Chem. Senses 26: 971–981, 2001

Volumetric and Horseradish Peroxidase Tracing Analysis of Rat Olfactory Bulb Following Reversible Olfactory Nerve Lesions

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Abstract

Olfactory receptor neurons can regenerate from basal stem cells. Receptor neuron lesion causes degenerative changes in the olfactory bulb followed by regeneration as new olfactory receptor axons innervate the olfactory bulb. To our knowledge, parametric analyses of morphometric changes in the olfactory bulb during degeneration and regeneration do not exist except in abstract form. To better characterize olfactory bulb response, we performed morphometric analysis in rats following reversible olfactory nerve lesion with diethyldithiocarbamate. We also performed anterograde tracing of the olfactory nerve with wheatgerm agglutinin linked to horseradish peroxidase. Results of morphometry and tracing were complementary. The glomerular layer and external plexiform layer showed shrinkage of 45 and 26%, respectively, at 9 days. No significant shrinkage occurred in any other layer. Individual glomeruli shrank by 40–50% at 3 and 9 days following lesion. These data show that degenerative changes occur both in the glomeruli and transneuronally in the external plexiform layer. Olfactory nerve regeneration (identified by WGA–HRP transport) paralleled volumetric recovery. Recovery occurred first in ventral and lateral glomeruli between 9 and 16 days followed by recovery in medial and dorsal glomeruli. These data indicate substantial transynaptic degeneration in the olfactory bulb and a heretofore unrecognized gradient in olfactory nerve regeneration that can be used to systematically study recovery of a cortical structure.

Introduction

The olfactory bulb (OB) presents a unique system for studying neuronal plasticity processes in the central nervous system. Numerous procedures have been described to lesion olfactory receptor neurons (ORN) which project to the glomerular layer of the olfactory bulb. Many of these procedures spare the basal stem cells that replace the injured ORN and reinnervate the OB (Margolis *et al.*, 1974; Harding *et al.*, 1977; Nadi *et al.*, 1981; Schwob *et al.*, 1995; Ravi *et al.*, 1997; Setzer and Slotnick, 1998). Changes occurring in the OB represent both responses to degeneration and regeneration of the olfactory nerve terminals. Of major significance is that degeneration and regeneration occur both in the terminal fields of the olfactory nerves and transneuronally (see below).

Transneuronal processes in the OB are reasonably well known (Pinching and Powell, 1972; Margolis *et al.*, 1974; Nadi *et al.*, 1981; Baker *et al.*, 1983; Stone *et al.*, 1991; Sashihara *et al.*, 1997; Schwob *et al.*, 1999; Weruaga *et al.*, 2000). Electron microscopic evidence from rats showed relatively minor changes (Pinching and Powell, 1972). However, strikingly decreased OB expression of tyrosine hydroxylase follows ORN lesion (Nadi *et al.*, 1981; Baker *et al.*, 1983; Stone *et al.*, 1991). Several studies report elevation of glial markers not only around the glomeruli, but in laminae that do not receive a direct input from the olfactory nerve (Barber and Dahl, 1987; Anders and Johnson, 1990; Ravi *et al.*, 1997; Schwob *et al.*, 1999). Glial activation suggests that some neuropil reorganization is occurring in these laminae, although the exact nature is cryptic. Growth cone-associated protein in the olfactory bulb declines after lesion but is elevated roughly 2-fold during regeneration (Verhaagen *et al.*, 1990; Struble *et al.*, 1998; Schwob *et al.*, 1999). Amyloid precursor-like protein immunoreactivity initially appears in degenerating ORN axons at 3 days after lesion, is then expressed by glia at several days post-lesion and subsequently is expressed in dendrites of neurons several weeks following the lesion (Struble *et al.*, 1998). Similar processes probably occur in other parts of the CNS, but are more difficult to visualize (Cotman *et al.*, 1998). These processes are obvious in the OB because: (i) ORN lesion removes a substantial part of the input to a single layer and (ii) recovery after lesion extends for weeks because receptors must regenerate and extend axons to the OB.

Distinct laminae with well-described cell types and

connectivity further facilitate interpretation of ORN lesions (Greer, 1991). The olfactory nerve layer (ONL) contains the axons of the ORN which terminate in the glomerular layer (GL), contacting dendrites of mitral and tufted neurons within the glomerulus. The external plexiform layer (EPL) does not receive ORN axons, but is composed of processes of neurons post-synaptic to the ORN and axons extrinsic to the OB. The mitral cell layer, which forms the inner limit of the EPL, surrounds the internal plexiform layer (IPL) and the granule cell layer (GCL). Granule cells have dendrites which extend to the EPL and engage in reciprocal synapses with dendrites of mitral cells (Rall *et al.*, 1966). Remnants of the ventricular layer and subependymal zone persist at the core of the bulb. In juvenile animals this layer contains numerous precursor cells that can differentiate into either glia or interneurons (see Peretto *et al.*, 1999). This ability may persist into adulthood.

We were unable to find published volumetric analyses of the adult OB following ORN lesion and during subsequent regeneration. These studies are necessary for reasonable interpretation of the OB as a model for plasticity. Therefore, in this study we have systematically evaluated the OB following a reversible lesion of ORN. We combined volumetric analyses of the OB with anterograde tracing studies of the olfactory nerve with wheatgerm agglutinin–horseradish peroxidase (WGA–HRP). Relatively simple methods for anterograde tracing of the olfactory nerve with WGA–HRP exist (Shipley, 1985; Stewart, 1985; Baker and Spencer, 1986; Itaya *et al.*, 1987; Yee and Costanzo, 1995; Moon and Baker, 1998; Setzer and Slotnick, 1998; Schwob *et al.*, 1999). Hence, properly performed tracing studies can visualize the distribution of regenerated ORN axons. We found transient shrinkage of both the GL and EPL following olfactory nerve lesion, supporting transneuronal changes in the OB. We also found that ventral and lateral glomeruli in the middle portions of the OB recovered both innervation and size more rapidly than did medial or dorsal glomeruli. These studies present important observations for utilization of the OB as a model to study degeneration and regeneration in the central nervous system.

Materials and methods

All procedures in these studies received prior approval by the Southern Illinois University School of Medicine Laboratory Animal Care and Use Committee. Rats were housed in the animal care facility, which is accredited by the AAALAC. They were maintained on a 12:12 light:dark cycle and food and water were available *ad libitum*.

ORN lesions

We performed reversible lesions of olfactory epithelium with diethyldithiocarbamate (DDTC) as previously described (Ravi *et al.*, 1997; Struble *et al.*, 1998). Wistar male rats (Harlan), 300–350 g body wt (60+ days of age), were sedated with 7.6 ml/kg 5% chloral hydrate in distilled water. When sedation was obtained, a 2.5 inch 20 gauge needle was inserted s.c. at the intrascapular level and the rats were injected with 600 mg/kg DDTC dissolved in normal saline (180 mg/ml). The skin surrounding the needle was pinched closed and the needle withdrawn. Rats were killed at 3, 9, 16, 28 and 42 days following DDTC injection.

WGA–HRP treatment

WGA–HRP (Sigma) solution was infused intranasally 48 h prior to death. Rats were anaesthetized with 40 mg/kg pentobarbital and placed on their back. Several differing concentrations of WGA–HRP and methods of applying WGA–HRP via the external nares were tested. Our final technique used 50 µl of 4.0% WGA–HRP (Sigma), diluted in 0.9% saline, slowly dripped into (1–2 mm) one external naris with a pipette. The rats were allowed to inspire each drop normally. During infusion (3–5 min) the rat was gently rolled from side to side while still on its back. A 3.5 mm^3 piece of gel foam (~1.5 mm/side), soaked in 4.0% WGA–HRP, was inserted 4–5 mm into the external opening of the naris using an 18 gauge Abbocath (Abbott Laboratories) catheter.

Tissue preparation

Forty-eight hours later the rats were deeply anesthetized with pentobarbital (80 mg/kg). When a pinch-withdrawal response was absent they were perfused with 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain was removed and placed in fresh fixative in phosphate buffer for a further 2 h, cryoprotected overnight in 30% sucrose in phosphate buffer and frozen with dry ice. The turbinates were freed from the surrounding bone and post-fixed for 2 h, placed in Cal-Ex (Fisher Scientific) for 3 h and rinsed in phosphate buffer twice. They were then infiltrated with 3% warm gelatin, placed in 30% sucrose in phosphate buffer overnight and frozen with dry ice.

For quantitative analysis of laminar volume and glomerular area, a total of 21 DDTC-treated rats were used: four non-lesioned rats and survival periods of 3 (n = 3), 9 (n = 3), 16 (n = 4), 28 (n = 3) and 42 (n = 4) days. The OB was serially cut with a freezing microtome at 30 µm collected in phosphate buffer and every sixth section was directly mounted and dried on chrome alum subbed slides, defatted and stained with cresyl violet. For volumetric analysis the area of each lamina was outlined using Neurolucida (Colchester, VT), which combines an operator-generated overlay image on the microscopic image. Figure 1A shows a Nissl stained section with the laminae outlined. The area of the outlined area was automatically calculated. Volume was estimated by summing the area from two adjacent sections, dividing by two and multiplying by the interval (180 µm) between each section. To estimate area of glomeruli we measured the area of every glomerulus in one section roughly midway through the rostral–caudal axis of the OB (Figure 1A). This section

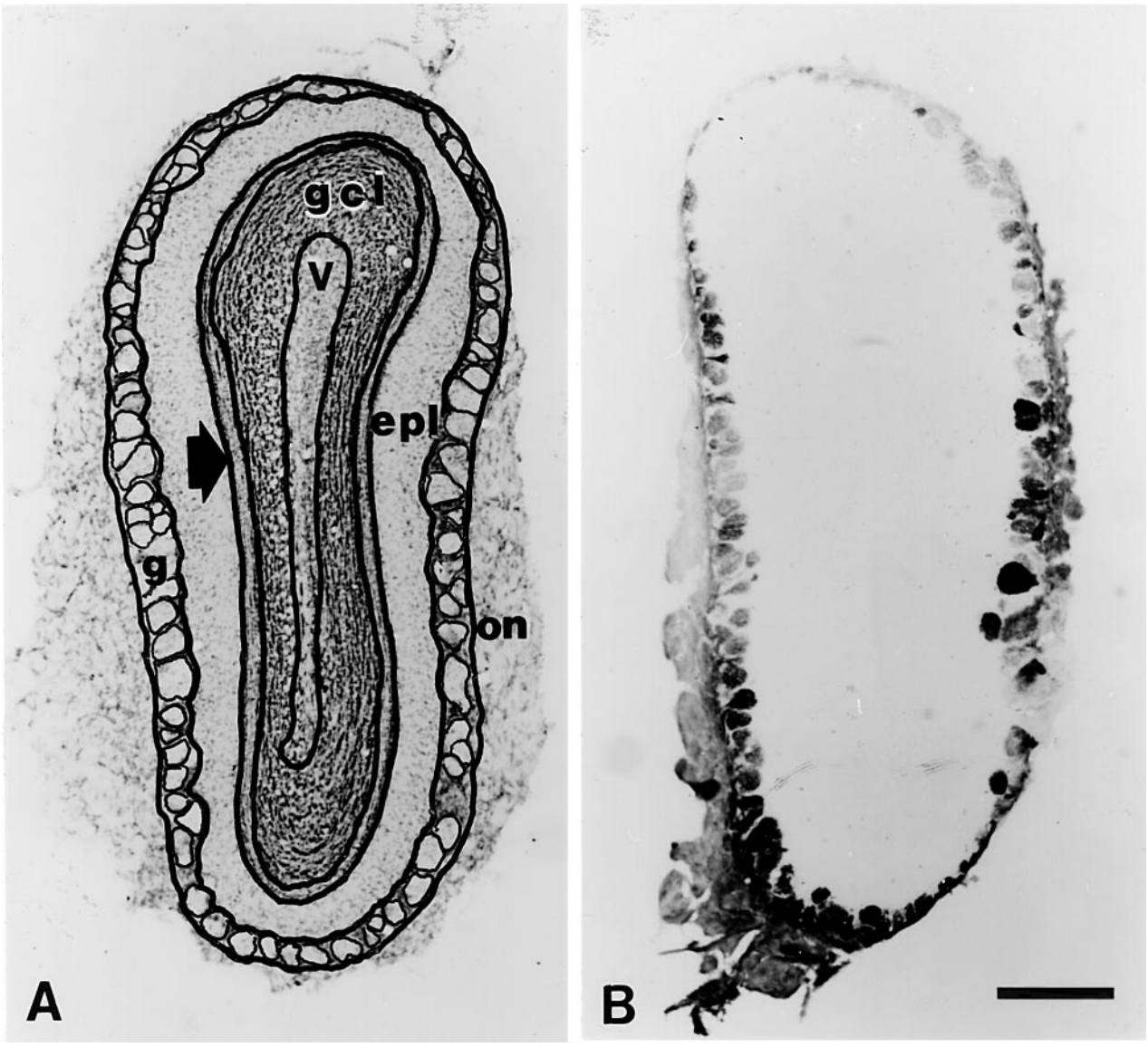

Figure 1 Low power photomicrographs of olfactory bulb stained for Nissl **(A)** or WGA–HRP **(B)**. Medial is to the center of the figure and dorsal to the top. (A) This coronal section, from approximately half-way through the OB, shows the quantified laminae (drawn lines). The approximate limits of each glomerulus are outlined. The mitral cell layer (arrow head) forms the innermost limit of the EPL. Beneath the mitral cell layer is the IPL (not labeled). on, olfactory nerve; epl, external plexiform layer; v, ventricular layer subependymal zone. (B) This coronal section of a WGA–HRP-reacted section represents approximately the same rostral–caudal level as (A). Most glomeruli contain label, including the dorsal-most glomeruli, although variability does exist in the density of reactivity. The bar in (B) represents 500 μm.

was adjacent to the section used for quantification in tracing studies (see below). Glomeruli were operationally divided into four groups (dorsal, ventral, lateral and medial) and the area traced with Neurolucida. The operational definition of ventral (or dorsal) was made with a line perpendicular to the bottom (or top) of the ventricular layer.

WGA–HRP development

One in six OB sections was saved in phosphate buffer for WGA–HRP development. Product was developed using a nickel intensification technique of the HRP-diaminobenzidine (DAB) product (Benzing and Mufson, 1995). Briefly, sections were rinsed three times for 10 min each in 0.01 M imidazole in 0.05 M acetate buffer (I/A buffer), adjusted to pH 7.4 with glacial acetic acid. A 0.05% DAB solution was made in I/A buffer that had not been pH adjusted. After sonication, nickel ammonium sulfate was added to make a 0.06 M solution, then 1% H_2O_2 was added to make a 0.0015% solution. Sections were reacted for 7 min then rinsed with buffered I/A, mounted, dehydrated and coverslipped.

Semi-quantification of glomerular labeling by WGA–

HRP was performed on sections adjacent to those used for glomerular area measurements. For this analysis we used a total of 21 rats (five controls and three at 3, three at 9, four at 16, two at 28 and four at 42 days). In most cases these were the same rats as used in the volumetric studies. However, in several cases the quality of the WGA–HRP staining in rats used for morphometry was inadequate for analysis. Sections from two control rats were selected from a comparable rostro-caudal region to replace two controls of the morphometric analysis showing no WGA–HRP reactivity and one rat at 28 days showed no WGA–HRP reactivity. Glomerular staining with WGA–HRP was categorized in three groups. (i) *Unstained* glomeruli could be easily identified (when the substage condenser was stopped down) as having a clear core surrounded by somata. (ii) *Partially* stained glomeruli were defined as either lightly stained throughout the extent of the glomerulus or having patchy depositions of reaction product in the glomerulus. (iii) *Fully* stained glomeruli were defined as containing reaction product throughout the extent of the glomerulus. Figure 1B shows a WGA–HRP-reacted section. Most of the glomeruli in this section would have been categorized as fully stained. The number of glomeruli of each type was counted and data expressed as a percentage of total glomeruli in that section. For statistical analysis we combined the number of partially filled with fully filled glomeruli.

Turbinates were cut on a cryostat at 10 μm and directly thaw-mounted on chrome alum subbed slides. Sections were stained with cresyl violet combined with periodic acid Schiff or reacted for WGA–HRP with either direct DAB or nickel intensification. Standard DAB development of WGA–HRP used 0.01% H_2O_2 and 0.05% DAB (w/v) (Sigma) in 0.1 M phosphate-buffered saline.

Electron microscopy

Finally, two rats were prepared for electron microscopy after WGA–HRP injection. We perfused the rats with either 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer or 1% paraformaldehyde and 1.25% glutaraldehyde in phosphate buffer. Sections were cut at 50 or 100 μm on a Vibratome and reacted with either DAB or DAB with nickel intensification. Intensely stained glomeruli were subsequently dissected from the coronal section, embedded in Epon and cut at 1 μm for light microscopic evaluation or at 90 nm (silver-gray) for electron microscopy. Sections were post-stained with lead citrate and uranyl acetate.

Statistical analysis

All statistical analyses were performed with SYSTAT9. Repeated measures analysis of variance (ANOVA) was used. Survival time following lesion (or no lesion) was the between-subjects measure and laminae or region (ventral, dorsal, medial or lateral) was the within-subjects measure. *Post hoc* analysis was performed with Bonferroni correction. In the event that a significant effect was found by ANOVA but the *post hoc* test did not detect a difference at the 0.05 level, the greatest mean difference was considered to be the only significant difference.

Results

Olfactory bulb volumes following lesions

Figure 2 graphically presents laminar volumes calculated from the histological sections following olfactory nerve lesion. The overall F test (repeated measures ANOVA) showed no significant effect of days post-lesion on total bulb volume ($F = 1.60$, d.f. = 5,15, $P = 0.22$). Total volumes (means ± SE) ranged from 17.38 ± 1.26 mm^3 in untreated rats to a minimum of 12.81 ± 1.45 mm^3 at 9 days, with a return to essentially control values at 42 days (17.31 ± 1.26), however, variance among animals at each time point obscured any group significance.

A significant difference between laminar volumes was found, as would be expected ($F = 523$, d.f. = 4,60, $P < 0.000$). A significant lamina × post-lesion survival interaction showed that some laminae were affected differentially by lesion ($F = 2.80$, d.f. = 20,60, $P < 0.001$). Subsequent ANOVA found that the GL shrank significantly ($F = 3.96$, d.f. = 5,15, $P < 0.02$). *Post hoc* analysis disclosed a significant 45% shrinkage of the GL on day 9 compared with both the control and 42 days post-lesion. The EPL did not reach statistical significance ($F = 1.83$, d.f. = 5,15, $P = 0.16$). Neither the IPL nor the GCL showed significant changes (IPL: $F = 0.48$, d.f. = 5,15; $P < 0.79$; GCL: $F = 0.65$, d.f. = 5,15, $P = 0.66$). The ventricular layer (VENT) appeared to enlarge following lesion but did not reach statistical significance ($F = 2.45$, d.f. = 5,15, $P = 0.08$).

A correlational analysis disclosed substantial correlation among the variables and, in particular, a significant correlation between EPL volume and the combined volume of IPL, GCL and VENT ($r = 0.90$). These latter three areas

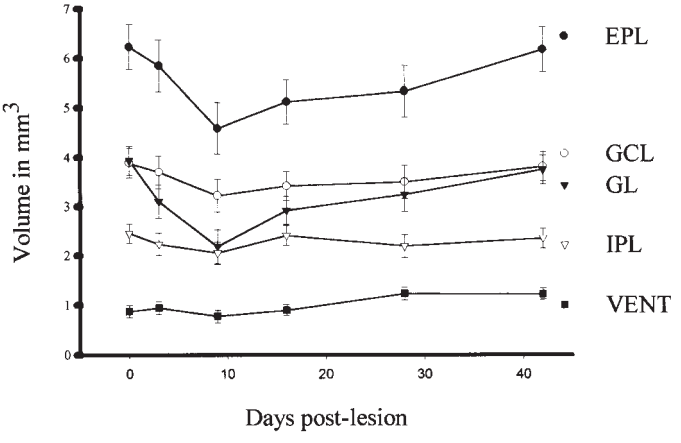

Figure 2 This graph shows the volumetric estimates as described in the text. The GL displayed statistically significant shrinkage. The other lamina did not show statistically significant shrinkage except for the EPL in an analysis of covariance. Analysis of covariance of the EPL is shown in Figure 3.

showed no lesion effect (see above). Therefore, an analysis of covariance was performed on EPL volume with the combined volume of IPL, GCL and VENT as the covariate. This analysis showed that the EPL volume decreased following lesion when volume was adjusted for more central areas ($F = 3.40$, d.f. = 5,14, $P < 0.03$). *Post hoc* analysis did not find 'significant' differences because of the conservative nature of Bonferroni contrasts. However, inspection showed a maximal decline of 26% at 9 days and a delayed recovery (Figure 3). Use of the same covariate did not change the statistical findings on GL shrinkage following ORN lesion. Conversely, use of the EPL as covariate eliminated the statistical significance of GL changes, which confirmed that EPL shrinkage was related to ONR lesion.

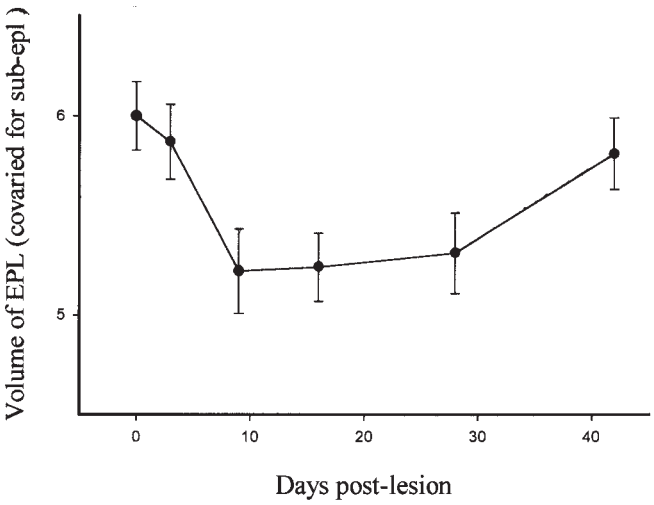

Figure 3 This graph presents the analysis of covariance data for the EPL. The EPL volume was analyzed with covariates of the IPL, GCL and VENT. The greatest shrinkage occurred at 9 days after lesion and recovery was delayed compared to the GL (Figure 2).

Glomerular areas

A factorial repeated measures ANOVA (with regional area as the repeated measure and time after lesion as the between-subjects factor) found both a significant main effect of time after lesion ($F = 7.35$, d.f. = 5,15, $P < 0.001$) and a significant regional effect ($F = 34.37$, d.f. = 3,45, $P < 0.000$). In addition, a significant region × time after lesion effect was found ($F = 2.27$, d.f. = 15,45, $P < 0.02$). *Post hoc* testing disclosed that dorsal glomeruli were smaller than glomeruli of other regions irrespective of lesion effects.

Equivalent lesion effects (i.e. shrinkage) occurred in all glomeruli (Figure 4) after ORN lesion. The interaction showed that glomerular area recovery was more rapid in ventral and lateral glomeruli than in medial and dorsal glomeruli. These differences were clearly obvious in Nissl stained sections (Figure 5). *Post hoc* testing of ventral glomeruli showed a 40–50% decline at day 9 from both the control and 42 day groups. Lateral glomeruli area differed significantly from the control and 42 days post-lesion groups at 3 and 9 days. Medial glomeruli area displayed a delayed recovery and was significantly different from the control and 42 day groups at both days 9 and 16. Finally, dorsal glomeruli were different from controls 3, 9 and 16 days following lesion. In sum, all glomeruli shrank equivalently following nerve lesion, but recovery was more rapid in ventral and lateral glomeruli than in medial and dorsal glomeruli.

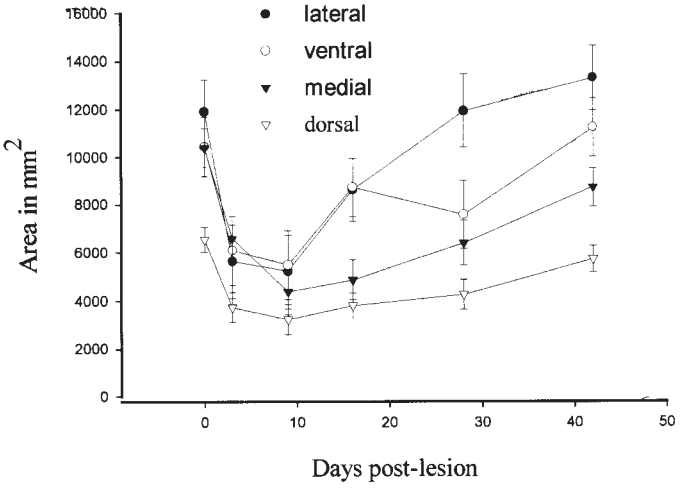

Figure 4 Glomerular areas. This graph shows that significant shrinkage of glomeruli occurred at 3 days. Recovery began after 9 days. The delay of recovery of dorsal and medial glomeruli is clearly obvious.

WGA–HRP tracing in the olfactory bulb

We evaluated several concentrations and methods of application of WGA–HRP described in previous reports. Highly variable labeling of glomeruli in untreated rats occurred with concentrations of 1–2% WGA–HRP. Some glomeruli stained well, but many displayed no observable labeling. Four percent WGA–HRP improved but did not solve this problem. All quantification and most observations were performed with 4% WGA–HRP and nickel-intensified reaction product. We also found that occlusion of the contralateral naris during WGA–HRP inspiration markedly reduced labeling. This suggests that decreasing the inspiration rate of the naris ipsilateral to the irrigation is important in obtaining more complete labeling, a conclusion implicit in a previous report (Schwob *et al.*, 1999).

We obtained labeling of most glomeruli (90%) in untreated rats using a slow drip technique and 4% WGA–HRP (Figure 1). No regional selectivity for labeling was identified in control rats (Figure 6). Very occasionally we observed WGA–HRP-labeled neurons adjacent to intensely labeled glomeruli. In contrast to controls, weak labeling was not a problem in the rats following lesion. A diffuse, weak accumulation of reaction product throughout the glomerulus, as seen in some control rats, was not present in the lesioned rats until 42 days post-lesion, when reinnervation in most glomeruli tended to be complete (judging by area measures).

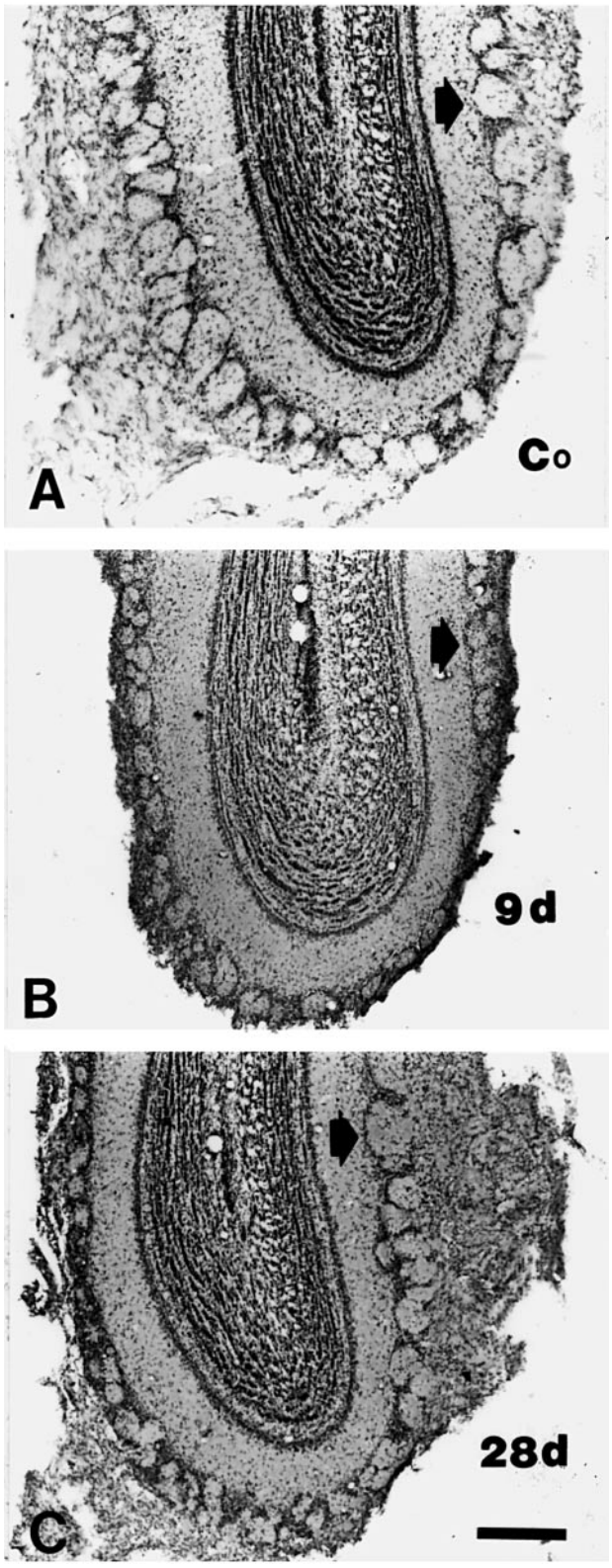

Figure 5 These are Nissl stained sections from a control rat **(A)**, 9 days following lesion **(B)** and 28 days following lesion **(C)**. Arrows point to the lateral side. Both medial and lateral (arrow) glomeruli show obvious shrinkage at 9 days (B) compared with the control rat (A). Note absence of the olfactory nerve. By 28 days (C) the lateral glomeruli (arrow) have recovered but the medial glomeruli are still shrunken. Bar in (C) represents 250 μm.

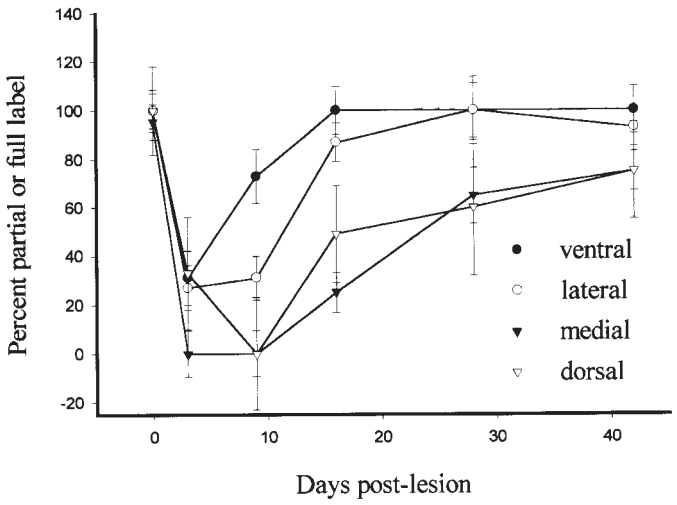

Figure 6 This graph shows the combined percentage of WGA–HRP partially and filled glomeruli. No regional difference exists in the control animals, in which ~100% of glomeruli are labeled by this procedure. Compare these data with Figure 1B. WGA–HRP labeling declines in all glomeruli 3 days following lesion, but shows recovery in ventral glomeruli at 9 days. Recovery is 'complete' in both ventral and lateral glomeruli by 16 days, but is significantly slower in dorsal and medial glomeruli.

Effects of lesion and glomerular labeling

Figure 6 displays the combined percentage of glomeruli either fully or partially labeled following olfactory nerve lesion. Statistical testing showed significant variation in labeling with region ($F = 13.67$, d.f. = 3,45, $P < 0.000$) and with days post-lesion ($F = 14.03$, d.f. = 5,15, $P < 0.000$). The interaction region × time post-lesion failed to achieve an accepted level of significance ($F = 1.80$, d.f. = 15,45, $P = 0.065$). The main effect of regional variability represented delayed recovery by medial and dorsal glomeruli compared with ventral and lateral glomeruli. *Post hoc* testing within each region showed that WGA–HRP labeling of ventral glomeruli decreased only at 3 days (27% of ventral glomeruli showed some labeling). Labeling increased at 9 days, when almost 70% of ventral glomeruli showed either full or partial labeling (raw data 30, 90, 100%). WGA–HRP labeled significantly fewer lateral glomeruli at both 3 and 9 days than either the control group or on days 16–42 post-lesion. Medial glomeruli showed a lower labeled percentage at 3, 9 and 16 days than either the control or 42 days post-lesion group. Very high inter-animal variation masked differences in the dorsal glomeruli versus the controls except at 9 days, but the pattern was similar to medial glomeruli. The graphs of these data (Figure 6) show that WGA–HRP labeling of ventral and lateral glomeruli recovered more rapidly than did medial or dorsal glomerular labeling.

The subjective WGA–HRP activity largely paralleled the morphometric analysis of glomerular size. At 3 days most glomeruli had little or no evidence of WGA–HRP labeling (Figure 7A). What little labeling was present in the bulb at 3 days was in the ventral or lateral glomeruli. At 9 days the

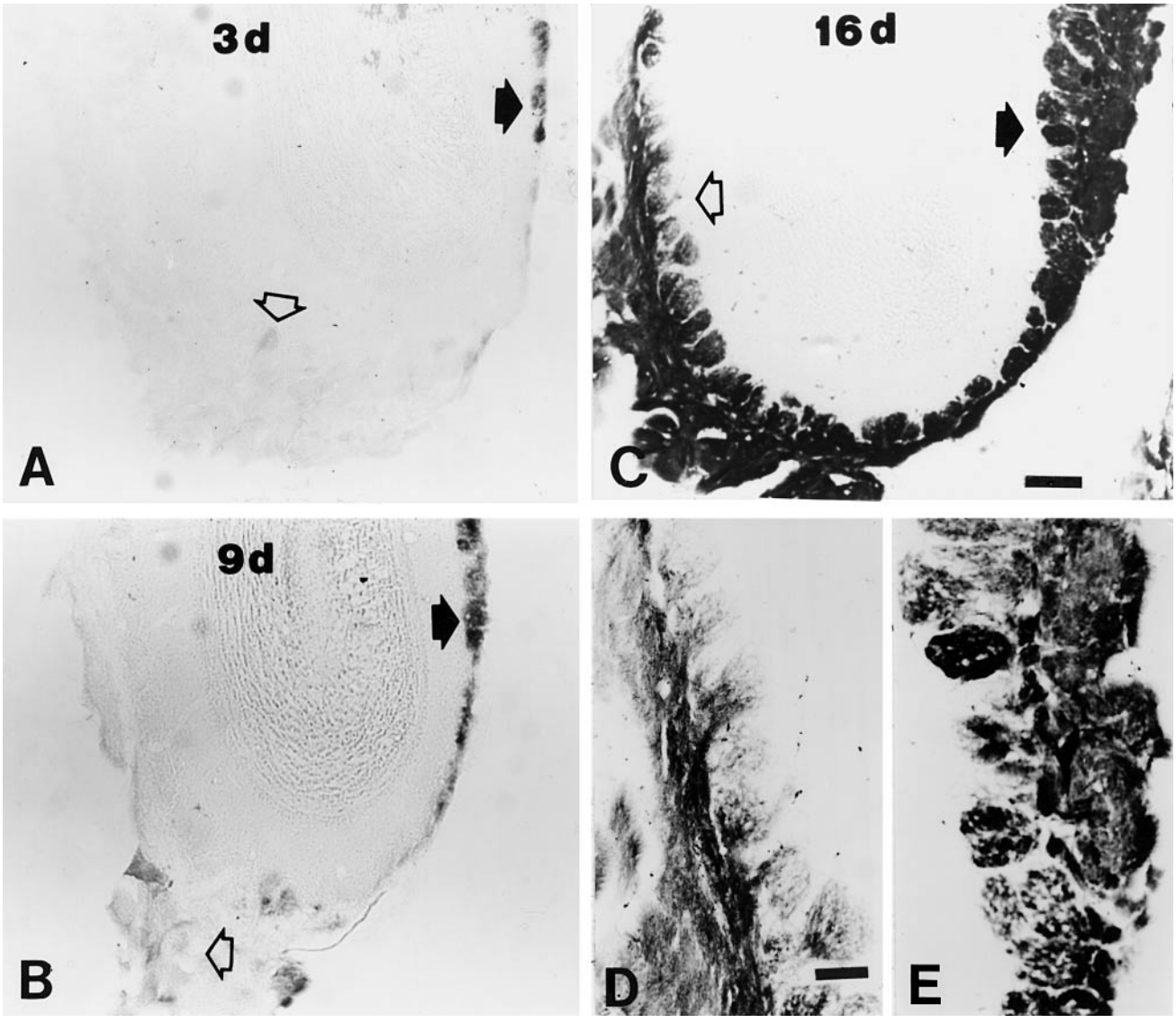

Figure 7 This plate shows WGA–HRP labeling in the OB at 3 days **(A)**, 9 days **(B)** and 16 days **(C)** post-lesion. In (A) (3 days) very weak staining can be seen in the lateral regions (filled arrow) and one very weakly labeled ventral glomerulus (open arrow). At 9 days (B) more labeling is obvious in lateral glomeruli (solid arrow). In addition, the olfactory nerve (open arrow) and some ventral glomeruli display partial labeling. In this particular case only 30% of glomeruli showed some WGA–HRP labeling. By 16 days (C) the ventral and lateral glomeruli (solid arrow) show more labeling than medial glomeruli (open arrow). Bar in (C) 100 μm. **(D)** An enlargement of medial glomeruli from (C). Bar in (D) 50 μm. **(E)** An enlargement of lateral glomeruli adjacent to the open arrow. The density of staining is substantially greater in lateral (E) than medial (D) glomeruli.

ventral glomeruli in the middle third of the bulb showed labeling, but label was still absent in other regions (Figure 7B). Substantial differences appeared at 16 days and later. At 16 days most ventral and lateral glomeruli contained WGA–HRP in the middle two-thirds of the bulb (Figure 7C). Conversely, medial glomeruli were less intensely labeled at this stage (Figure 7D,E).

The pattern of labeled glomeruli at 16 days clarified the pattern of regeneration. Analysis of serial stained sections, although not quantified, showed that most glomeruli at or behind the level of the anterior olfactory nucleus displayed label (Figure 8A,B) and label was present two-thirds of the way through the OB (from the caudal-most glomeruli; Figure 8C,D). Sections rostral to this level displayed WGA–HRP in the nerve, but few glomeruli were labeled (Figure 8E,F). This reconstruction suggests that new fibers enter the denervated OB about one-third of the way from the rostral tip in the ventral layer and progress rostrally and caudally.

We observed little glomerular labeling contralateral to the injected naris. This observation was not carefully explored, but our impression is that relatively little solution infused in one naris was able to gain access to the contralateral turbinates. These observations suggest that unilateral lesions

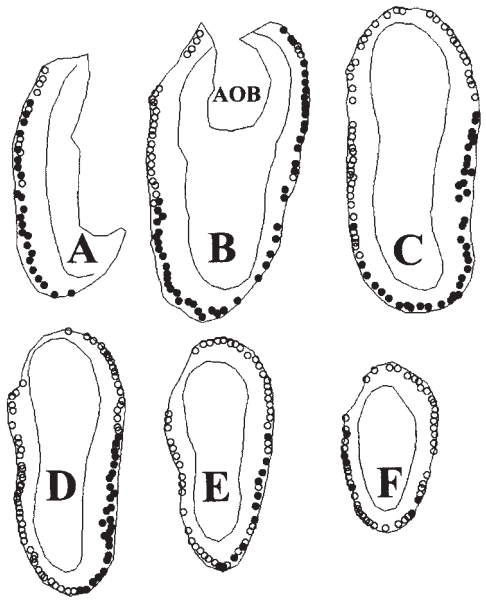

Figure 8 Sections (A)–(F) represent the caudal to rostral progression of glomerular labeling with WGA–HRP 16 days post-lesion. Solid circles represent glomeruli filled with WGA–HRP, open circles either partially or unlabeled glomeruli The mid-point of the bulb is between sections (C) and (D). In caudal regions **(A, B)** most glomeruli are fully labeled. A clear dichotomy between medial and lateral glomeruli is apparent In the middle portions of the bulb **(C, D)** such that medial glomeruli are less often fully labeled than lateral glomeruli. In the rostral third of the bulb **(E, F)** few glomeruli contain detectable amounts of WGA–HRP. AOB, accessory olfactory bulb.

of the rat olfactory epithelium are possible (Turner and Perez-Polo, 1993). It is worth noting that the same procedure in mice resulted in bilateral labeling (Struble, personal observation).

Electron microscopy

Either fixative resulted in good visualization of the WGA–HRP product. In thick plastic-embedded sections (1 μm) the DAB product was more visible than the nickel intensification product. At the electron microscopic level the reaction product could be seen in processes that contained large numbers of vesicles (Figure 9). On this basis we conclude that they were olfactory afferents. It was also obvious that not all olfactory afferents in a single densely labeled glomerulus displayed reaction product.

Olfactory epithelium

We were generally unable to detect WGA–HRP reaction product in olfactory epithelium (data not shown). When present, labeling was most common in the lateral turbinates. ORN, sustentacular cells and, presumably, macrophages in damaged areas of the olfactory epithelium displayed WGA–HRP activity. No labeling was identified in the portions of the turbinates that surrounded the central core of the olfactory turbinates. We attribute this lack of labeling to

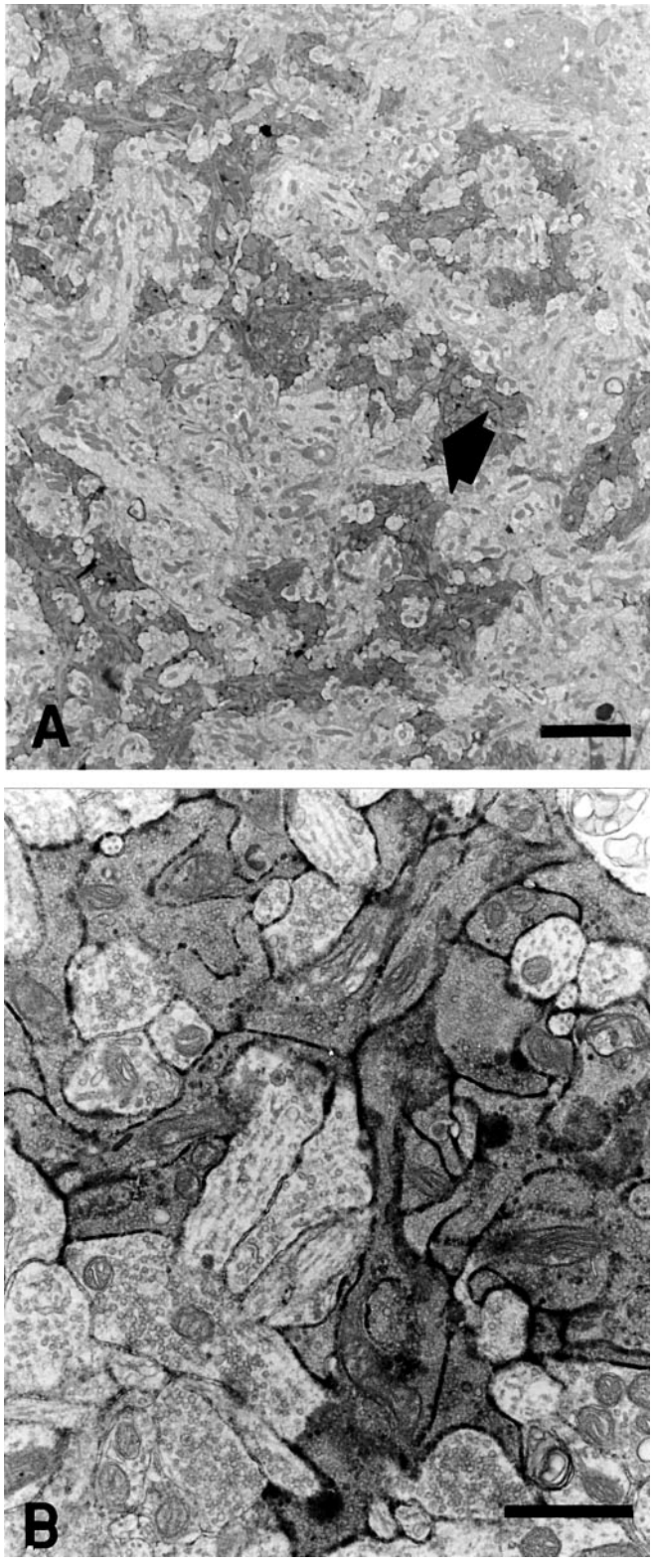

Figure 9 Electron micrographs of a fully filled glomerulus (as judged at the light microscopic level). Tissue was fixed with 4% paraformaldehyde and 0.1% glutaraldehyde as described in Materials and methods. **(A)** Note that the labeled terminals (dark) fill up relatively little of the total glomerular region. Bar 5 μm. **(B)** At a higher magnification the region marked by the filled arrow in (A) shows both dark and light terminals containing round vesicles identifying these as most probably olfactory nerve. Bar 1 μm.

xenobiotic activity, rapid clearance of WGA–HRP from the olfactory epithelium or some aspect of tissue preparation that is incompatible with maintaining WGA–HRP activity (Moon and Baker, 1998).

Discussion

Our studies extend previous reports of the effects of olfactory nerve lesion on the OB (Pinching and Powell, 1971; Margolis *et al.*, 1974; Harding *et al.*, 1977; Nadi *et al.*, 1981; Baker *et al.*, 1983; Stone *et al.*, 1991; Schwob *et al.*, 1995; Ravi *et al.*, 1997; Sashihara *et al.*, 1997; Setzer and Slotnick, 1998; Schwob *et al.*, 1999; Weruaga *et al.*, 2000). Our volumetric analyses extend these studies and show that the response of the OB can be basically broken into two well-separated time periods. The initial response is degeneration, which occurs until ~7–8 days. Subsequently, regenerative responses appear and continue until ~6–8 weeks.

Degeneration

Our data support both direct and transneuronal changes in the OB. Our previous analysis of freshly obtained OB identified a statistically significant 20% decrease at 3–7 days (Struble *et al.*, 1999). Our current data show a statistically non-significant bulb volume shrinkage of 26%. Non-significant changes in the histological analysis is largely a variance problem. In our previous study (Struble *et al.*, 1999) variance in the lesion group was quite small soon after lesion (see Table 1) (Struble *et al.*, 1999). The olfactory nerve in the current study sustained variable damage during dissection and histological processing and we could not reliably quantify olfactory nerve. Basically, the variance among rats in this study had increased relative to the variance under conditions resulting in a non-significant effect. However, both the GL and EPL shrank.

Nerve damage with DDTC clearly causes glomerular shrinkage. Individual glomeruli shrink by ~40% on day 3 and further decline to 50% of control at 9 days. This magnitude of individual glomerulus shrinkage at 3 days corresponds to our estimate that, on average, 75% of the olfactory epithelium is damaged by DDTC (Struble *et al.*, 1998) and that olfactory nerve terminals comprise ~50% of the glomerulus (Hinds and McNelly, 1981). In contrast to individual glomeruli, the GL shrink by a non-significant 22% at 3 days and a statistically significant 45% by 9 days after lesion. The implication of the temporal difference in shrinkage of glomeruli (3 days) versus the GL (9 days) is that the total glomerular layer shrinkage represents both the 40–50% shrinkage of glomeruli and subsequent shrinkage in the neuropil surrounding the glomeruli. This observation has precedent in studies reporting tyrosine hydroxylase decline in periglomerular neurons following nerve lesion (Nadi *et al.*, 1974; Baker *et al.*, 1983, 1984; Margolis *et al.*, 1983). Dendritic and axonal fields of tyrosine hydroxylase-containing periglomerular interneurons probably shrink and contribute to the total GL shrinkage (Baker *et al.*, 1984). Similar changes could occur in other neurons and in terminal dendritic fields of mitral and tufted neurons (Pinching and Powell, 1972) and account for the delayed EPL shrinkage discussed below.

The EPL follows the same temporal pattern as the GL, declining by only 6% on day 3 and 26% at day 9 after lesion. This difference is not statistically significant until we adjusted for volume by analysis of covariance with the subjacent laminae. Adjustment makes intuitive sense since the granule cells of the GCL provide much of the neuropil for the EPL. Use of EPL volume as a covariate for GL volume eliminates some inter-animal variability. However, it also eliminates the statistical significance of lesion on the GL. This result confirms that changes within the EPL are related to the same factor (nerve lesion) that caused GL shrinkage.

It is important to point out that laminar shrinkage occurs in the presence of extensive reactive gliosis following nerve lesion (Barber and Dahl, 1987; Anders and Johnson, 1990; Ravi *et al.*, 1997; Schwob *et al.*, 1999). Therefore, transneuronal changes may be more extensive than our data show. It is also interesting that the ventricular layer shows non-significant evidence of expansion several weeks after lesion and this difference became significant ($P = 0.02$) when the EPL is used as a covariate. The biological significance of this change may be related to functions of the subependymal layer in possible gliagenesis and neurogenesis occurring after ORN lesion (Peretto *et al.*, 1999)

The WGA–HRP tracing studies confirm and extend the volumetric aspects of this study. We initially had problems in obtaining OB labeling in control rats when using previously described techniques (Shipley, 1985; Stewart, 1985; Baker and Spencer, 1986), but these problems were usually ameliorated with a higher concentration of WGA–HRP. Nonetheless, our electron microscopic data from control rats showed unfilled olfactory terminals even when glomeruli were selected for intense WGA–HRP activity. It is clear that although WGA–HRP was transported into the OB, labeling with our procedure was incomplete. We had fewer problems with weak labeling after lesion, presumably because either airflow or the xenobiotic scavenging system was impaired in the lesioned rats. However, several of the control rats and one lesioned rat used in the morphometric studies showed no evidence of labeling even with 4% WGA–HRP, hence we had to supplement this group in the tracing study.

WGA–HRP tracing confirmed previous estimates of damage by DDTC (Struble *et al.*, 1998). Seventy to eighty percent of glomeruli are devoid of any visible WGA–HRP reactivity 3 days following lesion. It is noteworthy that the significant WGA–HRP labeling decline at 3 days compares well with glomerulus shrinkage but not with GL shrinkage. This further supports transneuronal changes underlying volumetric changes; loss of the nerve occurs by 3 days, but shrinkage continued for another 6 days.

Regeneration

Volumetric recovery of the OB occurred between 9 and 16 days after olfactory nerve lesion. Importantly, the morphometric estimates of glomerular recovery and the WGA–HRP transport study are complementary. Approximately 70% of ventral and lateral glomeruli are devoid of WGA–HRP activity at 3 days. Regenerating fibers appear to return via the ventral OB surface and ventral glomeruli show initial recovery of WGA–HRP labeling. At 9 days 73% of ventral, but only 31% of lateral, glomeruli showed some label. By 16 days 100% of ventral glomeruli and 86% of lateral glomeruli showed WGA–HRP. Medial glomeruli labeling lagged both ventral and lateral labeling. Our data are compatible with a previous study (Schwob *et al.*, 1999) that estimated that reinnervation occurred during weeks 2–3. Their estimate is correct for total glomeruli, but the ventral and some lateral glomeruli appear to be reinnervated earlier, i.e. at ~9 days. These studies are also congruent with recovery studies in the mouse (Verhaagen *et al.*, 1990). Any discrepancy between our data and data previously published most likely relates to differential reinnervation of glomeruli discussed below.

Obvious preferential reinnervation occurs when comparing both glomerular area and WGA–HRP labeling. Laterally placed glomeruli in the middle portions of the OB recover both area and WGA–HRP labeling before most medial glomeruli. Medial and dorsal glomeruli in the rostral two-thirds of the OB appear to recover much more slowly. This ventral–lateral–medial gradient is not an artifact of the technique. Close inspection of photomicrographs in three other reports shows a comparable gradient following either physical or chemical lesion of the nerve (Yee and Costanzo, 1995; Setzer and Slotnick, 1998; Schwob *et al.*, 1999). Preferential sparing of ORN is not an attractive hypothesis because different lesion techniques, which could selectively lesion epithelial regions, should result in different patterns of sparing. However, the same pattern of regeneration is seen with both chemical and mechanical lesions.

One possible explanation for a medial–lateral difference is the epithelial distribution of ORN cells projecting to glomeruli. The presence of very weak labeling of ventral and lateral glomeruli at 3 days presumably represents axons spared by the toxin, the number of which are sufficient to visualize WGA–HRP. It is possible that the ventral and lateral glomeruli may get a more dispersed projection from the epithelium than do medial and dorsal glomeruli. Lesion of epithelium, by any method, may result in sparing of some innervation and spared axons could act to guide growing axons to ventral and lateral glomeruli. It is also possible that the greater distance of dorsal and medial glomeruli from the olfactory epithelium may require more time for axons to arrive.

An alternative, although less simple, hypothesis is suggested by a previous report of a protein preferentially expressed in ventral and lateral olfactory epithelium (Schwob and Gottlieb, 1986) which project to the homologous region of the OB (Astic and Saucier, 1986; Saucier and Astic, 1986). Subsequent studies proposed that this protein is a putative neural cell adhesion molecule (Alenius and Bohm, 1997). Perhaps this protein facilitates growth of axons from these regions. Why preferential reinnervation occurs is not clear, but differential recovery permits within-animal use of the OB for systematic neuroanatomical studies of central changes during reinnervation.

Recovery of WGA–HRP nerve labeling appears to parallel recovery of the glomerular area except in ventral glomeruli, where a discrepancy may exist between reinnervation, assessed with WGA–HRP, and glomerular area recovery. In essence, WGA–HRP shows 'complete' labeling of ventral glomeruli at 2 weeks, although the size of ventral glomeruli had not recovered by 4 weeks (compare Figures 4 and 6). Full recovery requires 6 weeks. Axonal growth in young animals has been described as 'over-exuberant' (Cummings *et al.*, 2000) and one study of reinnervation of identified axons suggested axonal reorganization occurring after 10 weeks (Costanzo, 2000). We suspect that early arriving axons may initially enter ventral glomeruli (as shown with WGA–HRP) but may not establish a functional relationship which would be reflected by area recovery.

In sum, these studies present morphometric data on degeneration and regeneration of the OB subsequent to a reversible lesion of the olfactory epithelium. Both morphometric and tracing studies identify significant transneuronal changes, present a better time line of regeneration and identify a previously unrecognized gradient in OB reinnervation. These data further support the use of the OB as a unique experimental system for analysis of degeneration and regeneration-associated processes that occur in the central nervous system.

Acknowledgements

We wish to acknowledge the work of Stephanie Perry on aspects of this project. This work was supported by NIH grants AG08014 (R.G.S.) and DC03889 (B.P.N.) and a Southern Illinois University Central Research Committee grant (R.G.S.), an Illinois Department of Public Health AD grant (B.P.N.) and Eastern Illinois University CFR grants (B.P.N.). Part of these data was previously presented in abstract form at the American Association of Neuropathologists

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Accepted May 1, 2001