Chem. Senses 26: 605–610, 2001

Olfactory Marker Protein Immunohistochemistry and the Anterograde Transport of Horseradish Peroxidase as Indices of Damage to the Olfactory Epithelium

Burton Slotnick, Natalya Bodyak and Barry J. Davis[1]

Department of Psychology, American University, Washington, DC 20016 and [1]Department of Anatomy and Neurobiology and Program in Neuroscience, University of Maryland School of Medicine, Baltimore, MD 21201, USA

Correspondence to be sent to: Burton Slotnick, Department of Psychology, American University, Washington, DC 20016, USA. e-mail: slotnic@american.edu

Abstract

The present study compared the relative effectiveness of wheatgerm agglutinin–horseradish peroxidase (WGA–HRP) and olfactory marker protein (OMP) in detecting the presence of intact olfactory axons in glomeruli of the main olfactory bulb (MOB) in the rat. The olfactory epithelium was damaged by i.p. injections of the toxin 3-methyl indole and, after 5 or 6 days, the olfactory sac was injected with a 1% WGA–HRP solution. No anterograde labeling was observed in the dorsal and ventromedial quadrants of the MOB in the WGA–HRP material. However, in the same cases OMP immunostaining was observed throughout the MOB. In other rats the rostral olfactory epithelium was aspirated unilaterally and after 3, 11 and 16 days the olfactory sacs were injected with WGA–HRP and rats were perfused 1 day later. In these cases WGA–HRP reaction product was absent in the dorsolateral quadrant of the MOB on the aspirated side in all animals, but OMP immunostaining could be detected in the 4 and 12 day survival animals but not in the 17 day survival rat. These findings indicate that anterograde transport of WGA–HRP accurately reflects the presence of intact axons *en route* to the MOB. In contrast, OMP immunostaining persists in axon terminals severed from their parent cell body for at least 12 days and is a less useful marker of intact olfactory axons in experiments using short survival times.

Introduction

The anterograde transport of horseradish peroxidase (HRP) and wheatgerm agglutinin-conjugated horseradish peroxidase (WGA–HRP) has been used in a number of studies to assess the connections between the olfactory epithelium and the olfactory bulb in rats with restricted or widespread damage to the olfactory epithelium produced by olfactotoxins, metallic salts, detergents or surgical intervention (Meredith and O'Connell, 1988; Boehm *et al.*, 1991; Setzer and Slotnick, 1998a,b; Schwob *et al.*, 1999; Xu and Slotnick, 1999). As shown by Setzer and Slotnick and Schwob *et al.*, the decrease in or absence of WGA–HRP reaction product in olfactory bulb glomeruli can be used to measure both the severity of damage to the olfactory epithelium and the degree of recovery of connections between the epithelium and bulb over time (Setzer and Slotnick, 1998a; Schwob *et al.*, 1999). When combined with appropriate anatomical analysis the anterograde transport of WGA–HRP is a powerful tool in functional and anatomical studies of the olfactory system. However, the anterograde transport of HRP has certain limitations. If the enzyme, typically applied by intranasal lavage, does not contact all sensory neurons it would be difficult or impossible to distinguish treatment effects from false negatives due to inadequate application of the anterograde tracer. If, on the other hand, the enzyme was transported by axons severed from their cell bodies this would produce false positive responses.

The immunohistochemical identification of olfactory marker protein (OMP) in bulbar glomeruli provides an alternative and potentially more useful index of surgery- or toxin-induced disruption of such connections (Walters *et al.*, 1996). The protein is found normally in the terminals of mature olfactory sensory axons and, hence, the visualization of OMP in glomeruli does not require exogenous application of a transportable tracer (Hartman and Margolis, 1975; Monti-Graziadei *et al.*, 1977). However, one potential disadvantage of using OMP as a marker for intact olfactory axons is the possibility that the protein may degrade slowly after injury to the epithelium and its presence in bulbar glomeruli, especially in short survival studies, may produce false positive responses (Kream and Margolis, 1984). This report compares the usefulness of the WGA–HRP and OMP methods for detecting afferent connections to the

main olfactory bulb (MOB) after various survival times following damage to the olfactory epithelium.

Materials and methods

Subjects

Ten adult male Long Evans rats were housed individually or in groups of three on sawdust in plastic cages in a temperature and humidity controlled vivarium.

Toxin treatment

Seven rats were injected i.p. with vegetable oil (n = 3) or 250 mg/kg (n = 4) 3-methyl indole (3-MI) (Sigma M-2127). Intraperitoneal application of 3-MI has been shown to cause widespread damage in the olfactory epithelium (Peele *et al*., 1991). The toxin was dissolved in corn oil to a concentration of 25 mg/ml. Five or six days after treatment each rat was anesthetized with ketamine and xylazine and placed in a stereotaxic head holder. The nasal bones were removed and each olfactory sac was injected with 10 µl of 1% WGA–HRP (Sigma). After a 24 h survival period the animals were deeply anesthetized and killed by transcardial perfusion with saline and mixed aldehydes (Mesulum, 1982). Fifty micrometer thick frozen sections of the olfactory bulb were cut using a sliding microtome. Every other section was processed by the tetramethyl benzidine histochemical procedure (Mesulum, 1982), air dried, lightly stained with thionin, dehydrated quickly through a cold alcohols series, cleared in xylene and covered using Permount.

OMP immunohistochemistry

The alternate series of sections was processed using the OMP immunohistochemical procedure (Monti-Graziadei *et al*., 1977). The following steps were carried out in sequence on freely floating sections: (i) rinses in 0.02 M phosphate-buffered saline, pH 7.4 (PBS); (ii) a pre-soak in PBS containing 0.5% H_2O_2 in 70% methanol for 20 min; (iii) a pre-soak in 0.2% Triton X-100/10% normal rabbit serum (NRS) in PBS for 30 min; (iv) incubation in goat anti-OMP antiserum diluted 1:3000 in PBS containing 0.2% Triton X-100 and 2% NRS for 24–48 h at 4°C; (v) incubation in biotinylated rabbit anti-goat IgG (Vector Laboratories) diluted 1:200 in PBS for 2 h; (vi) incubation in avidin–biotin–horseradish peroxidase (Vector Laboratories Elite kit) diluted 1:100 in PBS for 1 h; (vii) rinses in 0.1 M Tris buffer, pH 7.6. A peroxidase reaction was visualized using 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) and 0.005% H_2O_2 in Tris buffer. In initial pilot studies the dilution of the OMP antiserum was varied from 1:500 to 1:5000 to determine the optimal dilution. Each dilution yielded usable immunostaining and the mid-range dilution of 1:3000 was chosen. As a control OMP antiserum was either omitted or replaced with normal goat serum. No immunostaining was ever observed in control sections. The sections were mounted, air dried, dehydrated in a graded series of ethanol, cleared in xylene and coverslipped with Permount. The OMP antiserum was kindly provided by Dr Frank Margolis.

Aspiration lesions

In three rats the right olfactory epithelium was exposed and visualized using a surgical microscope. All tissue in the rostral third of what was judged the olfactory epithelium was aspirated. The surgical site was covered with Gelfoam, the scalp was closed with wound clips and the rat was placed on a heating pad until it recovered its righting reflex.

Three, eleven or sixteen days later one of these rats was anesthetized, the intact olfactory sac on the left and the surgical site on the right side were exposed and each sac was injected with 10 µl of 1% WGA–HRP. After a 24 h survival period the animals were deeply anesthetized and killed by perfusion and the olfactory bulbs processed as described above.

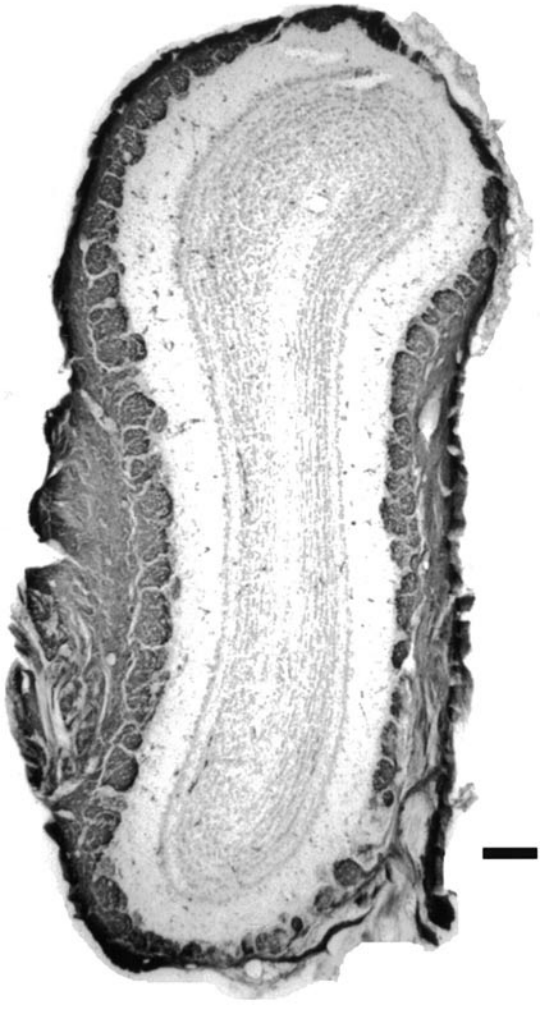

Figure 1 Coronal section through the olfactory bulb of a vegetable oil-treated control. Note that essentially every glomerulus is filled with dense WGA–HRP reaction product. Calibration bar 0.2 mm.

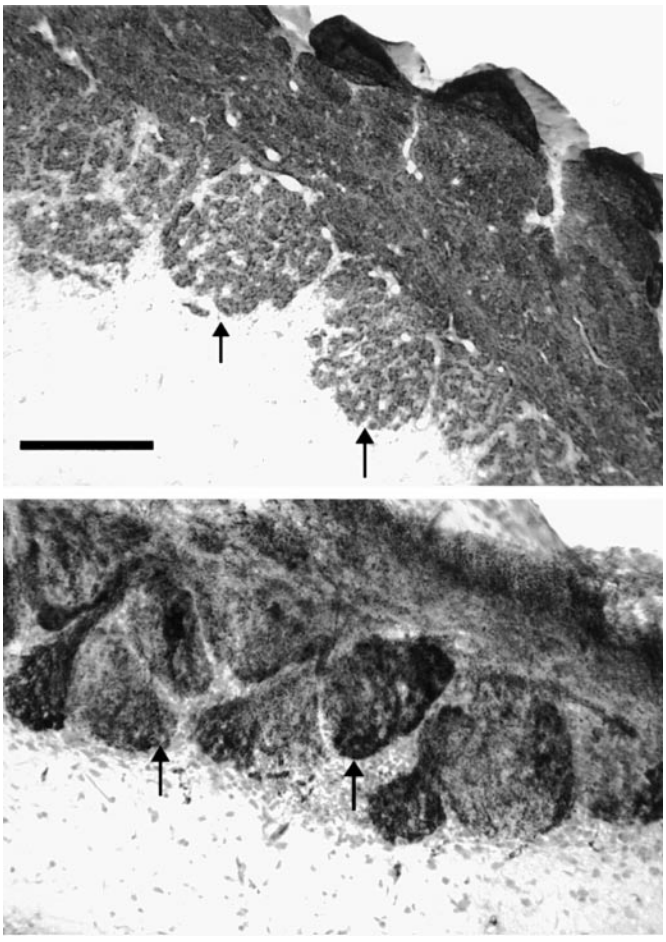

Figure 2 Olfactory bulb glomeruli in the dorsal part of the bulb of a control rat. (Top) OMP in glomeruli and nerve layer. (Bottom) A section ~0.15 mm more posterior showing glomeruli filled with WGA–HRP reaction product. Arrows in each figure identify selected glomeruli. Calibration bar 0.03 mm.

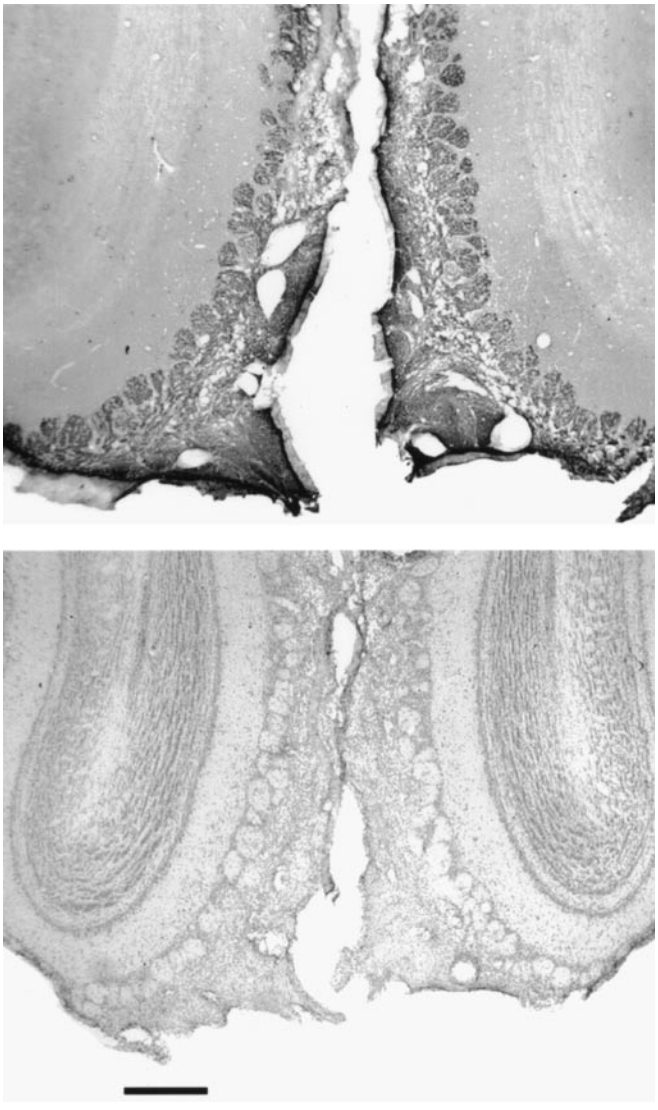

Figure 3 Ventromedial aspect of the olfactory bulb from a rat treated with 250 mg/kg 3-methyl indole 6 days prior to killing. (Top) A section processed to show OMP. Note moderate to dense OMP in each glomerulus. (Bottom) A section ~0.15 mm more posterior processed to show WGA–HRP reaction product. Note the absence of reaction product. Calibration bar 0.2 mm.

Data analysis

Each olfactory bulb section of control, 3-MI-treated and aspiration-lesioned rats was examined microscopically using bright field and polarized light microscopy at ×20–400. Selected sections were photographed using a RS Photometrics Cool Snap digital camera and captured in Adobe Photoshop. Representative photographs were assembled as individual panels in Figures 1–6 and printed using a Fujix Pictography 3000 at 400 d.p.i.

Results

Virtually all identified glomeruli from the WGA–HRP-reacted bulb sections of the three saline-treated control rats were filled with dense or moderately dense reaction product (Figure 1). Likewise, all identified glomeruli from the OMP-reacted bulb sections were darkly immunostained (Figure 2).

In each of the four 3-MI-treated rats there was a severe reduction in or complete loss of WGA–HRP reaction product in most bulbar glomeruli. The loss was most severe in the dorsal and ventromedial quadrants of the bulb and most glomeruli in these regions contained no detectable reaction product. However, in the alternate sections of the same rats virtually all glomeruli in sections processed for the presence of OMP had dense or moderate levels of staining. The different outcomes for these two indices of afferent input to the olfactory bulb are shown in Figures 3 (ventromedial area of the bulb) and 4 (dorsal bulb). Generally, in glomerular regions that contained no WGA–HRP reaction product OMP staining appeared somewhat less dense than in regions that contained dense WGA–HRP product, but these differences were not marked.

Aspiration of rostral epithelial tissue from the right

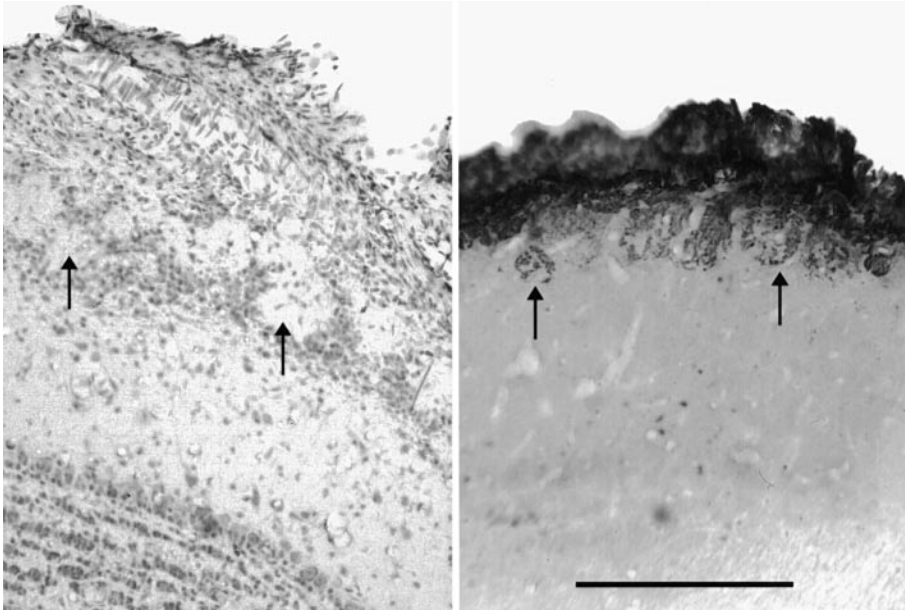

Figure 4 Glomeruli in the dorsal aspect of the olfactory bulb from a rat treated with 250 mg/kg 3-methyl indole 6 days prior to killing. (Left) A section processed to show WGA–HRP reaction product. Note the absence of reaction product. (Right) A section ~0.15 mm more posterior processed to show OMP. Note moderate to dense OMP immunostaining in each glomerulus. Arrows in each figure identify selected glomeruli. Calibration bar 0.2 mm.

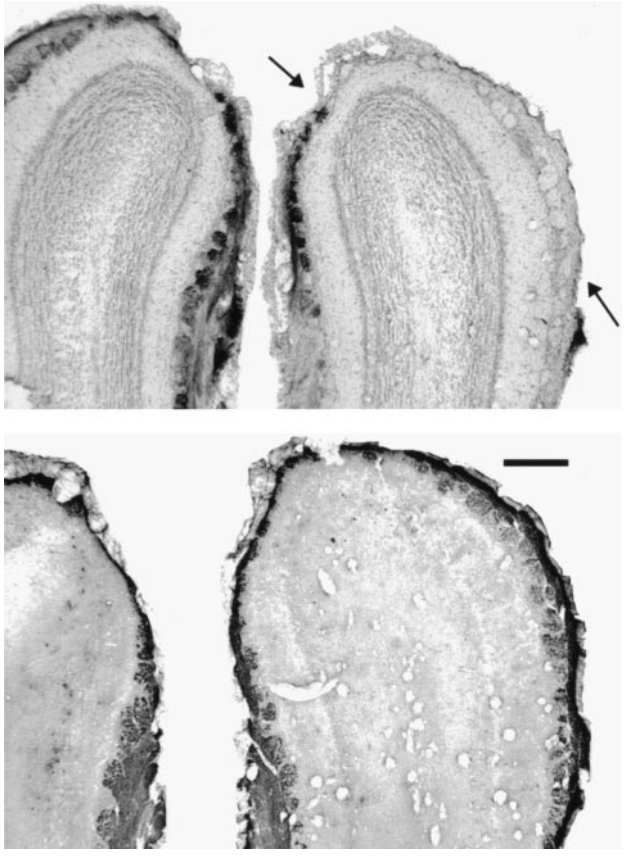

Figure 5 Sections from a rat killed 4 days after an extensive aspiration lesion of the rostral portion of the right olfactory epithelium. (Top) A section processed to show WGA–HRP reaction product. Glomeruli on the dorsal and dorsolateral surface (arrows) contained no detectable reaction product in this case or in the 12 and 17 day survival rats (see Figure 6). (Bottom) A section ~0.15 mm more posterior processed to show OMP. Note moderate to dense olfactory marker protein immunostaining in each glomerulus in the dorsal and dorsolateral aspects of the olfactory bulb. Calibration bar 0.2 mm.

olfactory sac resulted in a complete loss of WGA–HRP reaction product in the dorsolateral quadrant of the right olfactory bulb in the 4, 12 and 17 day survival cases (Figure 5). Normal levels of reaction product were observed in the corresponding area of the contralateral bulb.

The density of OMP immunostaining in the same dorsolateral quadrant on the experimental side in these three cases varied as a function of survival time. Glomeruli in the dorsolateral bulb in the 4 day survival animal stained darkly for OMP (Figure 5). Although the density of OMP immunostaining was less than in the same region in the contralateral control (left) bulb, each glomerulus was clearly stained. OMP immunostaining was appreciably less dense in the 12 day survival case. Nonetheless, almost all identifiable glomeruli in the dorsolateral quadrant on the experimental side contained discernable OMP. Little or no OMP immunostaining in this same region of the bulb could be detected in the 17 day survival case (Figure 6).

Discussion

The absence of WGA–HRP reaction product in a discrete region of the glomerular layer in the 3 day lesion cases indicates that at least some, if not all, of the axons of damaged receptor neurons failed to transport this enzyme. In other cases, not reported here, we have evidence that this

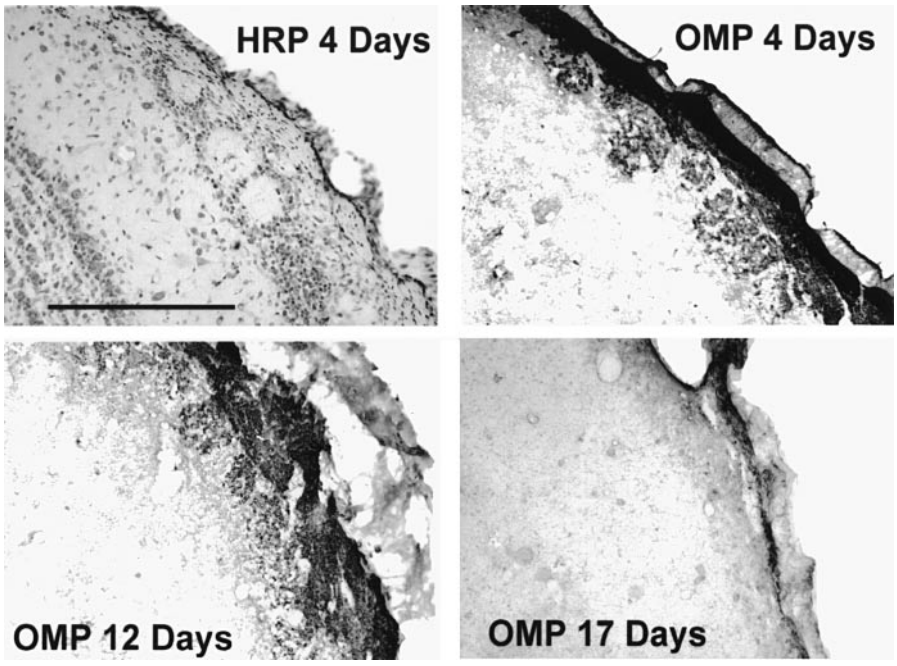

Figure 6 Sections illustrating glomeruli in the dorsolateral aspect of the olfactory bulb of rats that received aspiration lesions of the ipsilateral rostral olfactory epithelium. **(A)** A section from a 4 day survival rat that was processed to show WGA–HRP reaction product. None of the glomeruli in this area contained detectable reaction product. **(B)** A section ~0.15 mm more posterior from the same rat processed to show OMP. Note moderate to dense OMP immunostaining in each glomerulus. **(C)** A section from the same bulbar region processed to show OMP from a 12 day survival rat. Each glomerulus in this field contained light to moderately dense OMP immunostaining. **(D)** A section from the same bulbar region processed to show OMP from a 17 day survival rat. Glomeruli in this section contained no detectable OMP immunostaining. Calibration bar 0.2 mm.

failure in anterograde transport occurs within 24 h after damage to the epithelium. These results support the contention that anterograde transport of WGA–HRP from the olfactory epithelium to the olfactory bulb provides a valid measure of functional connections between the periphery and the olfactory bulb (Setzer and Slotnick, 1998a; Schwob *et al*., 1999). If some severed axons were still capable of anterograde transport then one might expect to find very light reaction product in some or most of the experimentally deafferented glomeruli. However, in the three rats with aspiration lesions of the rostral epithelium none of the glomeruli in the affected quadrant of the bulb contained detectable reaction product. Thus, it seems reasonable to assume that few if any such false positive outcomes would occur. Boehm *et al*. also found a complete absence of HRP transport in the rat olfactory bulb after multiple applications of zinc sulfate to the nasal vault (Boehm *et al*., 1991), a treatment known to destroy olfactory epithelium (Burd, 1993). This issue is relevant because Matsumoto and Scalia found that thin axons in the frog optic nerve transported HRP for several weeks after transection of the nerve (Matsumoto and Scalia, 1981). The present results indicate that this probably does not occur in the damaged mammalian olfactory nerve. Severed olfactory axons in mammals may degenerate much more quickly than those of the severed frog optic nerve or, alternatively, the damage produced by zinc sulfate (Boehm *et al*., 1991), aspiration lesions or treatment with 3-MI may block the ability of remaining axons to transport the enzyme.

In sharp contrast to the rapid and essentially complete loss of anterograde WGA–HRP transport after damage to the epithelium, the loss in OMP immunostaining was much more gradual. Our results indicate that OMP can be detected in bulbar glomeruli of 3-MI-treated and epithelial-lesioned rats for several days after deafferentation. Clearly, the degradation of the receptor cell axon and constituent proteins occurs only slowly and the presence of OMP in short survival cases may not provide a valid marker for functional connections between the periphery and the olfactory bulb.

Acknowledgements

We thank Drs Catherine Priest and Igor Kratskin for help with the OMP immunohistochemistry. This research was supported by NIH/NIDCD grant DC 00245 to B.J.D.

References

Boehm, N., Lazarus, C. and **Aron, C.** (1991) *Interactions of testosterone with the olfactory system in the display of mounting behavior in the female rat*. Physiol. Behav., 50, 1001–1006.

Burd, G.D. (1993) *Morphological study of the effects of intranasal zinc sulfate irrigation on the mouse olfactory epithelium and olfactory bulb*. Microsc. Res. Tech., 24, 195–213.

Hartman, B.K. and **Margolis, F.L.** (1975) *Immunofluoresence identification of the olfactory marker protein*. Brain Res., 96, 176–180.

Kream, R.M. and **Margolis, F.L.** (1984) *Olfactory marker protein: turnover and transport in normal and regenerating neurons*. J. Neurosci., 4, 868–879.

Matsumoto, D.E. and **Scalia, F.** (1981) *Long-term survival of centrally projecting axons in the optic nerve of the frog following destruction of the retina*. J. Comp. Neurol., 202, 135–155.

Meredith, M. and **O'Connell, R.J.** (1988) *HRP uptake by olfactory and vomeronasal receptor neurons: use as an indicator of incomplete lesions and relevance for non-volatile chemoreception*. Chem. Senses, 13, 487–515.

Mesulum, M. (1982) Tracing Neuronal Connections with Horseradish Peroxidase. Wiley, New York.

Monti-Graziadei, G.A., Margolis, F.L., Harding, J.W. and **Graziadei, P.P.C.** (1977) *Immunocytochemistry of the olfactory marker protein*. J. Histochem. Biochem., 25, 1311–1316.

Peele, D.B., Allison, S.D., Bolon, B., Prah, D., Jensen, K.F. and Morgan, K.T. (1991) *Functional deficits produced by 3-methylindole-induced olfactory mucosal damage revealed by a simple olfactory learning task*. Toxicol. Appl. Pharmacol., 107, 191–202.

Schwob, J.E., Youngentob, S.L., Ring, G., Iwema, C.L. and **Mezza, R.C.** (1999) *Reinnervation of the rat olfactory bulb after methyl bromide-induced lesion: timing and extent of denervation*. J. Comp. Neurol., 412, 439–457.

Setzer, A.K. and **Slotnick, B.** (1998a) *Disruption of axonal transport from olfactory epithelium by 2-methyl indole*. Physiol. Behav., 65, 479–487.

Setzer, A.K. and **Slotnick, B.** (1998b) *Odor detection in rats with 3-methyl indole-induced reduction of sensory input*. Physiol. Behav., 65, 489–496.

Walters. E., Grillo, M, Oestreicher, A.B. and **Margolis, F.L.** (1966) *LacZ and OMP are co-expressed during ontogeny and regeneration in olfactory receptor neurons of OMP promoter-lacZ transgenic mice*. Int. J. Dev. Neurosci., 14, 813–822.

Xu, W. and **Slotnick, B.** (1999) *Olfaction and peripheral olfactory connections in methimazole-treated rats*. Behav. Brain Res., 102, 41–50.

Accepted February 25, 2001