular mass (fcm) of extratoes (K) mice. oe, Olfactory neuroepithelium; nc, nasal cavity. Scale bar = 200 μ m in A, C, 50 μ m in B, D–F, 100 μ m in H–K.

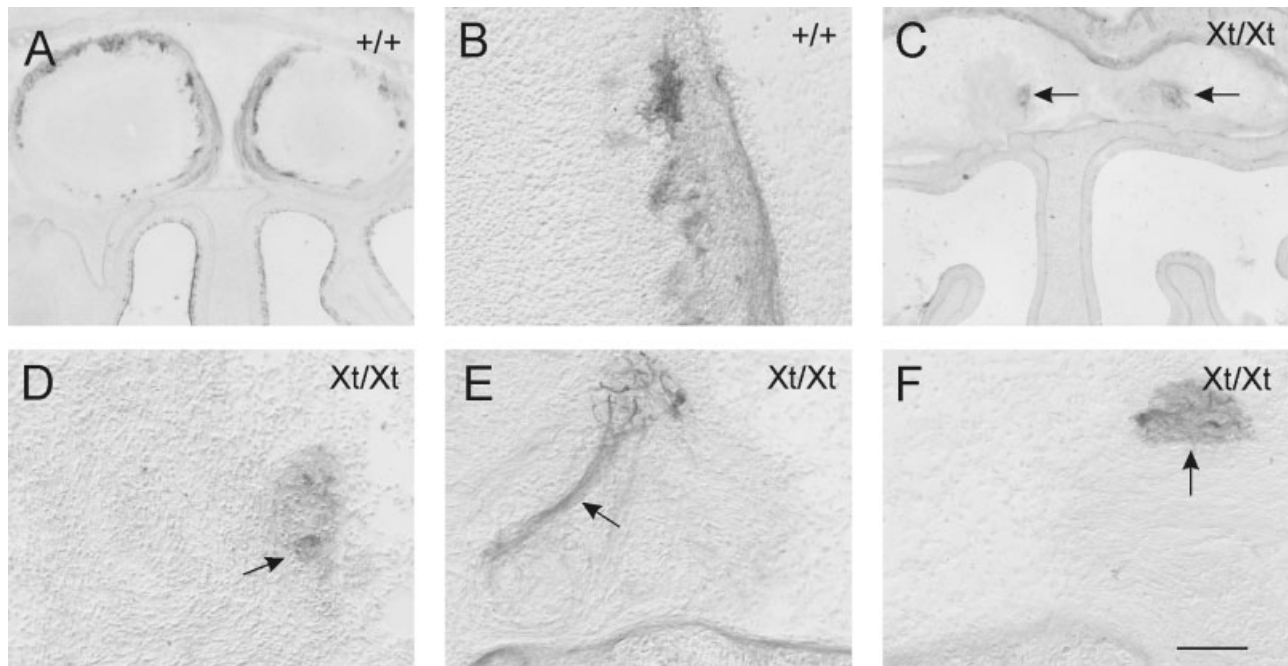


Fig. 4. Distribution of DBA-reactive primary olfactory axons in wild-type (+/+) and extratoes (Xt/Xt) mice. **A:** DBA-reactive primary olfactory axons converge on glomeruli located in the ventromedial and dorsolateral surfaces of the olfactory bulb. **B:** The left olfactory bulb is enlarged to show convergence of axons to glomeruli. **C:** DBA-reactive axons target discrete areas (arrows) in the FCM of extratoes mice. The

left fibrocellular mass is enlarged in **D**, showing the convergence of axons (arrow). **E:** A fasciculated bundle of DBA-reactive olfactory axons coursing through the FCM of another extratoes mouse. **F:** Higher magnification of a compact locus in the FCM of an extratoes mouse targeted by DBA-reactive axons. Scale bar = 200 μ m in A,C, 40 μ m in B,D–F.

DBA-stained axons could be seen to sort out into fascicles in the FCM (Fig. 4E) before targeting loci (Fig. 4F).

Axon convergence following bulbectomy

The results of the above-described analysis revealed that the olfactory nerve pathway in extratoes mice contained sufficient cues for the convergence of olfactory axons during embryonic development. Would convergence also occur in the absence of an olfactory bulb in postnatal animals? To address this question, we surgically removed the olfactory bulb (bulbectomy) from P4.5 P2-IRES-tau:LacZ mice and allowed the animals to recover for either 3 or 6 weeks. In these animals, the frontal pole of the telencephalon shifts forward to fill the space previously occupied by the olfactory bulb (Fig. 5A,B).

As previously demonstrated (Graziadei et al., 1978), primary olfactory neurons regenerate, and newly growing axons extend into the cranial cavity, where they form glomerular-like structures in the frontal pole. Despite the presence of these OMP-positive loci at 3 weeks (Fig. 5C) and the presence of P2-lacZ axons within the frontal pole, we did not observe any convergence of P2 axons at this time (not shown). At 6 weeks postbulbectomy, OMP-positive loci were widely distributed throughout the frontal pole that occupied the cavity left by the olfactory bulb (Fig. 5D). By this age, P2 axons had converged to form numerous loci at random, dispersed sites in the frontal pole (Fig. 5A,E,F), including sites that were deep within the frontal pole (Fig. 5B). We also examined, in addition to the P2 subset of axons, the convergence of the subpopulation of neurons reactive to the lectin DBA. These axons

normally project to a discrete subpopulation of glomeruli in the olfactory bulb (Fig. 4A,B). After bulbectomy, DBA-reactive axons also converged to discrete glomerular-like loci (arrows, Fig. 5G). Taken together, results from the extratoes and bulbectomized mice indicate that target bulb neurons were not needed for convergence of specific subpopulations of primary olfactory axons either during embryonic development or subsequently during regeneration of primary olfactory neurons in the postnatal animal.

DISCUSSION

What are the cues that allow primary olfactory axons to converge and target topographical positions in the mammalian olfactory bulb? Neurons expressing the same odorant receptor are mosaically distributed across a relatively large and convoluted epithelial sheet lining the nasal cavity. The axons of these neurons intermingle with many other olfactory axons expressing different odorant receptors as they grow toward the olfactory bulb. Each distinct subpopulation of axons then sorts out within the olfactory nerve fiber layer and converges to between one and several glomeruli at topographically fixed target sites in the olfactory bulb. We have shown here that convergence occurs independently of cues from the olfactory bulb. These results suggest that the olfactory bulb instead provides cues for targeting to topographic sites, indicating that convergence and targeting are clearly separable events.

Evidence from several sensory pathways indicates that the processes of convergence and targeting are inextricably linked and are dependent on target cues. For instance,

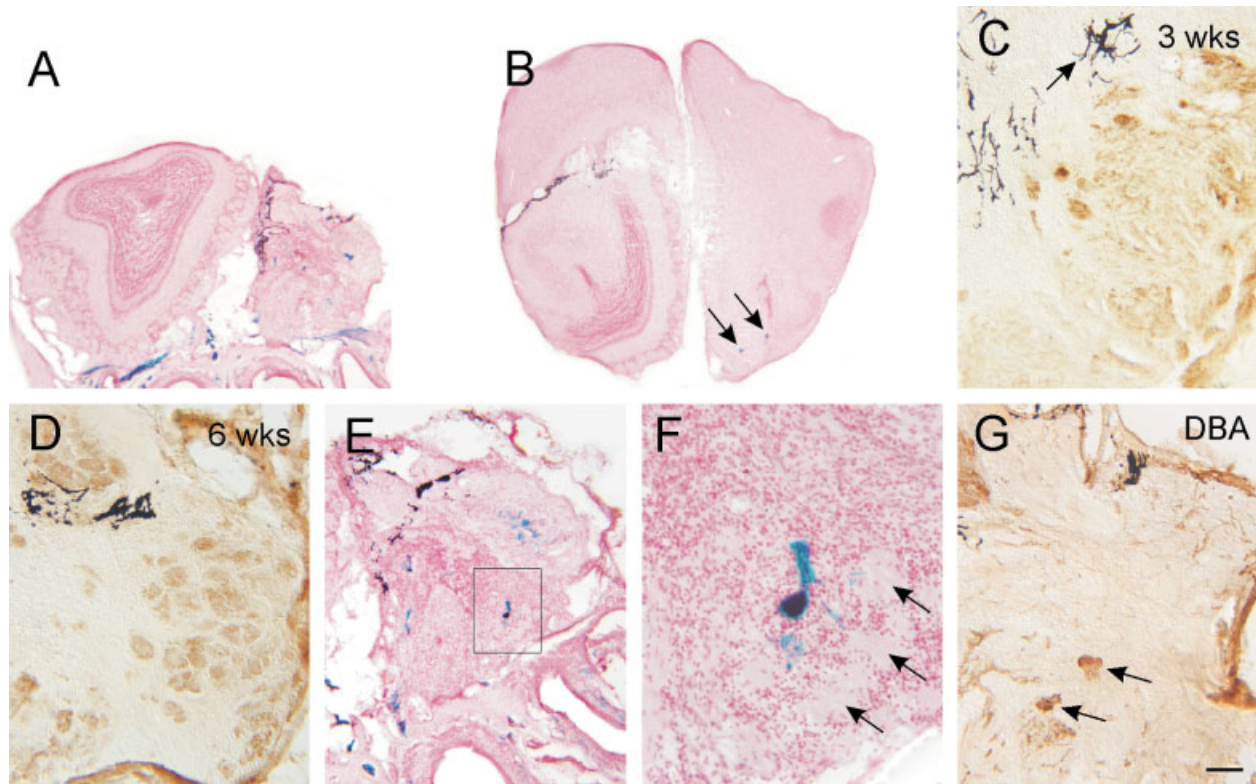


Fig. 5. Axon convergence following bulbectomy. Panels are coronal sections through the olfactory bulb region of adult mice following unilateral bulbectomy, which was performed at P4.5. C is at 3 weeks postbulbectomy; all other panels are 6 weeks postbulbectomy. **A:** Low-magnification micrograph at the level of the left rostral olfactory bulb. The unablated olfactory bulb is at left. **B:** Section at the level of the left caudal olfactory bulb. On the right, the bulbectomized side has protruded rostrally. P2-lacZ axons terminate in two distinct locations (arrows) in the ventral forebrain. **C:** OMP immunostaining at 3 weeks following bulbectomy revealed labeled glomerular-like loci within the frontal pole. Melanocytes (arrow) were

present in the tissue. **D:** At 6 weeks following bulbectomy, OMP clearly reveals loci of primary olfactory axons within the frontal pole. **E:** At 6 weeks postbulbectomy, P2-lacZ axons had penetrated the frontal pole and converged to form loci. The boxed area in E is shown in F. **F:** P2-lacZ axons terminate in a locus within the frontal pole. Unstained glomerular-like structures (arrows) can be seen adjacent to the P2 locus. **G:** Lectin histochemistry using *Dolichos biflorus* agglutinin labeled a subset of glomerular-like structures, further confirming the convergence of like axons. A, B, D, and F are counterstained with nuclear fast red. Dorsal is to the top in all panels. Scale bar = 400 μ m in A,B, 200 μ m in E, 80 μ m in C,D,G, 40 μ m in F.

in the retinotectal pathway, retinal axons use cues distributed in gradients across the surface of the tectum as navigational cues (Frisen et al., 1998). In the absence of appropriate tectal cues, retinal axons fail to converge and instead spread out over the surface of the tectum (Chien et al., 1995). When the caudal tectum is ablated, the severed retinal axons regenerate and grow diffusely over the remaining rostral tectum without converging (Udin, 1977), again indicating that convergence in this pathway is target-tissue dependent. Thus, the axons of the primary olfactory pathway behave very differently with regard to the role of the target tissue in axon navigation.

The topographic refinement of terminations of retinal axons in the tectum is dependent on functional synaptic connections (Zhang et al., 1998). This refinement is mediated by the convergent retinal inputs on single tectal neurons. In the absence of activity, axon terminals spread diffusely over the tectal surface (Cline and Constantine-Paton, 1989). In contrast to the case in the retinotectal pathway, electrical activity appears to have a minimal role in convergence of olfactory axons. In mice lacking the olfactory cyclic nucleotide-gated channel subunit olfactory-specific channel (Lin et al., 2000; Zheng et al.,

2000), P2 axons continued to converge and target topographically appropriate sites in the olfactory bulb. Different results, however, were found for the subpopulation of olfactory axons expressing the M72 olfactory receptor (Zheng et al., 2000). In heterozygous animals, M72-expressing olfactory axons with functional channels converged on glomeruli separate from M72 neurons with non-functional channels. These results indicated that functional connections within the bulb were important in the topographic mapping of glomeruli but were not involved in the ability of olfactory axons to converge. These observations do not, however, preclude a role for the postsynaptic partners in providing guidance cues during convergence. This issue had begun to be addressed previously by Bulfone et al. (1998), when they demonstrated that P2 olfactory axons continued to converge and target topographically correct glomeruli in mutant mice lacking either many mitral cells or GABAergic interneurons. However, the presence of remaining subpopulations of bulbar neurons in these mice precluded ruling out their role in convergence and topographic mapping.

In the present study, we rigorously assessed the role of postsynaptic olfactory bulb projection neurons in conver-

gence by analysing the extratoes mutant mouse. These mice are deficient in the Gli3 transcription factor and lack the normal complement of olfactory bulb neurons. The deficit is so severe in these mice that the olfactory bulb is replaced by an FCM lacking any laminar cytoarchitecture. Despite this aberrant morphology, primary olfactory axons continued to converge and form discrete loci within the FCM, albeit in random mediolateral and dorsoventral positions. These results, at least, suggest that the convergence of primary olfactory axons is independent of olfactory bulb projection neurons and most likely the local interneurons. Although extratoes mutants had a small subpopulation of GABA-expressing neurons (several neurons per section) in the FCM, it is unlikely that they influenced convergence by P2 axons insofar as we never observed any spatial relationship between these neurons and the P2 loci. Moreover, periglomerular neurons cannot be involved in the initial stages of convergence; they are generated only at E18.5 (Hinds, 1968), 2 days after the P2 axons have already begun to form loci (Royal and Key, 1999). Our interpretation that olfactory bulb neurons have no role in convergence would be incorrect if olfactory bulbs appeared transiently during embryogenesis in extratoes mice and then subsequently regressed. However, our analysis revealed the absence of the olfactory bulb early in the development of these mice.

In marked contrast to the disruption in the topographical positioning of P2 loci about the mediolateral and ventrodorsal axes, these loci were restricted in their distribution along the rostrocaudal axis. P2 loci were always restricted to one of two $\sim 240\text{-}\mu\text{m}$ zones located between 160 and 400 μm and between 320 and 560 μm from the rostral pole of the FCM. The specificity in the positioning of these loci is similar to the 180- μm zone to which P2 glomeruli are restricted in adult animals (Royal and Key, 1999). Thus, it appears that P2 axons are responding to the presence of topographical cues along the rostrocaudal axis that are less affected by the loss of either olfactory bulb neurons or laminar organisation in the FCM. At present, the identity of these topographical cues or the cells that express them remains unknown. We know that these cues are not associated with radial glia, in that they are absent from the FCM. They could instead be associated with cells that migrate with the axons into the FCM. One possibility is that olfactory ensheathing cells arising from different locations along the rostrocaudal axis of the nasal cavity are positionally coded. These cells may then migrate into the olfactory bulb and establish an independent rostrocaudal axis. This could explain why the rostrocaudal axis appears less affected in extratoes mice. Thus targeting about the mediolateral and dorsoventral axes may depend on endogenous bulbar cues, whereas rostrocaudal cues could be imparted to the bulb, possibly by the exogenous ensheathing cells. Although, in receptor swap experiments, olfactory axons expressing inappropriate receptors converged at aberrant dorsoventral positions in the olfactory bulb, they did reach the correct rostrocaudal position (Wang et al., 1998). Similarly, ectopic glomeruli arising from neurons expressing odorant receptor transgenes form at the correct rostrocaudal level but are misplaced along the dorsoventral axis in comparison with their cognate endogenous receptors (Vassalli et al., 2002). Together these observations support our present results in extratoes mice, indicating the separation of targeting mechanisms along dorsoventral and rostrocaudal axes.

The olfactory bulb or some remnant form of this structure, such as the FCM, does not appear to be essential for convergence of primary olfactory axons expressing the same odorant receptor. When we physically removed the olfactory bulb from early postnatal mice, primary olfactory axons regenerated and extended into the frontal pole of the telencephalon, where they again sorted out and converged in glomerular-like loci. Thus, neither olfactory bulbs nor the FCM possesses any particular cell type that is essential for the convergence of olfactory axons. Although we have examined only the P2 odorant receptor expressing axons, there is no reason to expect that convergence does not also occur for axons expressing other odorant receptors. It seems that convergence is dependent on axon-axon interactions involving odorant receptors. We know this because the M71 odorant receptor is able to rescue the aberrant targeting phenotype of vomeronasal organ axons in mice deficient in the pheromone receptor Vr_2 (Rodriguez et al., 1999). In $Vr_2^{-/-}$ mice, axons normally expressing this receptor now terminate broadly throughout the glomerular layer of the accessory olfactory bulb rather than converging on a discrete subpopulation of glomeruli. When the coding region for the Vr_2 receptor is replaced by the coding region of the M71 odorant receptor, convergence of axons is restored. Thus, the M71 receptor is autonomously responsible for axon convergence, most likely via axon-axon interactions, but probably only after the axons have been presorted by other mechanisms. Similar conclusions have recently been reached through transgenic approaches involving the expression of two receptors, MOR23 and M71, by primary olfactory neurons in inappropriate regions of the nasal cavity (Vassalli et al., 2002). These ectopic neurons continued to converge and form glomeruli, consistently with the role of the receptor in convergence, but they did so in regions appropriate for their new location in the nasal cavity. This later observation indicates the importance of the zonal organisation of the nasal cavity in determining targeting. The contrast between convergence and targeting is also seen with regenerating primary sensory olfactory axons that fail to target their original topographically correct glomerular sites when the olfactory nerve is physically lesioned (Costanzo, 2000). Instead, axons converge at inappropriate positions in the bulb, suggesting that, in this model, convergence is intrinsic to axons and is an event separate from targeting.

In summary, we have shown that select subpopulations of primary olfactory axons are able to sort out and converge to form loci without involvement of the olfactory bulb. Although these results are consistent with homotypic interactions between axons being critical for convergence of olfactory axons, they do not preclude the role of ensheathing cell-axon interactions. It should be remembered that primary olfactory axons begin to sort out and converge into discrete chemically distinct fascicles only as they enter the olfactory nerve fiber layer (Key and Akeson, 1993; Treloar et al., 1997). Perhaps the nerve fiber layer contains a trigger that initiates this axon sorting and provides the opportunity for axons expressing the same receptor to interact.

ACKNOWLEDGMENTS

We thank Dr. P. Mombaerts for providing the P2-IRES-tau:lacZ mice. Linh Nguyen provided excellent technical assistance, and Victoria Hunter provided valuable assistance with the maintenance of the mouse colony.

LITERATURE CITED

- Aoki K, Nakahara Y, Yamada S, Eto K. 1999. Role of polysialic acid on outgrowth of rat olfactory receptor neurons. *Mech Dev* 85:103–110.
- Bulfone A, Wang F, Hevner R, Anderson S, Cutforth T, Chen S, Meneses J, Pedersen R, Axel R, Rubenstein JL. 1998. An olfactory sensory map develops in the absence of normal projection neurons or GABAergic interneurons. *Neuron* 21:1273–1282.
- Chien CB, Cornel EM, Holt CE. 1995. Absence of topography in precociously innervated tecta. *Development* 121:2621–2631.
- Chuah MI, Au C. 1991. Olfactory Schwann cells are derived from precursor cells in the olfactory epithelium. *J Neurosci Res* 29:172–180.
- Cline HT, Constantine-Paton M. 1989. NMDA receptor antagonists disrupt the retinotectal topographic map. *Neuron* 3:413–426.
- Cloutier JF, Giger RJ, Koentges G, Dulac C, Kolodkin AL, Ginty DD. 2002. Neuropilin-2 mediates axonal fasciculation, zonal segregation, but not axonal convergence, of primary accessory olfactory neurons. *Neuron* 33:877–892.
- Costanzo RM. 2000. Rewiring the olfactory bulb: changes in odor maps following recovery from nerve transection. *Chem Senses* 25:199–205.
- D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T. 1995. A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature* 374:719–723.
- Danneman PJ, Mandrell TD. 1997. Evaluation of five agents/methods for anesthesia of neonatal rats. *Lab Anim Sci* 47:386–395.
- Franz T. 1994. Extra-toes (Xt) homozygous mutant mice demonstrate a role for the Gli-3 gene in the development of the forebrain. *Acta Anat* 150:38–44.
- Frisen J, Yates PA, McLaughlin T, Friedman GC, O'Leary DD, Barbacid M. 1998. Ephrin-A5 (AL-1/RAGS) is essential for proper retinal axon guidance and topographic mapping in the mammalian visual system. *Neuron* 20:235–243.
- Graziadei PP, Levine RR, Graziadei GA. 1978. Regeneration of olfactory axons and synapse formation in the forebrain after bulbectomy in neonatal mice. *Proc Natl Acad Sci USA* 75:5230–5234.
- Hinds JW. 1968. Autoradiographic study of histogenesis in the mouse olfactory bulb. I. Time of origin of neurons and neuroglia. *J Comp Neurol* 134:287–304.
- Jenkinson KM, Morgan JM, Furness JB, Southwell BR. 1999. Neurons bearing NK(3) tachykinin receptors in the guinea-pig ileum revealed by specific binding of fluorescently labelled agonists. *Histochem Cell Biol* 112:233–246.
- Johnson DR. 1967. Extra-toes: a new mutant gene causing multiple abnormalities in the mouse. *J Embryol Exp Morphol* 17:543–581.
- Keller A, Margolis FL. 1975. Immunological studies of the rat olfactory marker protein. *J Neurochem* 24:1101–1106.
- Key B, Akeson RA. 1993. Distinct subsets of sensory olfactory neurons in mouse: possible role in the formation of the mosaic olfactory projection. *J Comp Neurol* 335:355–368.
- Lin DM, Wang F, Lowe G, Gold GH, Axel R, Ngai J, Brunet L. 2000. Formation of precise connections in the olfactory bulb occurs in the absence of odorant-evoked neuronal activity. *Neuron* 26:69–80.
- Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M, Edmondson J, Axel R. 1996. Visualizing an olfactory sensory map. *Cell* 87:675–686.
- Morse WR, Whitesides JG 3rd, LaMantia AS, Maness PF. 1998. p59fyn and pp60c-src modulate axonal guidance in the developing mouse olfactory pathway. *J Neurobiol* 36:53–63.
- Nagao H, Yoshihara Y, Mitsui S, Fujisawa H, Mori K. 2000. Two mirror-image sensory maps with domain organization in the mouse main olfactory bulb. *Neuroreport* 11:3023–3027.
- Pasterkamp RJ, De Winter F, Holtmaat AJ, Verhaagen J. 1998. Evidence for a role of the chemorepellent semaphorin III and its receptor neuropilin-1 in the regeneration of primary olfactory axons. *J Neurosci* 18:9962–9976.
- Pohl TM, Mattei MG, Ruther U. 1990. Evidence for allelism of the recessive insertional mutation add and the dominant mouse mutation extratoes (Xt). *Development* 110:1153–1157.
- Puche AC, Poirier F, Hair M, Bartlett PF, Key B. 1996. Role of galectin-1 in the developing mouse olfactory system. *Dev Biol* 179:274–287.
- Ramón-Cueto A, Avila J. 1998. Olfactory ensheathing glia: properties and function. *Brain Res Bull* 46:175–187.
- Redmond L, Hockfield S, Morabito MA. 1996. The divergent homeobox gene PBX1 is expressed in the postnatal subventricular zone and interneurons of the olfactory bulb. *J Neurosci* 16:2972–2982.
- Ressler KJ, Sullivan SL, Buck LB. 1993. A zonal organization of odorant receptor gene expression in the olfactory epithelium. *Cell* 73:597–609.
- Ressler KJ, Sullivan SL, Buck LB. 1994. Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. *Cell* 79:1245–1255.
- Ribak CE, Vaughn JE, Saito K, Barber R, Roberts E. 1977. Glutamate decarboxylase localization in neurons of the olfactory bulb. *Brain Res* 126:1–18.
- Rodriguez I, Feinstein P, Mombaerts P. 1999. Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system. *Cell* 97:199–208.
- Royal SJ, Key B. 1999. Development of P2 olfactory glomeruli in P2-internal ribosome entry site-tau-LacZ transgenic mice. *J Neurosci* 19:9856–9864.
- Royal SJ, Gambello MJ, Wynshaw-Boris A, Key B, Clarris HJ. 2002. Laminar disorganization of mitral cells in the olfactory bulb does not affect topographic targeting of primary olfactory axons. *Brain Res* 932:1–9.
- Royet JP, Souchier C, Jourdan F, Ploye HJ. 1988. Morphometric study of the glomerular population in the mouse olfactory bulb: numerical density and size distribution along the rostrocaudal axis. *J Comp Neurol* 270:559–568.
- Schimmang T, Lemaistre M, Vortkamp A, Ruther U. 1992. Expression of the zinc finger gene Gli3 is affected in the morphogenetic mouse mutant extra-toes (Xt). *Development* 116:799–804.
- Schwartz GA, Kostek C, Ahmad N, Dibble C, Pays L, Puschel AW. 2000. Semaphorin 3A is required for guidance of olfactory axons in mice. *J Neurosci* 20:7691–7697.
- Strotmann J, Wanner I, Helfrich T, Beck A, Breer H. 1994a. Rostro-caudal patterning of receptor-expressing olfactory neurones in the rat nasal cavity. *Cell Tissue Res* 278:11–20.
- Strotmann J, Wanner I, Helfrich T, Beck A, Meinken C, Kubick S, Breer H. 1994b. Olfactory neurones expressing distinct odorant receptor subtypes are spatially segregated in the nasal neuroepithelium. *Cell Tissue Res* 276:429–438.
- Sullivan SL, Bohm S, Ressler KJ, Horowitz LF, Buck LB. 1995. Target-independent pattern specification in the olfactory epithelium. *Neuron* 15:779–789.
- Theil T, Alvarez-Bolado G, Walter A, Ruther U. 1999. Gli3 is required for Emx gene expression during dorsal telencephalon development. *Development* 126:3561–3571.
- Treloar H, Tomasiewicz H, Magnuson T, Key B. 1997. The central pathway of primary olfactory axons is abnormal in mice lacking the N-CAM-180 isoform. *J Neurobiol* 32:643–658.
- Ubins R, Hokfelt T. 2000. Expression of neuropeptide Y in olfactory ensheathing cells during prenatal development. *J Comp Neurol* 423:13–25.
- Udin SB. 1977. Rearrangements of the retinotectal projection in *Rana pipiens* after unilateral caudal half-tectum ablation. *J Comp Neurol* 173:561–582.
- Vassalli G, Rothman A, Feinstein R, Zapotocky M, Mombaerts P. 2002. Minigenes impart odorant receptor-specific axon guidance in the olfactory bulb. *Neuron* 35:681–696.
- Vassar R, Ngai J, Axel R. 1993. Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. *Cell* 74:309–318.
- Vassar R, Chao SK, Sitcheran R, Nunez JM, Vosshall LB, Axel R. 1994. Topographic organization of sensory projections to the olfactory bulb. *Cell* 79:981–991.
- Walz A, Rodriguez I, Mombaerts P. 2002. Aberrant sensory innervation of the olfactory bulb in neuropilin-2 mutant mice. *J Neurosci* 22:4025–4035.
- Wang F, Nemes A, Mendelsohn M, Axel R. 1998. Odorant receptors govern the formation of a precise topographic map. *Cell* 93:47–60.
- Zhang LL, Tao HW, Holt CE, Harris WA, Poo M. 1998. A critical window for cooperation and competition among developing retinotectal synapses. *Nature* 395:37–44.
- Zhang X, Firestein S. 2002. The olfactory receptor gene superfamily of the mouse. *Nat Neurosci* 5:124–133.
- Zheng C, Feinstein P, Bozza T, Rodriguez I, Mombaerts P. 2000. Peripheral olfactory projections are differentially affected in mice deficient in a cyclic nucleotide-gated channel subunit. *Neuron* 26:81–91.