

Metamorphic Remodeling of the Primary Olfactory Projection in *Xenopus*: Developmental Independence of Projections from Olfactory Neuron Subclasses

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ABSTRACT: In adult *Xenopus*, the nasal cavity is divided into separate middle (MC) and principal (PC) cavities; the former is used to smell water-borne odorants, the latter air-borne odorants. Recent work has shown that olfactory neurons of each cavity express a distinct subclass of odorant receptors. Moreover, MC and PC axons project to distinct regions of the olfactory bulb. To examine the developmental basis for this specificity in the olfactory projection, we extirpated the developing MC from early metamorphic (stage 54–57) tadpoles and raised the animals through metamorphosis. In most lesioned animals, the

MC partly regenerated. Compared with the unlesioned side, reduction of the region of the glomerular layer of the olfactory bulb receiving MC afferents ranged from 70% to 95%. PC afferents did not occupy regions of the olfactory bulb deprived of MC afferents. These results support a model in which intrinsic cues in the olfactory bulb control the projection pattern attained by ingrowing olfactory axons. © 1997 John Wiley & Sons, Inc. *J Neurobiol* 32: 213–222, 1997

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INTRODUCTION

It has long been known that vertebrate olfactory receptor cells located in the olfactory epithelium send axons back through the olfactory nerves to the olfactory bulbs of the brain. Here they synapse with dendrites of secondary olfactory cells (mitral, tufted, and periglomerular) within knots of dense neuropil called glomeruli. Until recently, however, the organization of this primary olfactory projection was unclear. In the visual and auditory systems, the intrinsic organization of the stimulus is reflected in

the retinotopic and tonotopic organization of their respective projections (Kandel et al., 1991). By contrast, the lack of any obvious scale for odorant quality made it difficult to understand how the olfactory projection might be organized.

This situation has changed greatly in the last 5 years, with the cloning of odorant receptor genes in mammals (Buck and Axel, 1991), fishes (Ngai et al., 1993a; Barth et al., 1996; Byrd et al., 1996), salamanders (Zhao et al., 1995), frogs (Freitag et al., 1995), and birds (Leibovici et al., 1996). The number of individual receptor genes is estimated to range from 1000 in mammals (Buck and Axel, 1991) down to 100 in catfish (Ngai et al., 1993a). Studies using *in situ* hybridization have shown that olfactory receptor cells expressing a given receptor protein are widely dispersed: in fishes and birds throughout the entire olfactory epithelium (Ngai et al., 1993b; Byrd et al., 1996; Leibovici et al., 1996),

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and in mammals within one of four domains (Ressler et al., 1993; Vassar et al., 1993; Strotman et al., 1994). However, although they are dispersed in the epithelium, mammalian receptor cells expressing particular odorant receptors have axons that converge to only a few highly stereotyped glomeruli within the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994). Circumstantial evidence suggests that a similar situation exists in fishes (Baier et al., 1994). Moreover, dispersed subclasses of receptor neurons defined either morphologically or physiologically may also project to distinct regions of the olfactory bulb (Jia and Halpern, 1995; Morita et al., 1996). Thus, the general organization of the primary olfactory projection appears to involve physiologically defined classes of receptor neurons that are dispersed in the epithelium, but converge within the olfactory bulb.

Given such an organization, the developmental problem is clear: Somehow, specificity of the projection must be attained despite the lack of topologic mapping from epithelium to bulb. *Xenopus laevis*, the African clawed frog, provides a unique system to examine the developmental basis of such specificity, because in *Xenopus* adults there is an overall topology to the olfactory projection. In the periphery, the nasal cavity is divided into two chambers—a principal cavity (medial diverticulum) and a middle cavity (lateral diverticulum)—separated by a valve. Principal cavity (PC) receptor cells sense airborne odorants; middle cavity (MC) receptor cells sense water-borne odorants (Altner, 1962). It has recently been shown that the odorant receptor genes of the PC and MC belong to distinct sequence subclasses (Freitag et al., 1995). In the olfactory bulb, PC afferents project dorsomedially and MC afferents ventrolaterally, with no apparent overlap (Weiss, 1986, 1987). The PC and MC thus provide a coarse model of the specificity seen in the olfactory projection of other vertebrates. They are perhaps most closely comparable to the epithelial domains of mammals, which also appear to project to distinct regions of the olfactory bulb (see Discussion). They have the advantage of allowing for selective experimental ablation of one subclass of receptor.

Development of the olfactory system in *Xenopus* is complicated by the phenomenon of metamorphosis (Fig. 1). In premetamorphic larvae, the MC is absent and the PC is used to sense water-borne odorants. During early metamorphosis, the MC arises as a bud at the rostral end of the nasal sac (Föske, 1934). At this time MC and PC projections overlap in the ventral part of the olfactory bulb. As meta-

morphosis proceeds, and the MC develops further, the PC projection to the ventral bulb disappears (Weiss, 1986, 1987) (see below).

To determine whether interactions between PC and MC axons are required for the normal metamorphic change in the PC projection, we ablated the developing MC in early metamorphic tadpoles, and then allowed the animals to progress through the end of metamorphosis. We used the lectin soybean agglutinin (SBA) as a marker to distinguish axons from the PC (weakly staining) and MC (strongly staining) (Key, 1986; Key and Giorgi, 1986; Hoffman and Meyer, 1991; Franceschini et al., 1992). Prelabeling of the ventral olfactory bulb with [^3H]-thymidine provided an internal marker for the dorsal-ventral boundary of the bulb (Fritz et al., 1996). Operated animals showed little sign that PC projections to the ventromedial olfactory bulb are retained when MC projections to this region are removed. Thus, it appears that intrinsic determinants are sufficient to result in a normal PC projection pattern, even in the absence of interaction with MC neurons.

MATERIALS AND METHODS

Animals

Tadpoles were obtained from our laboratory colony, or purchased from NASCO (Fort Atkinson, WI). They were raised at 20°C and fed on a suspension of boiled nettle powder daily. Tanks were cleaned weekly.

Dil Labeling

To trace the olfactory projections, small crystals of DiIC₁₈(3) (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR) were applied to the individual epithelia (PC, MC) or olfactory bulbs of tadpoles fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The tadpoles were returned to fix for approximately 10 days in the dark at 25°C, then were embedded in gelatin-albumin and sectioned at 50 μm on a Vibratome. Sections were counterstained with bisbenzimidazole (25 $\mu\text{g}/\text{mL}$) to reveal nuclei, and examined using rhodamine filters on a Zeiss epifluorescence microscope.

[^3H]Thymidine Labeling

Stage 46 (Nieuwkoop and Faber, 1994) animals were anesthetized in 1:5000 MS222 (tricaine methane sulfonate; Sigma Chemical Co., St. Louis, MO) and injected intraperitoneally (i.p.) with 1 μL (100 nCi) [^3H]-thymidine (New England Nuclear, Boston, MA) in dH₂O, using a 33-gauge needle in a 10 μL Hamilton syringe.

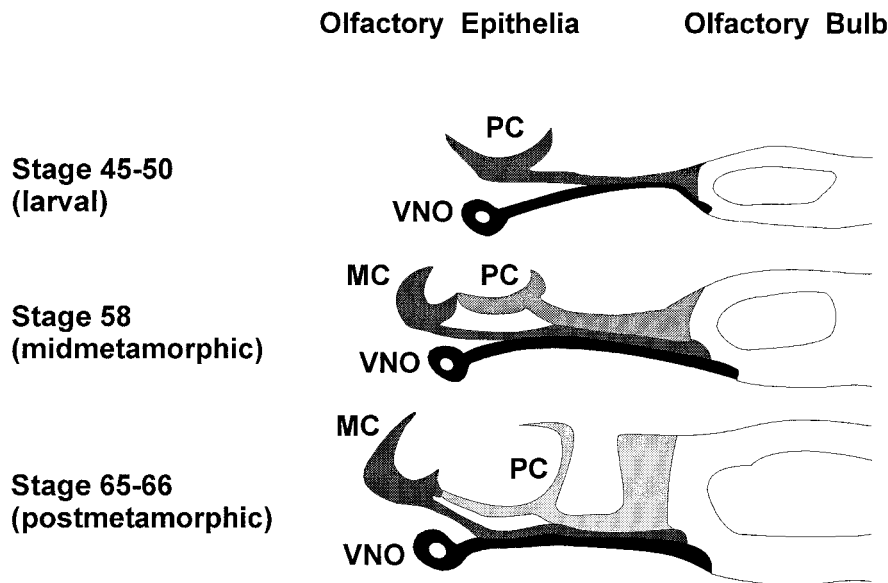


Figure 1 Schematic parasagittal sections through the developing olfactory system of *Xenopus*. The epithelium of the principal cavity (PC) projects to the entire olfactory bulb in larvae, but only to the dorsal olfactory bulb in postmetamorphic animals. The dorsal bulb first forms during metamorphosis. The middle cavity (MC) is absent in young larvae (stage 45–50), but present in midmetamorphic (stage 58) and postmetamorphic (stage 65–66) animals. Its epithelium projects to the ventral olfactory bulb at all stages. The vomeronasal (VNO) epithelium projects to the accessory olfactory bulb at all stages. Shading indicates strong (black or dark gray), moderate (medium gray) or weak (light gray) staining by the lectin SBA. Modified after Key (1986).

One day after the injection they were transferred to fresh medium and raised as above.

Surgeries and Tissue Processing

The majority of surgeries were carried out on stage 54–57 tadpoles anesthetized in 1:5000 MS222 ($N = 40$). A few additional surgeries were performed on stage 51–52 tadpoles ($N = 8$). Surgeries involved removal of the MC (or its precursor) using a homemade cautery gun consisting of the tip of a no. 11 scalpel blade mounted in an X-Acto knife chuck screwed into a Weller soldering iron (Cooper Tools, Apex, NC). Control animals of the same stages were anesthetized and allowed to recover. The animals were raised through the end of metamorphosis (stage 65–66) and perfused with 4% paraformaldehyde in PB, and the heads were cut off. After 4–6 h additional fixation, specimens were transferred to 30% sucrose in PB and sectioned horizontally at 20 μm on a cryostat. Alternate sections were mounted on Vectabond (Vector Labs, Burlingame, CA) coated glass slides to create two sets: one for SBA staining and one for autoradiography.

Soybean agglutinin staining was modified from Hofman and Meyer (1991). Briefly, slides were treated 30

min with 1% H_2O_2 , 0.3% Triton-X in PB to destroy endogenous peroxidase, blocked 30 min with 1% bovine serum albumin, 0.3% Triton-X in PB, incubated 45 min in 10 $\mu\text{g}/\text{mL}$ peroxidase-labeled SBA (Sigma), 0.3% Triton-X in PB, rinsed in PB, then transferred to 0.1 M acetate buffer (pH 6.0) for 15 min, and finally reacted for 20 min in 0.05% 3,3'-diaminobenzidine tetrahydrochloride, 2% nickelous ammonium sulfate, 0.4% H_2O_2 in acetate buffer, then rinsed in buffer, dehydrated, and mounted.

For [^3H]thymidine autoradiography, slides were dipped in Kodak NTB-2 emulsion at 42°C, slowly air-dried for a day at 25°C, then boxed and allowed to expose 30 days at 4°C. Slides were developed in Kodak D-19 developer for 3 min at 15°C, fixed, then counterstained with Toluidine Blue, dehydrated, and mounted.

Analysis of SBA-Labeled Slides

To determine the volume of the olfactory bulb occupied by projections from each cavity, the glomerular layer of the bulb was divided into regions staining weakly or strongly for SBA, and the outline of each traced using a video-equipped microscope connected to a microcomputer running Bioquant-OS2 software (R & M Biomet-

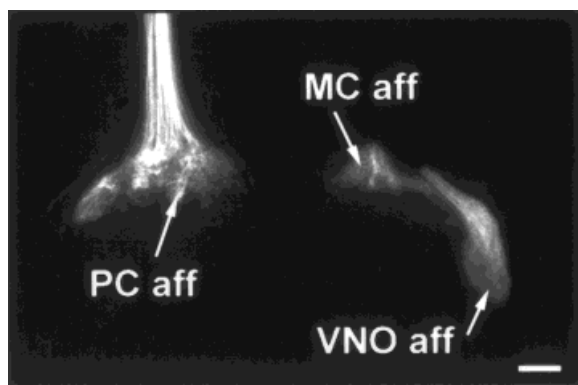


Figure 2 DiI labeling of olfactory afferents. Horizontal section of the olfactory bulb, anterior at top. Stage 55 animal. The lipophilic dye DiI₁₈(3) (Molecular Probes) was applied to the epithelium of the principal cavity (PC) on the left side, and the middle cavity (MC) and vomeronasal organ (VNO) on the right side. Note that the PC and MC afferents (PC aff, MC aff) partially overlap in this region of the ventral bulb. Scale bar = 63 μ m.

rics, Inc., Nashville, TN). A centerline for the fused left and right bulbs was determined by graphically extending the midline of the brain forward. Alternate sections were digitized. Values for the occasional section that could not be measured (due to folding, etc.) were estimated as the mean of the adjacent sections. The volume of each region of the bulb was calculated as the total section area multiplied by the distance between sections. Three-dimensional reconstructions of the olfactory bulbs were prepared using the same software. A total of 12 lesioned and four control animals were analyzed (only the left side of control animals was used). Statistical analysis was conducted using Sigma-Stat for Windows (Jandel Scientific, San Rafael, CA).

RESULTS

DiI Labeling of Projections

DiI backlabeling from the olfactory bulb revealed that MC axons reach the bulb by stage 52 (data not shown). Through stage 57 both MC axons and PC axons can be found in the ventral olfactory bulb (Fig. 2). By stage 58, however, PC axons no longer project to the ventral olfactory bulb. These results confirm previous reports (Weiss, 1986, 1987).

Effectiveness of MC Removal

Removal of the MC appeared to be complete, as determined by examination of animals fixed imme-

diately after surgery (Fig. 3). Despite this, an MC was present on the lesioned side of most animals raised through metamorphosis. This MC (presumably regenerated) was usually of reduced size; the epithelium was greatly reduced in thickness compared with that of control animals or the unlesioned side of the same animal [Fig. 4(A,B)].

Projection Patterns as Revealed by SBA Staining

As noted in the Introduction, MC afferents stain strongly with SBA, whereas PC afferents stain only weakly. As expected, reduction of the MC on the lesioned side was correlated with a reduction in the region of the glomerular layer of the olfactory bulb strongly labeled by SBA (Fig. 4; see also Fig. 6). Quantitatively, the volume of this region was reduced by 70–95% compared with the unlesioned side or control animals (Fig. 5), a significant reduction (t test, $p < 0.0001$). In addition to reduction of MC afferents going to the main olfactory bulb, a pronounced reduction of the SBA positive fibers of the extrabulbar olfactory projection (Hofman and Meyer, 1991, 1992) was always apparent on the lesioned side (not shown).

Surprisingly, strong SBA staining was never completely absent on the lesioned side, even when

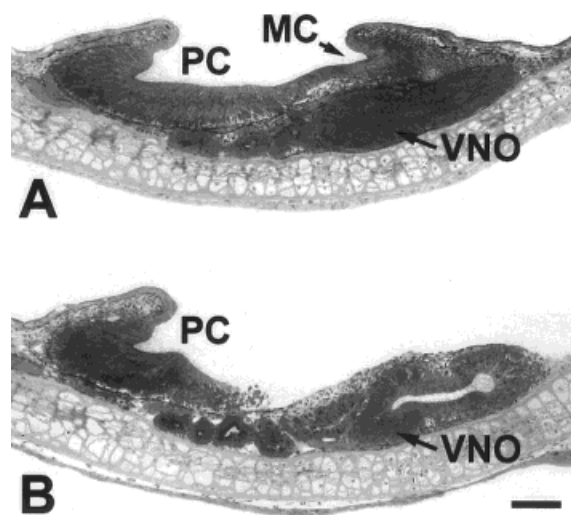


Figure 3 Parasagittal 1- μ m sections of the olfactory epithelia on the unlesioned (A) and lesioned (B) side of a stage 57 animal, fixed immediately after surgery. The MC is entirely absent on the lesioned side. There is also some damage to the PC epithelium, but the VNO is intact. Scale bar = 100 μ m.

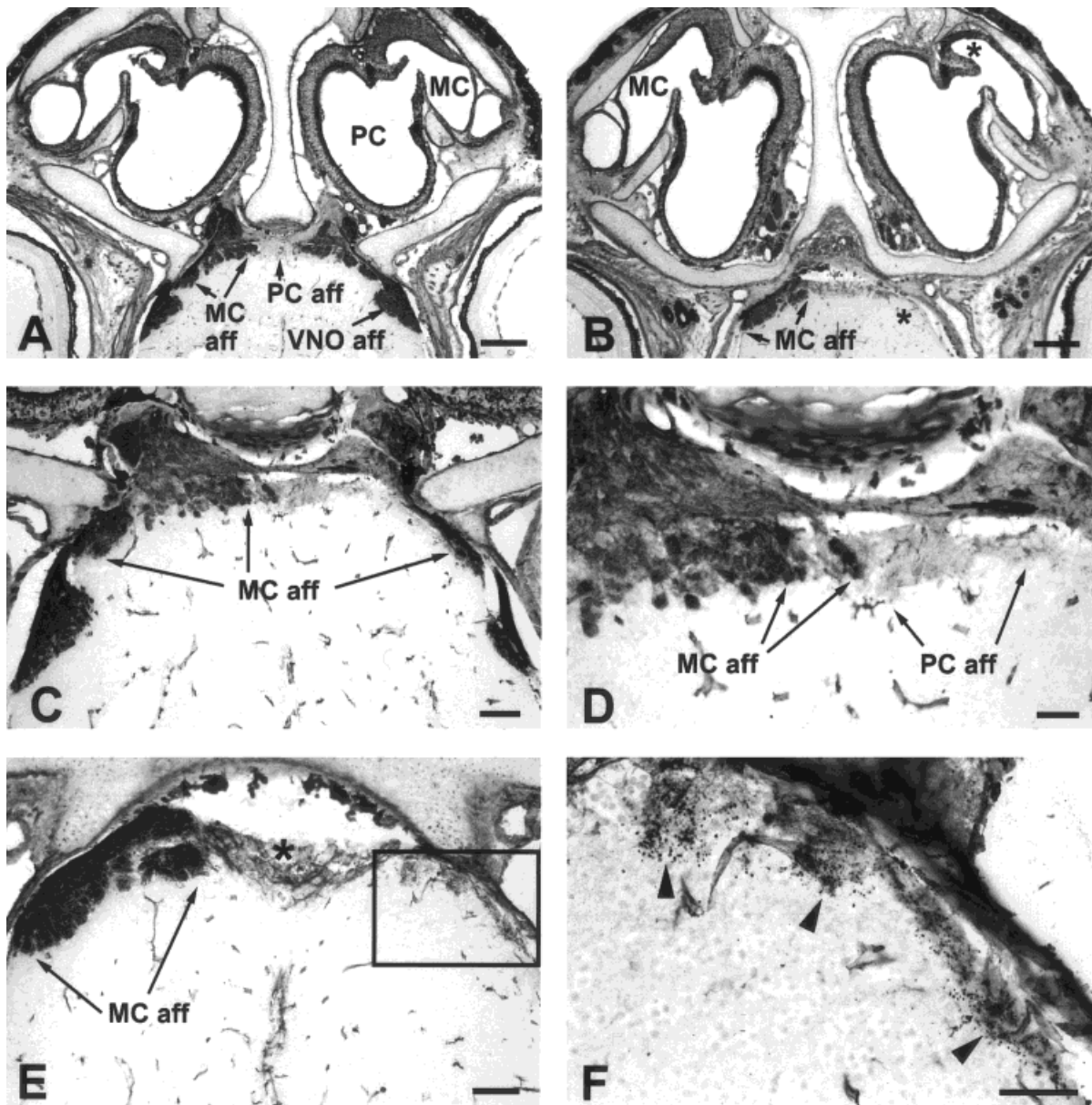


Figure 4 SBA stain of the olfactory system. Horizontal sections, anterior at top. (A) Control animal. Note valve separating PC and MC. SBA strongly labels MC and VNO afferents in the olfactory nerve and bulb, but only weakly labels PC afferents. (B) Lesioned animal. The MC epithelium is greatly reduced in thickness on the lesioned (right) side (asterisk). MC afferents are absent on the lesioned side of the olfactory bulb (asterisk). (C) Lesioned animal. MC afferents enter the olfactory bulb far laterally on the right (lesioned) side, but are absent more medially. The olfactory bulb of this animal is reconstructed in Figure 6(B). SBA-stained structures scattered throughout the brain are blood vessels. (D) Higher magnification of (C), to show PC afferents innervating olfactory bulb on the lesioned side, in the region occupied by MC afferents on the unlesioned side. (E) Lesioned animal. Note absence of MC afferents on the lesioned (right) side. Asterisk indicates SBA staining of pia mater, not olfactory afferents. (F) Higher magnification of boxed area in (E), to show punctate SBA staining in region that would normally receive MC afferents (arrowheads). Scale bars in (A,B) = 250 μm , in (C,E) = 100 μm , in (D,F) = 50 μm .

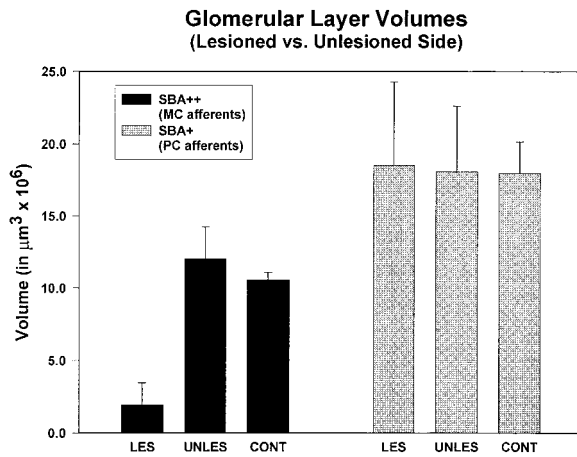


Figure 5 Comparison of volume of glomerular layer on lesioned and unlesioned sides. PC afferent regions of the glomerular layer show no significant difference from the unlesioned side or controls. This argues against any effect of the slight damage to the PC epithelium in the surgery. It also argues against any change in the number of PC afferents crossing between the unlesioned and unlesioned sides. Not surprisingly, MC afferent regions are significantly reduced on the lesioned side, compared both with the unlesioned side and control animals. Error bars indicate 1 S.D.

no morphologic MC was apparent. In most animals, such staining occurred in reduced regions, but these regions were still strongly labeled [Fig. 4(C)]. In some animals, however, a much looser tangle of individual heavily stained fibers could be seen in the ventral bulb. Because the entire area was digitized regardless of the density of stained fibers, volume measurements (Fig. 5) and reconstructions (Fig. 6) do not fully reflect the extent of afferent reduction. In a number of cases, a punctate SBA staining pattern was apparent in the olfactory bulb of the lesioned side, in the proper location for MC afferents [Fig. 4(F)]. Because no labeled fibers could be seen, these areas were not included in the volume estimates. The significance of such staining is discussed below.

By contrast with the MC region, the region of the glomerular layer receiving PC afferents appeared almost identical on lesioned and unlesioned sides (Fig. 6). Volume estimates also showed no significant differences between the lesioned side and either the unlesioned side or control animals (Fig. 5). In some animals it did appear that the PC afferents extended slightly further ventrolaterally on the lesioned than on the unlesioned side, into the dorsal part of the region normally occupied by the MC

projection [Figs. 4(C,D) and 6]. Nevertheless, in animals with the greatest reduction in MC afferents, a gap was often left between strongly staining and weakly staining regions [Fig. 6(C)].

Of animals operated on at younger stages, only one (operated on at stage 51) showed notable reduction of the MC. The projection pattern of PC and MC afferents in this animal was similar to that of animals operated on at older stages.

[^3H]Thymidine Labeling

To serve as an internal control for the position of afferent fibers with respect to the normal dorsoventral boundary of the bulb, animals were injected with [^3H]thymidine at stage 46, well before the surgery. Previous work had shown that all cells labeled by injections at this stage are located in the ventral olfactory bulb, and these authors suspected that such labeling is restricted to cells associated with the MC projection (Fritz et al., 1996). Comparison of SBA-labeled sections with adjacent sections processed for autoradiography supports this hypothesis (Fig. 7). In control animals and on the unlesioned side of experimental animals, only cells deep to regions of the glomerular layer strongly labeled by SBA were labeled by [^3H]thymidine [an exception being cells in the so-called "lateral quadrant" of Fritz et al. (1996)].

Examination of lesioned animals revealed no apparent differences in [^3H]thymidine labeling between the lesioned and unlesioned sides. Even in the absence of MC afferents, the ventral bulb contained many labeled cells (Fig. 7). Most important, at the somewhat crude level of resolution provided by this technique, there was no evidence that PC afferents extended unusually far down into the region of [^3H]thymidine-labeled cells in the ventral bulb.

DISCUSSION

A number of developmental mechanisms have been proposed to explain the specificity of mapping in the primary olfactory projection (Vassar et al., 1994; Ressler et al., 1994). In one model, olfactory receptor neurons project randomly to the olfactory bulb, and the receptor type expressed by the neuron depends on retrograde influences from the bulb. This model has been discredited by results showing that odorant receptor expression in proper regions of the epithelium occurs prior to the time olfactory axons reach the bulb (Strotman et al., 1995) and can occur even in the absence of an olfactory bulb (Sullivan

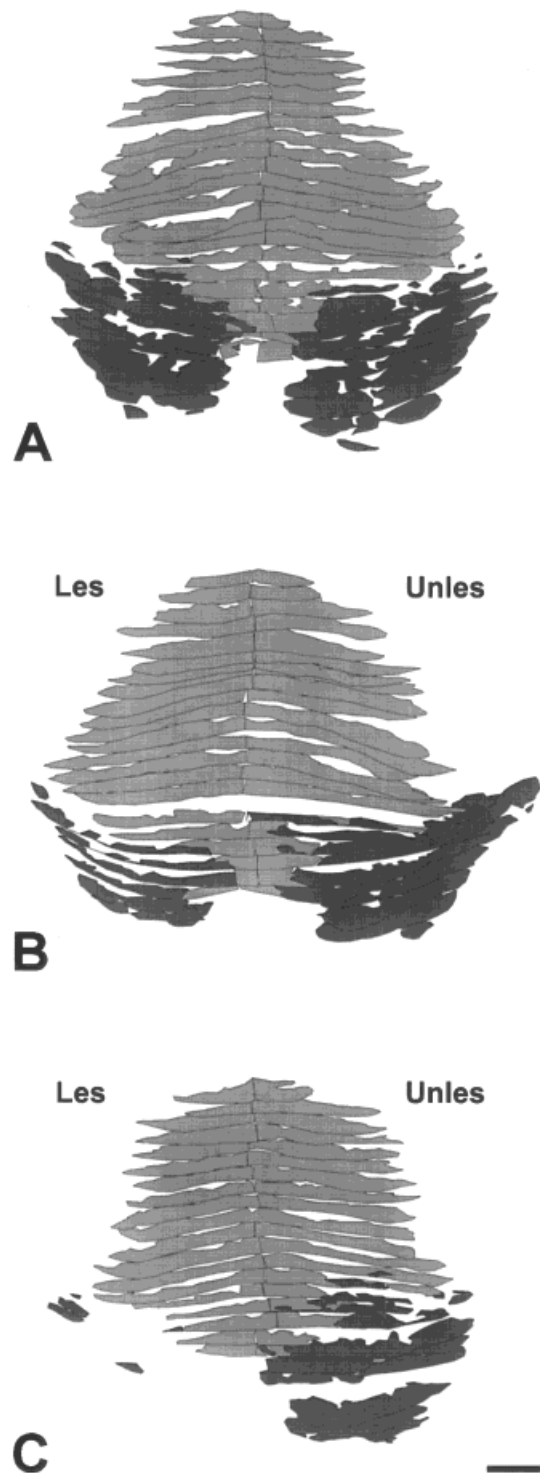


Figure 6 Reconstructions of glomerular layer of olfactory bulb. Anterior view, looking down at a 30° angle. Dorsal at top. (A) Control animal. MC afferent regions are located ventrolaterally on either side, the PC afferent region dorsomedially. Note fusion of left and right bulbs in the PC afferent area. (B) Lesioned animal. The MC afferent area is moderately reduced on the lesioned side.

et al., 1995). Alternatively, it has been suggested that a system of positional markers exists within the olfactory bulb. Axons of olfactory neurons expressing a given odorant receptor would be guided to the proper location within the bulb by a receptor that recognizes these markers, perhaps the odorant receptor itself (Vassar et al., 1994; Ressler et al., 1994; Singer et al., 1995). Finally, it has been suggested that an activity-dependent mechanism could be involved, based on the coactivation of olfactory neurons expressing a given odorant receptor. A combination of the latter two mechanisms is also possible: for example, a coarse map established by positional markers, later sharpened by activity-dependent mechanisms. There is good evidence for such a dual mechanism in the well-studied retinotectal system [reviewed by Kaprielian and Patterson (1994)].

The present study has shown that removal of the MC epithelium in *Xenopus* tadpoles has little effect on the postmetamorphic projection pattern of axons from the PC, even though PC and MC axons both project to the ventral olfactory bulb at the time of surgery. While in some cases a slight ventral extension of PC afferents did seem to occur on the lesioned side, this could be explained if there is normally a slight overlap in MC and PC projections in the region, which is masked by heavy SBA labeling of MC afferents. Regardless of the explanation of this anomaly, it is clear that interactions between PC and MC receptor neurons are not necessary for establishing an almost normal olfactory projection pattern. These results argue against a model in which activity-dependent mechanisms are the primary determinant of the projection pattern, because such mechanisms can only explain positive or negative assortment within a population of axons. Instead, these results support a model in which extrinsic cues within the olfactory bulb act to guide olfactory axons to their proper location. Within such a general model, moreover, one can distinguish models involving absolute cues (e.g., only neurons expressing receptor *X* can bind in a given region) from those involving relative cues (e.g., neurons

Note also the slight apparent ventral extension of PC afferents on this side. (C) Lesioned animal. MC afferents are greatly reduced on the lesioned side. Nevertheless, there is little difference apparent between PC afferent regions on lesioned and unlesioned sides. Dark gray = MC afferent region; light gray = PC afferent region. Scale bar = 200 μ m.

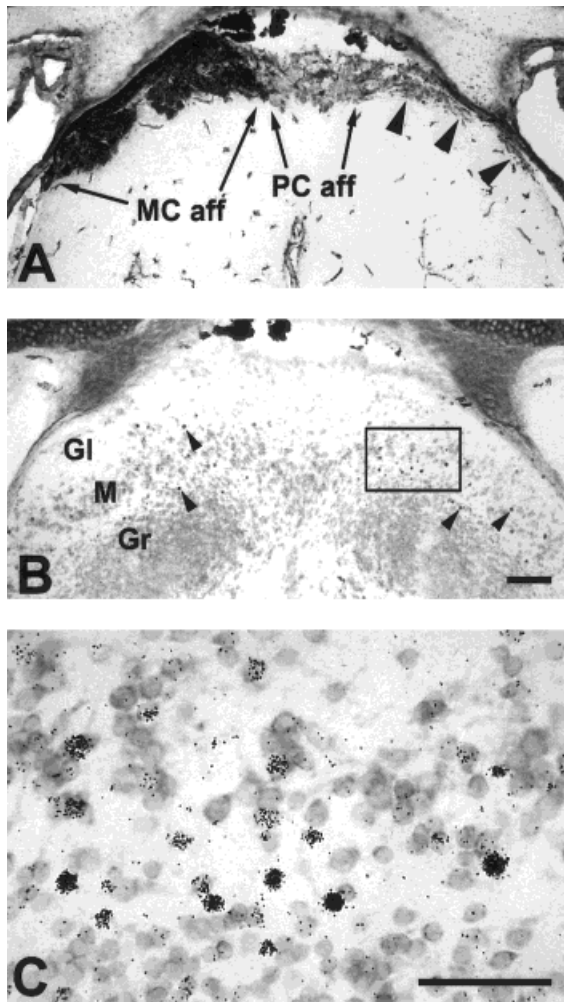


Figure 7 $[^3\text{H}]$ Thymidine labeling of ventral olfactory bulb. Horizontal sections, anterior at top. (A) SBA-labeled section. MC afferents are labeled on unlesioned side, absent on lesioned (right) side. Arrowheads indicate region of punctate SBA staining on the lesioned side. PC afferents are present medially; this is their most ventral extent. (B) Adjacent section processed for autoradiography. Note $[^3\text{H}]$ -labeled cells scattered through mitral cell (M) and granule cell (Gr) layers of both sides (several are indicated by arrowheads). Note absence of labeled cells medially. Very few cells (labeled or unlabeled) are present in the glomerular (GI) layer. (C) Higher magnification of boxed area in (B) to show silver grains. Scale bar for (A,B) = 100 μm ; for (C) = 50 μm .

expressing receptor *X* bind more strongly than those expressing receptor *Y*). The present results support an absolute model, since even in the absence of MC axons, PC axons do not project to the ventral bulb. This contrasts with results described for the fish optic tectum, where ingrowing ganglion cell axons appear to drive older terminals caudally (Wilm and Fritsch, 1992).

Several caveats to this conclusion are necessary, however, since the very features that make the *Xenopus* system amenable to experimental analysis may limit the generality of conclusions drawn from it. First, it is not clear to what degree the PC and MC are good models for olfactory neuron subclasses in other taxa. The facts that the epithelia are separate in the periphery and that the projections are separate in the nerve and bulb of the adult stand in contrast to the situation in most vertebrates. As noted in the introduction, the *Xenopus* system is most similar to the four domains of receptor expression in rodents, which also appear to map to distinct regions of the olfactory bulb (see Astic and Saucier, 1986; Astic et al., 1987; Schoenfeld et al., 1994; Vassar et al., 1994). Another analogy would be the projection of olfactory and vomeronasal neurons to the main and accessory olfactory bulbs, respectively, in all tetrapods. It is certainly quite possible that different mechanisms are involved in such coarse topologic mapping and the fine mapping to individual glomeruli.

Second, some important features of the biology of olfactory metamorphosis in *Xenopus* must be taken into account (Fig. 1). Previous work has demonstrated that the entire dorsal olfactory bulb is first generated during metamorphosis, although additional neurons are added throughout the bulb (Fritz et al., 1996). The present study confirms these results, and comparison with the pattern of SBA labeling shows that the newly formed dorsal bulb is the same region to which PC olfactory neurons project. Moreover, evidence from morphologic changes (Key, 1986; Weiss, 1986, 1987) and the presence of apoptotic bodies (Reiss, unpublished observation) suggests that the PC neurons which project to the ventral olfactory bulb in the larva die during metamorphosis, to be replaced by PC neurons projecting to the dorsal bulb in the juvenile. If this is true, then newly formed PC axons could be targeted to new dorsal olfactory bulb (perhaps even inducing it) while MC axons could be targeted to old ventral bulb. The mapping problem remains the same either way, but the context is different.

Finally, there is the question of the time course of degeneration of MC axon terminals after the surgery. The frequent presence of punctate SBA labeling on the lesioned side in the region occupied by MC afferents on the unlesioned side [Fig. 4(F)] suggests that the cell membrane of the MC axon terminals may be retained well after the time of surgery. Alternatively, the punctate staining seen in the ventral bulb could represent the membrane of degenerating PC axon terminals. Although it ap-

pears unlikely, if this membrane were located extracellularly it might continue to interact both with cells of the ventral bulb and any newly invading PC axons.

To conclude, this study provides evidence that the organization of the primary olfactory projection is determined by extrinsic cues within the olfactory bulb, and not by interactions among subclasses of olfactory afferents. Nevertheless, further work is clearly needed to determine the limits of the applicability of this finding, as well as to elucidate the molecular basis of such cues and their receptors.

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