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Rhinotopy is Disrupted During the Re-innervation of the Olfactory Bulb that Follows Transection of the Olfactory Nerve

Marc D. Christensen[1,2], Eric H. Holbrook[3,4], Richard M. Costanzo[3,4] and James E. Schwob[1]

[1]Department of Cell and Developmental Biology and [2]Department of Neurosurgery, SUNY Upstate Medical University, Syracuse, NY 13210 and [3]Department of Physiology and [4]Department of Otolaryngology–Head and Neck Surgery, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298, USA

Correspondence to be sent to: James E. Schwob, Department of Anatomy and Cellular Biology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111, USA. e-mail: jim.schwob@tufts.edu

Abstract

Re-innervation of the olfactory bulb was investigated after transection of the olfactory nerve using monoclonal antibody RB-8 to assess whether rhinotopy of the primary olfactory projection is restored. In normal animals RB-8 heavily stains the axons, and their terminals, that project from the ventrolateral olfactory epithelium onto glomeruli of the ventrolateral bulb (termed RB-8$^+$). In contrast, axons from dorsomedial epithelium are unlabeled (RB-8$^-$) and normally terminate in the dorsomedial bulb. Sprague–Dawley rats underwent unilateral olfactory nerve transection and survived for 6 weeks prior to perfusion, sectioning and immunostaining with RB-8. Nerve lesion does not shift the position of the boundary between RB-8$^+$ and RB-8$^-$ regions of the epithelium. However, following transection and bulb re-innervation, the distribution of RB-8$^+$ and RB-8$^-$ axons is markedly abnormal. First, in all 10 experimental animals RB-8$^-$ axons displace RB-8$^+$ axons from anterior glomeruli. Furthermore, the usual target of the RB-8$^-$ fibers, i.e. the dorsomedial bulb at more posterior levels of the bulb, remains denervated, judging by the lack of staining with antibodies that label axons derived from all epithelial zones. Finally, RB-8$^+$ fibers invade foreign territory in the dorsolateral bulb on the lesioned side in some cases. The shifts in terminal territory in the bulb after transection contrast with the restoration of the normal zonal patterning of the projection after recovery from methyl bromide lesion, but is consistent with reports of mistargeting by a receptor-defined subset of neurons after transection.

Introduction

It is a cornerstone of our understanding of olfactory biology that the primary olfactory projection, consisting of the olfactory epithelium and its axonal input to the olfactory bulb via the olfactory nerve, can regenerate after injury, restoring anatomical connectivity and some level of function [reviewed by Graziadei and Monti Graziadei (Graziadei and Monti Graziadei, 1985) and Costanzo (Costanzo, 1991)]. That the system can recover both anatomically and functionally after injury to the epithelium or the nerve has been demonstrated by many laboratories, including both of ours (Schwob *et al.*, 1995, 1999; Yee and Costanzo, 1995, 1998; Koster and Costanzo, 1996). Nonetheless, olfactory dysfunction develops and persists in several clinical settings, including patients who have suffered significant head trauma (Doty, 1979). Is the failure to recover full function due to deficits in reconstitution of the epithelium or in regeneration of the projection onto the bulb, or both? The key to understanding the discrepancy between anatomical recovery after lesion and persistent functional deficits is likely to reflect the highly organized nature of the projection of the olfactory epithelium onto the bulb in normal individuals and the degree to which the projection remains disorganized after regeneration of the periphery.

At the first level of organization the epithelium's projection onto the bulb is roughly zonal, relating broad areas of the receptor surface to broad areas of the glomerular sheet: dorsal epithelium projecting to dorsal bulb, lateral epithelium projecting to lateral bulb, etc. (Land and Shepherd, 1974; Costanzo and O'Connell, 1978, 1980; Schoenfeld *et al.*, 1994). The zone-to-zone mapping has been termed rhinotopy (Schoenfeld *et al.*, 1994). The results obtained with conventional neuroanatomical techniques, which first demonstrated the zonal map, have been extended by studies of the molecular phenotypes of olfactory neurons and their axons. The latter have demonstrated that the epithelium can be divided into sharply bounded zones on the basis of the differential expression of odorant receptor genes and cell surface molecules that are concentrated on olfactory axons (Mori *et al.*, 1985; Schwob and Gottlieb, 1986, 1988; Schwarting *et al.*, 1992; Ressler *et al.*, 1993; Strotmann *et al.*, 1994; Alenius and Bohm, 1997; Yoshihara

et al., 1997). The existence of sharply bounded zones was first demonstrated using monoclonal antibodies 2B5 (Mori *et al.*, 1985; Yoshihara *et al.*, 1997) and RB-8 (Schwob and Gottlieb, 1986, 1988), which differentially label the dorsomedial versus ventrolateral olfactory epithelium and the axonal projection onto the bulb. Subsequent studies demonstrated that the cell surface protein recognized by 2B5 and RB-8 is a member of the Ig superfamily of cell adhesion molecules which has been given several names: 'OCAM' (mouse) (Yoshihara *et al.*, 1997), 'RNCAM' (mouse) (Alenius and Bohm. 1997), 'NCAM2' (human) (Paoloni-Giacobino *et al.*, 1997) and, finally, 'mamFas II', to designate it as the mammalian homolog of *Drosophila* cell adhesion molecule fasciclin II, to which it is more closely related than is NCAM on sequence and protein structure homologies (rat) (H. Fang and J.E. Schwob, unpublished results).

OCAM/mamFas II mRNA and protein are expressed at low or undetectable levels in the dorsomedial part of the olfactory epithelium and the axons elaborated by neurons in this part of the epithelium, respectively (Mori *et al.*, 1985; Schwob and Gottlieb, 1986, 1988; Alenius and Bohm, 1997; Yoshihara *et al.*, 1997). The OCAM/mamFas II$^-$ area of the epithelium closely corresponds to zone I, the most dorsomedial of the four zones defined by odorant receptor expression [we use the terminology of Buck (Ressler *et al.*, 1993) and Breer (Strotmann *et al.*, 1994) when designating epithelial zones] (Alenius and Bohm, 1997; Yoshihara *et al.*, 1997) (C.L. Iwema, H. Fang and J.E. Schwob, unpublished observations) (Figure 1). In contrast, OCAM/mamFas II mRNA and protein are expressed at high levels in ventral and lateral epithelium, which encompasses odorant receptor-defined zones II–IV (Ressler *et al.*, 1993; Strotmann *et al.*, 1994; Alenius and Bohm, 1997; Yoshihara *et al.*, 1997; C.L. Iwema, H. Fang and J.E. Schwob, unpublished observations).

The differential pattern of gene expression in the epithelium translates to differential levels of protein on olfactory axons that project from the periphery onto the bulb, including their termination in the glomerular layer (as summarized in Figure 1) (Schwob and Gottlieb, 1986, 1988). Thus, the monoclonal antibody RB-8, which binds to both the transmembrane and GPI-linked forms of OCAM/mamFas II, stains heavily those axons and their terminals in the glomerular layer of the bulb that originate from neurons in the ventral lateral part (odorant receptor-defined zones II–IV) of the epithelium. Thus, the glomeruli in the ventral and lateral bulb, which are innervated by ventrolaterally derived olfactory axons, are replete with fibers that densely stain with the anti-OCAM/mamFas II antibody RB-8, while the terminal axons in glomeruli in the dorsomedial bulb, which are innervated by axons from dorsolateral epithelium,

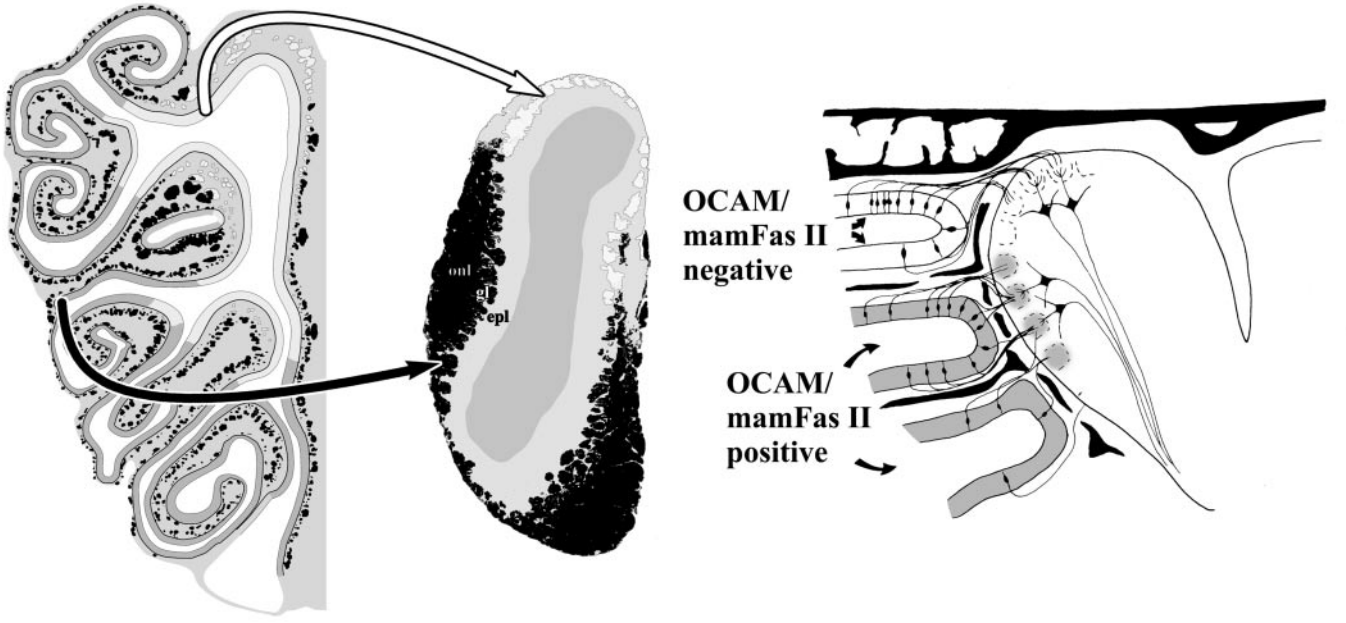

Figure 1 Staining with the anti-OCAM/mamFas II monoclonal antibody RB-8 can be used to distinguish the axonal projections of dorsomedial versus ventrolateral olfactory epithelium onto the olfactory bulb. The drawings of the olfactory epithelium **(left)** and olfactory bulb **(middle)** are abstractions of the distribution of labeling in actual sections immunohistochemically stained with RB-8. Dorsal is up and the midline is to the right. The density of shading in the olfactory epithelium (left) indicates whether neurons in that area express OCAM/mamFas II at high levels (moderately shaded ventrolateral epithelium) or at undetectable levels (lightly shaded dorsomedial epithelium). Fascicles of the olfactory nerve that conduct axons from the ventrolateral epithelium are black, while those that conduct axons from dorsomedial epithelium are light. The density of shading in the glomerular layer of the bulb (middle) correlates with the intensity of RB-8 labeling. The origin of the innervating axons is indicated by the curved arrows. The division of the olfactory projection into OCAM/mamFas II-positive and OCAM/mamFas II-negative components is schematized in the image to the right.

are left largely unstained (Figure 1). Hence, RB-8 is a valuable marker for distinguishing the projections of the ventrolateral versus dorsomedial olfactory epithelium and can be used to study the zonal organization of the primary olfactory projection after recovery from lesions.

Previous studies from one of our laboratories focused on the time course and extent of re-innervation of the bulb following the lesion caused by inhalation of the selectively olfactotoxic gas methyl bromide (MeBr) (Schwob *et al.*, 1995, 1999). The lesion destroys neurons and sustentacular cells in >95% of the epithelium but spares a large proportion of the basal cell population. As a consequence, the epithelium recovers to near normal after lesion, as judged on morphological and phenotypic criteria. In addition, the zonality, defined by either OCAM/mamFas II or odorant receptor expression, re-emerges after lesion (Schwob and Youngentob, 1991; Iwema *et al.*, 1997) (J.E. Schwob, H. Fang, J.M. Hershberger, J.A. Hamlin, C.L. Iwema and S.L. Youngentob, manuscript in preparation). Indeed, the boundary defined by RB-8 staining in the reconstituted epithelium is indistinguishable from that in normal epithelium (Schwob and Youngentob, 1991) (J.E. Schwob, H. Fang, J.A. Hamlin and C.L. Iwema, manuscript in preparation). Furthermore, the zonal projection onto the olfactory bulb as defined by RB-8 labeling or retrograde transport of fluorescent latex microspheres is restored after reconstitution of the directly damaged epithelium (Schwob and Youngentob, 1991) (J.E. Schwob, H. Fang, J.M. Hershberger, J.A. Hamlin, C.L. Iwema and S.L. Youngentob, manuscript in preparation).

In contrast to MeBr lesion, knife-cut transection of the olfactory nerve spares the epithelium any type of direct damage, but does mechanically disrupt the course of the nerve on the intracranial side of the cribriform plate. A degree of bulbar re-innervation and recovery of function are achieved (Graziadei and Monti Graziadei, 1980; Yee and Costanzo, 1995, 1998; Koster and Costanzo, 1996; Costanzo, 2000). However, the ability of the olfactory nerve to re-establish appropriate target innervation at a zonal or rhinotopic level during regeneration following nerve injury is currently not known and is the subject of this investigation, using immunostaining with RB-8 to identify dorsomedial versus ventrolateral components of the regenerated projection.

Materials and methods

Animals

Ten adult male Sprague–Dawley rats weighing 250–300 g were subjected to traumatic unilateral olfactory nerve transection. The rats were anesthetized by i.p. injection of sodium pentobarbital at a dose of 70 mg/kg. The dorsal surface of the olfactory bulb was exposed via craniotomy. A small Teflon blade was used to cut all olfactory fibers on the intracranial side of the cribriform plate according to an established procedure (Costanzo, 2000). The nerve was transected by inserting the Teflon blade down along the medial and ventral surface of the olfactory bulb, across the anterior and ventral surface and then along the lateral surface of the bulb. Deflection of the bulb with a small stream of saline solution improved visualization and access for blade insertion. The contralateral bulb was left intact and served as an internal control. After completion of the nerve transection, animals were allowed a 6 week recovery prior to death, at which time the animals were injected with a lethal dose of sodium pentobarbital and killed by intracardiac perfusion with a fixative solution containing periodate-lysine, 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). All animal use protocols were approved by the Institutional Animal Care and Use Committees at SUNY Upstate Medical University and VCU–MCV, respectively.

Tissue processing

After additional fixation by immersion in the perfusate for 4–6 h, the skull was removed from the surface of the nose and bulbs. The block was decalcified by immersion in saturated EDTA for 5 days, cryoprotected, frozen and cryosectioned at a thickness of 8 μm. Sections were stained with one of the following antibodies: the monoclonal anti-OCAM/mamFas II antibody designated RB-8 (Schwob and Gottlieb, 1986, 1988), polyclonal anti-NCAM antiserum (a gift of Dr Jonathan Covault) (Covault and Sanes, 1986) and a mouse monoclonal anti-GAP-43 antibody, designated 10E8 (Meiri *et al.*, 1991). Bound primary antibody was visualized using the avidin–biotinylated horseradish peroxidase technique according to standard labeling protocols that are described in detail elsewhere (Schwob *et al.*, 1995, 1999).

Densitometric analysis

RB-8 and NCAM stained sections from the anterior olfactory bulb were analyzed using the image analysis package IPLab Spectrum v.3.2 (Scanalytics, Vienna, VA). Mosaics of the bulbs were assembled from tiled images collected at 40× total magnification using a Hamamatsu CCD. Staining density in the glomerular layer of the anteromedial olfactory bulb was assessed by extracting the density along a 40 μm wide bar drawn through those glomeruli that were discrepant in their labeling on the transected side as compared with the intact side. Care was taken to ensure that the same region of the glomerular layer was being assessed on the two sides. Alternative measures, such as sum of pixel intensity or area occupied by pixels that reach a particular criterion, are a less satisfactory way of determining the source of the axons innervating the assayed glomeruli. Both of the latter measures could be confounded by whether a full complement of fibers was restored, which was not the issue under investigation.

Results

OCAM/mamFas II expression in the olfactory epithelium

The population of olfactory neurons recovered substantially by 6 weeks after transection of the olfactory nerve, consistent with previous results (Monti Graziadei and Graziadei, 1979; Yee and Costanzo, 1995, 1998; Koster and Costanzo, 1996; Costanzo, 2000). The epithelium on the transected side is nearly as thick as on the non-operated, contralateral control and contains numerous olfactory neurons as judged by NCAM labeling (Figure 2). Moreover, the pattern of differential OCAM/mamFas II expression is restored; immunostaining with RB-8 demonstrates that high levels of antigen are restricted to the ventrolateral epithelium and the axons originating from there. Thus, the neurons in the ventrolateral olfactory epithelium are labeled on the transected side at the same intensity of staining as on the control side and axon bundles of all sizes that derive from this region on the transected side are densely labeled along their trajectory from the basal lamina central-ward. Conversely, the epithelium lining the dorsal recess and tips of the turbinates is unstained on the transected side as on the control side and labeling in the axon bundles deep to the epithelium is markedly lower than in ventrolateral areas. The boundary between the RB-8$^+$ and RB-8$^-$ area is sharp on the lesioned side and is marked by a transition in the staining of the olfactory epithelium and of the small bundles of olfactory axons situated just deep to the basal lamina. Indeed, the boundary between high expressing and low expressing zones defined by RB-8 staining is located in the same position in the lesioned epithelium as compared with the contralateral, unoperated side (Figure 2). Alternative explanations for the difference in staining of dorsal- versus ventral-derived fascicles on the lesioned side can be excluded. For example, there is no evidence for relatively incomplete reconstitution dorsally as compared with ventrally, since the two zones are alike in their expression of NCAM and GAP-43 (Figures 2 and 3). These data indicate that neuron density is not differentially reduced in dorsal versus ventral epithelium, nor is the proportion of immature neurons markedly higher dorsally on the lesioned side.

Nonetheless, there is a subtle difference in the RB-8 staining pattern between the two sides. The labeling in the nerve fascicles derived from the dorsomedial epithelium, which is normally light but real, is weaker on the lesioned side than either control or unoperated normals (Figures 2

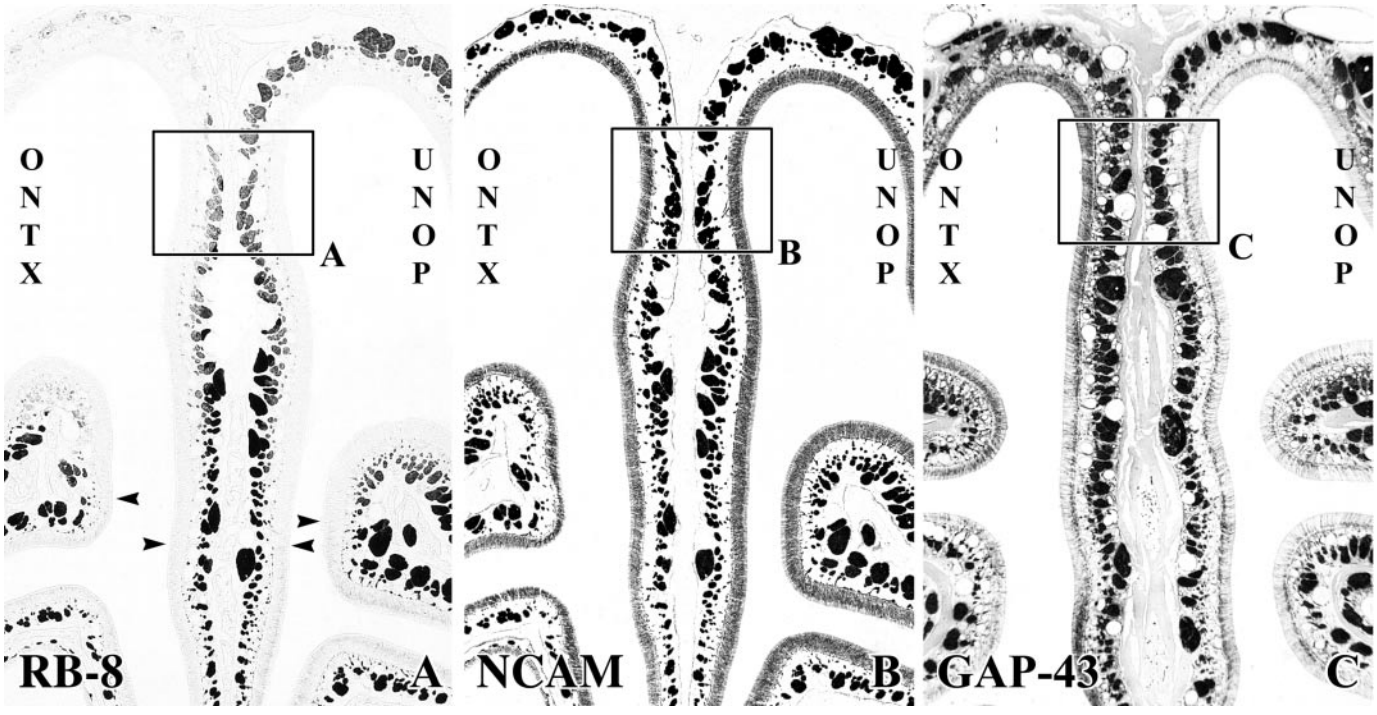

Figure 2 Differential labeling with RB-8 is restored after olfactory nerve transection. Dorsal is up in these coronal sections. ONTX indicates the side on which the olfactory nerve was transected 6 weeks prior to death and UNOP designates the unoperated side. **(A)** Staining with RB-8. Note the lower level of labeling in dorsal fascicles of the olfactory nerve as compared with ventral on both sides. The boundaries between high and low RB-8 labeling, defined by the density of stain in the small fascicles immediately deep to the epithelium, are indicated by the arrows and are in equivalent positions on the two sides. The differential between dorsal and ventral is more striking on the lesioned side due to a lower level of staining in the dorsal fascicles on the lesioned side, for example the fascicles immediately dorsal to the asterisk. **(B)** Staining with anti-NCAM antiserum. Section adjacent to the one illustrated in (A). Note the abundant population of neurons on the lesioned side and the equivalent density of NCAM labeling in the dorsal fascicles on both sides. **(C)** Staining with monoclonal antibody 10E8 directed against GAP-43. The section is displaced 0.5 mm from the level illustrated in (A) and (B). Note that the number of GAP-43$^+$ neurons is higher on the lesioned side. Boxed areas are shown at greater magnification in Figure 3.

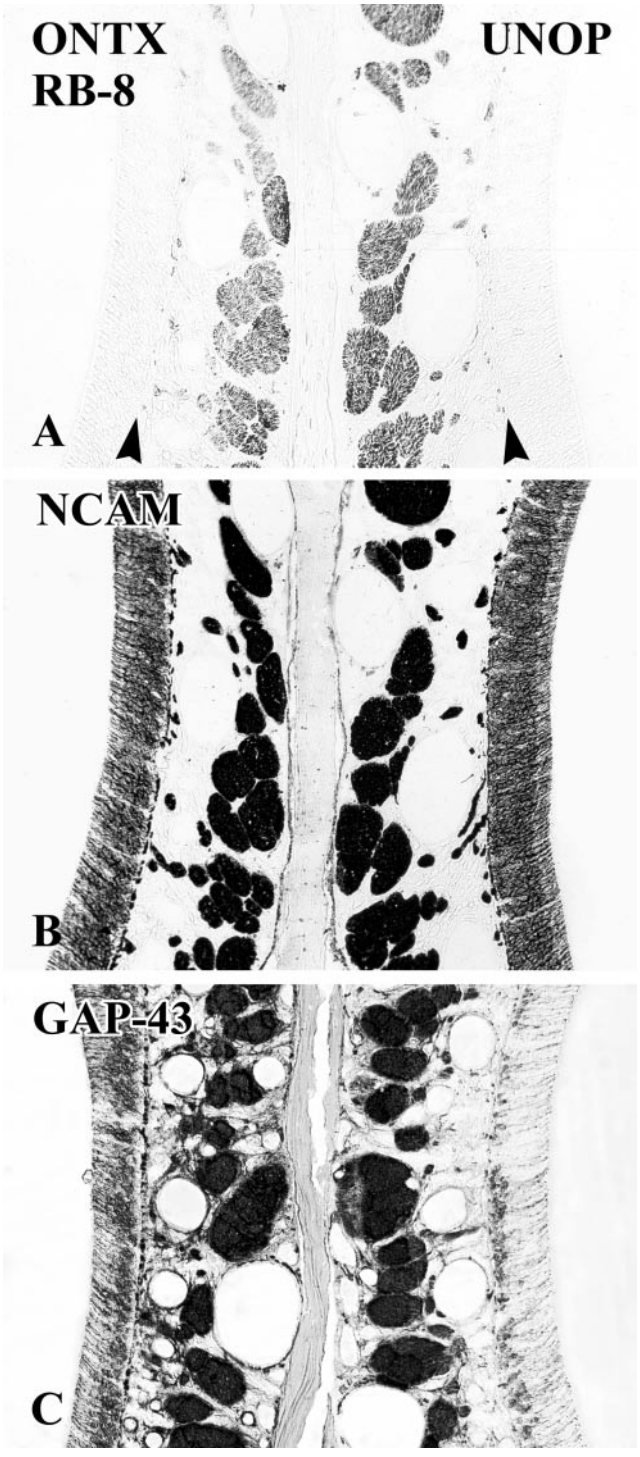

Figure 3 The dorsomedial RB-8$^-$ zone of the olfactory epithelium contains both mature and immature neurons. **(A)** Staining with RB-8. The small fascicles just below the basal lamina (indicated by the arrowheads) are very lightly labeled. The differential between lesioned (ONTX) and unoperated (UNOP) sides is perceptible, although less striking than in the lower power images. **(B)** Staining with anti-NCAM antiserum. Section adjacent to the one illustrated in (A). **(C)** Staining with monoclonal antibody 10E8 directed against GAP-43. Note the abundant population of NCAM$^+$ neurons on the lesioned side in (B), which is more numerous than the population of GAP-43$^+$ ones seen in (C). Hence, half or more of the neurons on the lesioned side are not GAP-43$^+$ and are, therefore, phenotypically mature.

and 3). The staining differential between the two sides of the experimental animals may have been due to the persistent decrease in total number of neurons as a consequence of the transection. An alternative explanation for the differential staining cannot be fully eliminated, namely that neurons on the transected side, of which a higher proportion are immature (Figures 2 and 3), express a lower level of OCAM/mamFas II. Against that possibility, other experiments suggest that the staining density of individual neurons labeled by *in situ* hybridization with OCAM/mamFas II cRNA probes doesn't differ between immature versus mature neurons (J.A. Hamlin, H. Fang and J.E. Schwob, unpublished observations). Specifically, the density of *in situ* hybridization label is equivalent when comparing neurons on the two sides in animals subject to unilateral bulbectomy; on the ablated side virtually all of the neurons were immature. Nonetheless, unilaterally bulbectomized rats also show less labeling of the dorsomedially derived nerve fascicles on ablated as compared with non-ablated sides (Schwob *et al.*, 1994) and the differential between the sides was even more pronounced than the present results. That the persistent reduction in neuron number on the operated side is also more pronounced in the bulbectomized animals is consistent with the suggestion that cell number is a possible explanation for the differential staining in dorsal fascicles. Alternatively, there may be a difference in the physical form of the antigen in immature axons derived from dorsomedial epithelium that interacts with the immunostaining protocol to generate the difference in labeling of dorsally derived fascicles between the two sides.

The pattern of staining in the epithelium after recovery from nerve transection indicates that RB-8 can be used as a marker for distinguishing axons that project from dorsomedial versus ventrolateral fibers and their terminations in the olfactory bulb in this experimental setting as in normal animals. In particular, none of our observations suggest that the level of staining of RB-8$^+$ fibers is reduced on the transected side to an extent that would render them unstained and indistinguishable from fibers that derive from the dorsomedial, i.e. RB-8$^-$, part of the epithelium. Thus, any glomerulus being innervated by olfactory axons that are poorly stained with RB-8 is receiving its projection from dorsomedial olfactory epithelium.

The projection of the epithelium onto the olfactory bulb after re-innervation

The pattern of innervation by fibers from the dorsomedial and ventrolateral epithelium to the olfactory bulb on the unlesioned side of transected animals is unaffected and indistinguishable from that seen in normal animals. It is worth reviewing the characteristics of the staining patterns with RB-8 and anti-NCAM antibodies as these are the measures that will be used to assess whether glomeruli are re-innervated and from which part of the epithelium they derive. On the unoperated side, as in normal animals, the

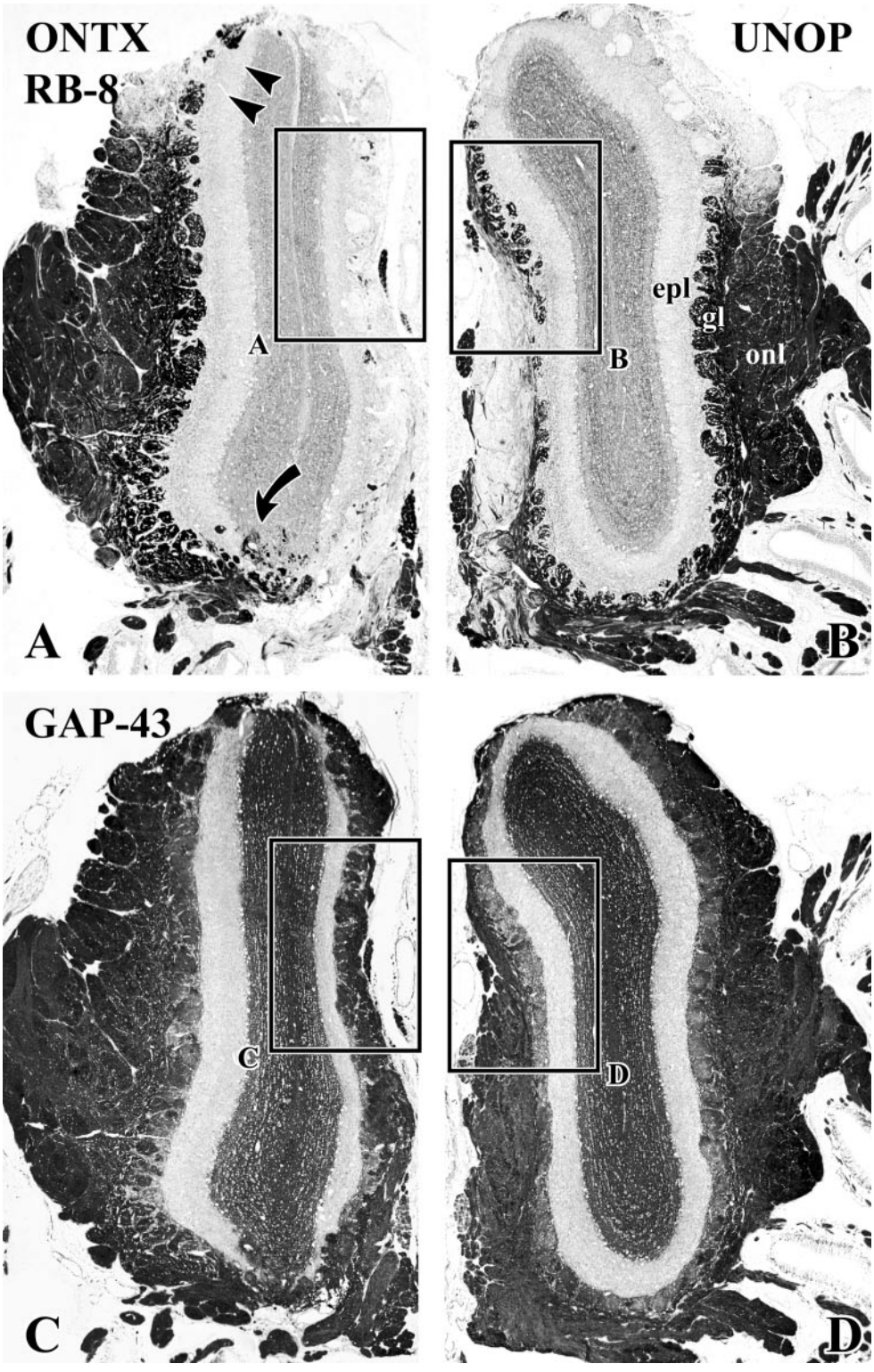

Figure 4 The axonal projection of the dorsomedial epithelium is mistargeted to the medial side of the anterior olfactory bulb. **(A)** RB-8 stained section of olfactory bulb, transected side (ONTX). **(B)** RB-8 stained section of olfactory bulb, unoperated side. Dorsal is up. The glomeruli along the medial aspect of the bulb on the transected side (ONTX) at this level are not stained with RB-8, in contrast to the unoperated side (UNOP). Boxed areas are shown at greater magnification in Figure 5. Arrowheads indicate an area of dorsolateral bulb that receives aberrant innervation by RB-8^+ fibers on the transected side. The curved arrow indicates an area where RB-8^+ fibers invade the parenchyma of the bulb, most likely as a consequence of direct damage during the transection procedure. **(C)** 10E8 (monoclonal anti-GAP-43 antibody) stained section of olfactory bulb, transected side. **(D)** 10E8 stained section of olfactory bulb, unoperated side. The section is adjacent to that illustrated in (A) and (B). Note that glomeruli of the medial bulb receive an increased projection of 10E8 stained, i.e. immature, fibers, but not more so than the lateral bulb which is appropriately re-innervated by RB-8^+ olfactory axons.

glomeruli in the ventrolateral part of the olfactory bulb are densely labeled with RB-8, while those in dorsomedial bulb are either unstained or less heavily stained than the neuropil of the external plexiform layer just deep to them, which is a convenient standard for comparing the density of staining between animals (Figure 4). Nonetheless, the dorsomedial glomeruli on the unlesioned side are innervated by olfactory axons, as documented by the higher density of NCAM staining of the glomerular neuropil. The greater density of axonal membrane per unit area due to tight packing of the olfactory axons may be responsible for the accentuation of NCAM staining in innervated glomeruli above the other layers of the bulb. Whatever the underlying reason, more intense NCAM labeling is a consistent feature and a useful criterion for identifying glomeruli that are well innervated by olfactory axons. On the unoperated side the boundary between the part of the glomerular layer receiving high expressing, i.e. RB-8^{+}, axons and that part innervated by low expressing, i.e. RB-8^{-}, axons is in a similar position to that in normals and has the same sharpness as the control bulb. In short, the unlesioned side of operated animals is not different from the pattern seen in unoperated animals and serves as an ideal, intra-animal control for the pattern observed 6 weeks after transection.

In contrast to the apparently normal labeling on the unoperated side, the RB-8 staining pattern on the transected side is markedly distorted. In experimental animals a substantial patch of glomeruli on the medial side of the bulb on the lesioned side is unstained with RB-8, unlike the corresponding ones in the unoperated bulb, which are heavily labeled (Figures 4 and 5). In the absence of RB-8 label, innervation of glomeruli can be demonstrated by dense staining on adjacent sections with either NCAM, which labels all olfactory axons from all zones, or GAP-43, which labels immature ones from all zones. By these criteria the RB-8 unlabeled glomeruli in the anteromedial bulb on the transected side are innervated by olfactory axons (Figures 4 and 5). The patch of glomeruli that contains mistargeted fibers begins at the anterior edge of the bulb and extends over ~2 mm, which is somewhat less than half of the bulb's length. Mistargeting of fibers from RB-8^{-}, i.e. dorsomedial, epithelium to this area of the bulb on the transected side is seen in all 10 experimental animals.

The visual impression of decreased RB-8 staining in glomeruli along the medial side of the anterior–mid bulb was confirmed by a densitometric comparison of the immunohistochemical staining with RB-8 versus anti-NCAM on the two sides (Table 1). The analysis was intended to establish that the visual difference between glomeruli in matched areas of the bulb was significant. Thus, we did not extend the comparison to the whole circumference of the bulb, nor its full anteroposterior extent. The data are expressed on a scale from 0 to 255. A lower number indicates a higher density of staining (i.e. less light transmittance), while a higher number indicates lighter

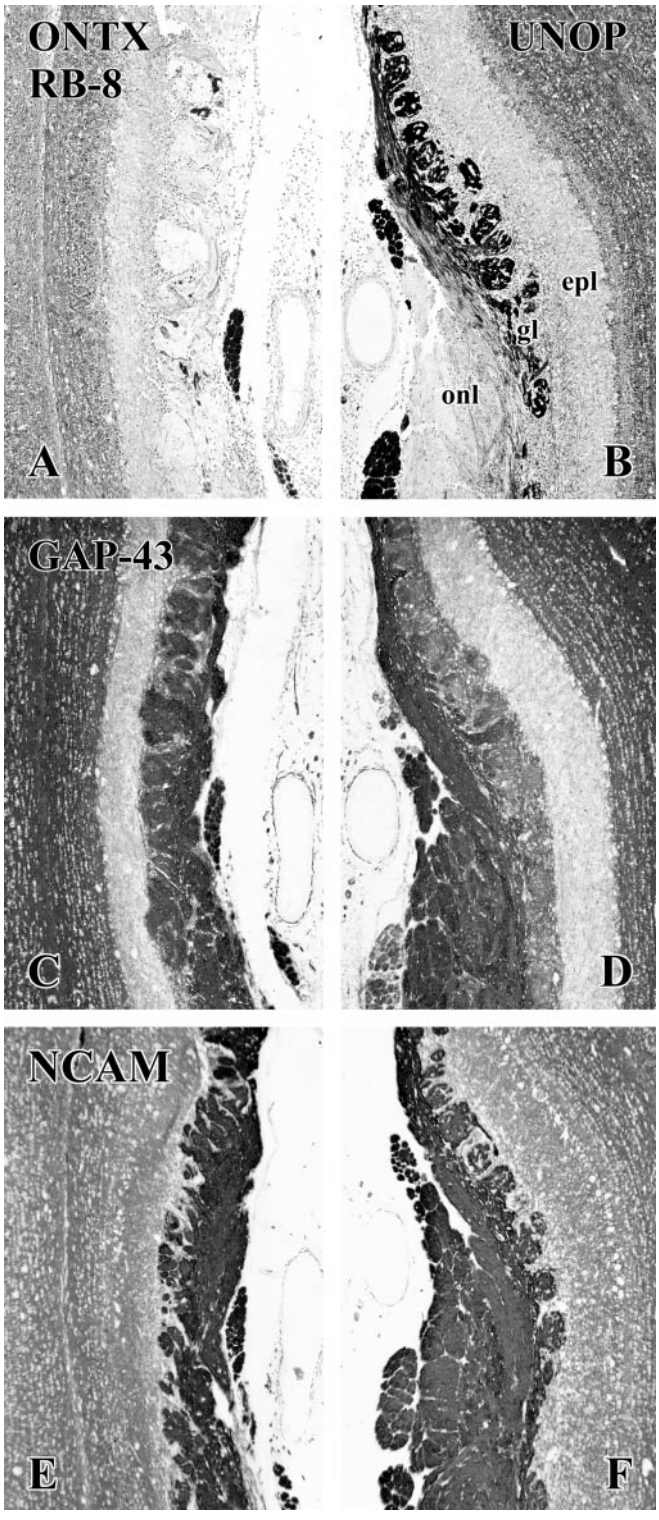

Figure 5 Higher magnification views of the sections illustrated in Figure 3. **(A,B)** RB-8 stained sections. **(C,D)** Monoclonal anti-GAP-43 stained sections adjacent to those in (A) and (B). **(E,F)** Anti-NCAM stained sections located 80 μm from (A)–(D). Note that the glomeruli on the lesioned side (ONTX) that lack RB-8 staining in (A) contain abundant GAP-43^{+} and NCAM^{+} axons in (C) and (E), respectively. Note also that the glomeruli are outlined by periglomerular cells. onl, olfactory nerve layer; gl, glomerular layer; epl, external plexiform layer.

Table 1 Densitometric comparison of RB-8 and anti-NCAM labeling of glomerular layer of anteromedial olfactory bulb

Case number	Sections analyzed	RB-8 transmittance			NCAM transmittance		
		ONX side	Unoperated side	Difference	ONX side	Unoperated side	Difference
ONX12	3	118.2	66.9	51.3	27.7	27.4	0.3
ONX17	4	78.7	41.7	37.0	19.3	21.8	–2.5
ONX18	4	96.0	57.8	38.2	27.8	24.7	3.1
ONX21	3	100.0	55.9	44.1	25.8	24.6	1.2
ONX22	3	80.0	33.1	46.9	19.8	23.2	1.2
ONX23	3	67.8	31.9	35.9	19.7	20.3	–0.6
Mean ± SD		90.1 ± 18.2	47.9 ± 14.4	42.2 ± 6.2	24.2 ± 3.9	23.9 ± 2.5	0.5 ± 1.9

Transmittance is reported on a scale of 0–255, with 255 signifying no light absorbance. Thus, higher numbers are an indication of less dense peroxidase-visualized immunostaining, e.g. glomeruli characterized by higher numbers show little immunostaining with RB-8 and are classified as RB-8$^-$, while those with lower numbers are more densely stained and are classified as RB-8$^+$. Statistical analysis was performed on the difference measures in order to minimize the slight variability due to differences in staining between cases.

staining (i.e. more light transmittance). The average density of RB-8 staining in the glomeruli in this region of the bulb was 90.1 ± 18.2 (mean ± SD) on the transected side versus 47.9 ± 14.4 on the unoperated side, i.e. the RB-8 staining in the glomeruli in the region under investigation is markedly less dense on the transected side, permitting greater light transmission. When the disparity in density between the two sides is compared on a case-by-case basis, the average decrease in density of RB-8 staining on the transected side is 42.2 ± 6.2. The difference is statistically significant, i.e. the density of RB-8 staining is significantly decreased, after recovery from transection (Student's $t = 16.7$, $P < 0.005$, $n = 6$). No difference in RB-8 staining was apparent when glomeruli in the ventral bulb were compared by densitometry on the two sides.

In contrast to the result with RB-8 and consistent with the light microscopic observations described above, densitometric analysis of NCAM staining of glomeruli in this region finds no difference between the two sides. The average density of NCAM staining was 24.4 ± 3.9 on the transected side versus 23.9 ± 2.5 on the control side, which is not a significant difference ($t = 0.65$, $P > 0.05$, $n = 6$). Thus, the densitometric analysis confirms that the glomeruli on the lesioned side that show a discrepant lack of labeling with RB-8 are indeed re-innervated, but with RB-8$^-$ axons. Furthermore, the glomeruli receiving the mistargeted fibers are re-innervated to the same apparent density as on the unoperated side.

Three other abnormalities were observed on the lesioned side. First, in some animals (2/10) RB-8$^+$ fibers invade glomeruli along the lateral side of the bulb on the transected side that are positioned further dorsal than the boundary between RB-8$^+$/RB-8$^-$ glomeruli on the control side or in bulbs from normal animals (Figure 4). Second, some RB-8$^+$ axons invade the deeper layers of the bulb in the region that is apposed to the cribriform plate (Figure 4). Third, the usual RB-8$^-$ territory in the dorsomedial zone of the posterior bulb is severely hypo-innervated or frankly not re-innervated on the transected side in all 10 of the lesioned animals (Figure 6). In this region of the bulb glomerular labeling with RB-8 does not exceed the density observed in the underlying external plexiform layer, but neither is there any NCAM staining that is denser than the deeper layers in most glomeruli in the posterodorsal bulb. The lack of dense NCAM labeling indicates that olfactory fibers are either absent from those glomeruli or extremely sparse. The question of innervation density of the posterior mediodorsal bulb was also addressed by performing immunohistochemistry with two additional antibodies that label axons from all zones of the epithelium, namely staining with anti-GAP-43 and anti-OMP. The density of anti-GAP-43 or anti-OMP label in the glomeruli of the posterior mediodorsal bulb, like that of anti-NCAM, is far below the level observed in the homologous glomeruli of the control bulb (data not shown), thus confirming the interpretation that the normal RB-8$^-$ territory at this level of the bulb is poorly re-innervated, if at all, in the experimental animals. Relatively sparse innervation of the posterior dorsomedial bulb has been observed previously in animals in which the nerve was transected (Koster and Costanzo, 1996).

Discussion

In the current investigation we have assessed re-innervation of the olfactory bulb by newly generated olfactory sensory neurons after traumatic olfactory nerve transection. Specifically, we used a combination of RB-8, anti-NCAM and anti-GAP-43 staining to assess the zonality and maturity of axons that project onto the bulb. We have demonstrated that the division of the epithelium into two fundamental zones is restored to normal after MeBr lesion, judged on the spatially restricted pattern of OCAM/mamFas II expression. The findings in the epithelium are

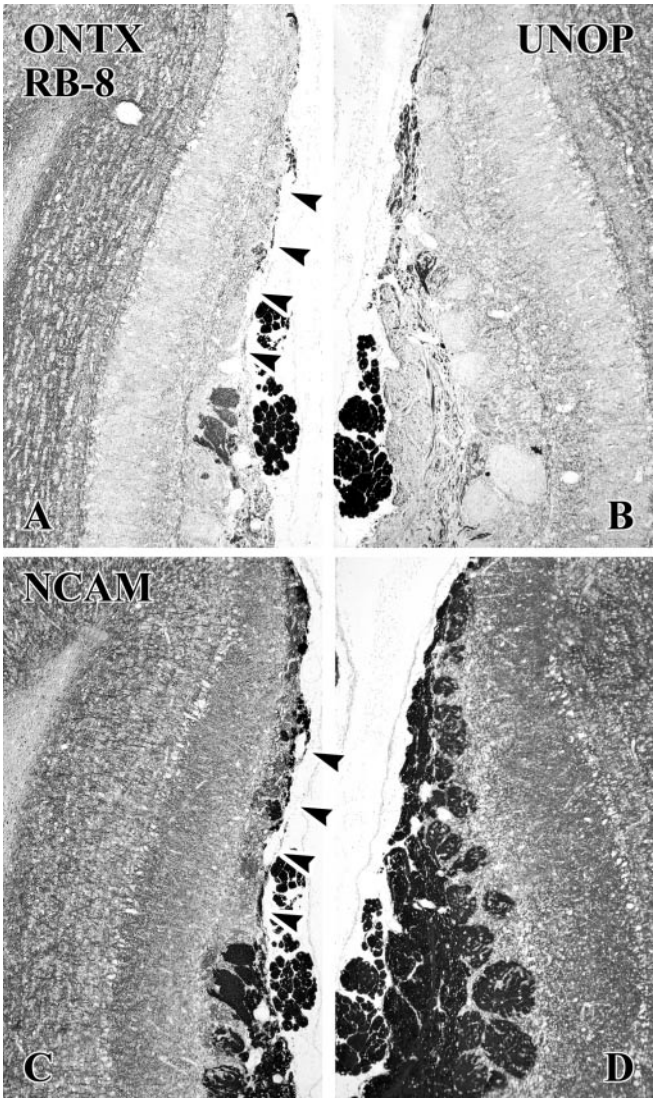

Figure 6 Posterodorsal olfactory bulb is poorly re-innervated, if at all. **(A,B)** RB-8 stained sections. **(C,D)** Anti-NCAM stained section adjacent to those in (A) and (B). Arrowheads designate a group of glomeruli that contain few $NCAM^+$ olfactory axons on the transected side (ONTX), as shown by their shrunken appearance relative to the unoperated side (UNOP) and the rough equivalence of staining density to that in the external plexiform layer. A few glomeruli on the transected side that are innervated by $RB\text{-}8^-$ axons are evident at the ventral margin of the photograph.

entirely analogous to published data indicating that ablation of the bulb, which permits less complete reconstitution of the epithelium than nerve transection due to absence of the target, does not disrupt the zonality of the epithelium with respect to either spatially restricted odorant receptor expression (Konzelman *et al.*, 1998) or OCAM/mamFas II expression (Schwob *et al.*, 1994). However, the results presented here demonstrate that the pattern of the projection onto the bulb is not restored to normal after transection. Instead, immunohistochemical staining with RB-8, used as a means of differentiating projection of fibers from dorsomedial versus ventrolateral epithelium, suggests that axons from the dorsomedial olfactory epithelium innervate glomeruli along the anteromedial surface of the bulb that are not normally their targets in all lesioned animals and do not reach their usual territory of glomeruli in the dorsomedial bulb at more posterior levels. Both changes are strikingly consistent across the experimental group. Fibers from ventrolateral olfactory epithelium are also mistargeted and in some cases invade dorsolateral glomeruli that are normally innervated by axons from dorsomedial epithelium. Thus, the present results extend previous demonstrations that the bulb is re-innervated after transection of the olfactory nerve but indicate that the projection is not restored to normal despite the capacity for epithelial recovery. For example, in work by Graziadei and Monti Graziadei the newly restored innervation is demonstrably lighter than that on the control side, when assessed by anti-OMP staining (Graziadei and Monti Graziadei, 1980). Furthermore, tauLacZ-marked P2 neurons also re-innervate the bulb, but terminate in other glomeruli than their usual target (Costanzo, 2000). Nonetheless, the present observations are the first demonstration that the rhinotopy of the reconstituted projection is disordered after recovery from transection, i.e. that projections from dorsomedial versus ventrolateral epithelium are aberrantly distributed onto the bulb. Thus, nerve transection is accompanied by a disruption in axon targeting at both the zonal and individual glomerular levels. The findings stand in stark contrast to the restoration of normal rhinotopy after recovery from MeBr lesion. After recovery from peripheral lesion, zonality is restored to normal in the epithelium [RB-8 expression (Schwob and Youngentob, 1991) (J.E. Schwob, H. Fang, J.M. Hershberger, J.A. Hamlin, C.L. Iwema and S.L. Youngentob, manuscript in preparation) and odorant receptor expression (Iwema *et al.*, 1997)], as we have shown here for nerve transection and previously for bulb ablation (Schwob *et al.*, 1994). However, unlike the present results, during re-innervation that follows MeBr exposure the boundaries in the bulb between the projections from the various zones of the epithelium are restored to positions that are indistinguishable from normal, as revealed by either staining with RB-8 or by using retrograde transport of fluorescently labeled latex microspheres from injection sites in the bulb to define patterns of connectivity (Schwob and Youngentob, 1991) (J.E. Schwob, H. Fang, J.M. Hershberger, J.A. Hamlin, C.L. Iwema and S.L. Youngentob, manuscript in preparation).

Our conclusion that targeting during re-innervation of the bulb is disrupted by prior transection is predicated on the notion that differential staining with the monoclonal antibody RB-8 serves as a reliable marker for the projection of dorsomedial- versus ventrolateral-derived fibers. Other work from one on our laboratories, namely the retrograde transport experiments refered to above, demonstrates that the staining characteristics of axons do not shift in the

course between epithelium and bulb in either normal rats or in animals that have recovered after previous lesion with MeBr (Schwob and Youngentob, 1991) (J.E. Schwob, H. Fang, J.M. Hershberger, J.A. Hamlin, C.L. Iwema and S.L. Youngentob, manuscript in preparation). Hence, bead injections into dorsomedial glomeruli innervated by RB-8$^-$ axons label neurons in dorsomedial RB-8$^-$ olfactory epithelium and not in ventrolateral RB-8$^+$ olfactory epithelium, while the converse is true of injection into lateral or ventrolateral RB-8$^+$ glomeruli (Schwob and Youngentob, 1991) (J.E. Schwob, H. Fang, J.M. Hershberger, J.A. Hamlin, C.L. Iwema and S.L. Youngentob, manuscript in preparation). Thus, differential staining of olfactory axons in the bulb does indeed reflect where the fibers originate. A second aspect of the issue focuses specifically on the identification of glomeruli innervated by RB-8$^-$ axons. Immunostaining with RB-8 does not provide a positive marker for these fibers. However, as indicated in the results, a glomerulus is classified as innervated by RB-8$^-$ fibers on conservative criteria, i.e. only if other markers, such as anti-GAP-43 or anti-NCAM, indicate that the glomerulus in question receives a major input of olfactory axons, identified by the increased density of staining with those antibodies relative to the external plexiform layer. Finally, that RB-8 densely labels the nerve fascicles from the ventrolateral epithelium on the lesioned side, despite the greater than normal proportion of fibers that are immature, indicates that immaturity of axons is not inconsistent with dense staining. Thus, the increased numbers of immature olfactory axons on the lesioned side does not preclude their identification as RB-8$^-$. The validity of our interpretation is further buttressed by the retrograde tracing experiments summarized above (Schwob and Youngentob, 1991) (J.E. Schwob, H. Fang, J.M. Hershberger, J.A. Hamlin, C.L. Iwema and S.L. Youngentob, manuscript in preparation).

Two other features of the altered pattern of innervation deserve additional comment. First, glomeruli that are innervated inappropriately by dorsomedially derived, RB-8$^-$ axons appear to be wholly innervated by RB-8$^-$ axons, without any substantial contingent of RB-8$^+$ axons. Thus, in the aberrantly innervated glomeruli the level of staining of the glomerular neuropil is as low as in the dorsomedial glomeruli in the unoperated bulb. RB-8 labeling is a sensitive measure for the presence of a mixed RB-8$^+$/RB-8$^-$ population of axons, as we demonstrated previously for a small subset of glomeruli located in the boundary region between RB-8$^-$ and RB-8$^+$ territories in the normal olfactory bulb (Schwob and Gottlieb, 1986). Hence, the principle of glomerular innervation by fibers that are alike in terms of their level of OCAM/mamFas II expression is adhered to despite the abnormal location. The result is certainly consonant with a potential role for differential expression of the Ig superfamily member OCAM/mamFas II in selective fasciculation of ventrolateral axons with ventrolateral ones and dorsomedial ones with dorsomedial ones.

Second, the consistency of location of the aberrantly innervated glomeruli along the medial surface of the anterior and mid bulb is striking. While we cannot provide, at the present time, a definitive explanation of why the aberrant fibers target that location, it is noteworthy that glomeruli in that part of the normal (and unoperated) bulb underlie a substantial bundle of RB-8$^-$ fibers that are coursing through the olfactory nerve layer. The fibers of passage normally target glomeruli located in more posterior dorsomedial bulb, most of which are not re-innervated or very incompletely re-innervated after nerve transection. Thus, we suggest that the RB-8$^-$ fibers that are normally destined for more posterior glomeruli are diverted aberrantly into glomeruli that are more proximal to the epithelium. However, coursing near a denervated glomerulus is not a sufficient explanation for the mistargeting by RB-8$^-$ axons, since that area of the bulb is appropriately re-innervated by RB-8$^+$ fibers during recovery from MeBr lesion, which has the effect of consistently and completely denervating those anterior glomeruli (Schwob and Youngentob, 1991; Schwob *et al.*, 1999, 2000). One hypothesis suggests that a difference in the timing of re-innervation of the anteromedial part of the bulb by fibers from dorsomedial versus ventrolateral epithelium may offer the dorsomedial fibers an opportunity to re-innervate those glomeruli first after transection. There are other data to suggest that timing and access of fibers may divert them from their usual target to more readily available glomeruli. For example, the number of fibers that re-innervate the bulb is substantially reduced in animals where the severity of lesion after MeBr exposure is accentuated by food restriction (Schwob *et al.*, 1999). In these cases a rim of typical (i.e. non-necklace) glomeruli at the posterior margin of the bulb remains permanently denervated (Schwob *et al.*, 1999). An alternative hypothesis is that the transection procedure itself may disrupt guidance cues that are preserved by the MeBr lesion paradigm. For example, physical disruption of the olfactory nerve may lead to invasion of blood-derived phagocytes or to a difference in activation of olfactory glia or to disruptions in channels formed by the apposition of ensheathing cells, as compared with MeBr lesion, any of which might have an effect on axon guidance. The observation that the axons of P2 neurons are mistargeted during re-innervation after nerve cut (Costanzo, 2000) is not inconsistent with the mistargeting that we observe when assessing connectivity using RB-8, however, the data do not help us distinguish among the alternative explanations for either form of mistargeting. Further experimentation will be required to discriminate between them.

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