Chem. Senses 23: 171–179, 1998

Replacement of Receptor Cells in the Hamster Vomeronasal Epithelium after Nerve Transection

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Abstract

Chemoreceptor cells in the vomeronasal and olfactory epithelium are replaced following experimentally induced degeneration. This study analyzes quantitatively the time course and degree of vomeronasal receptor cell replacement. Unilateral transection of the vomeronasal nerves in adult hamster was used to induce a retrograde degeneration of receptor cells in the vomeronasal organ. Histological measurement of both number of receptor cells and epithelial thickness were made for recovery times from 0 to 60 days. After nerve transection, there was a gradual degeneration of receptor cells, the number decreasing to 50% of control by day 2 and 16% by day 6. During days 7–15 maximum receptor cell replacement was observed. Cell number increased rapidly and reached a peak on day 15. At recovery times of 40–60 days, cell number returned to the control level. Epithelial thickness, however, decreased to 60–70% during the degeneration period (days 4–6) and did not return to control levels. After 40–60 days epithelial thickness remained at 70% of control. These results demonstrate that vomeronasal receptor cells are replaced following degeneration, but epithelial thickness does not return to control levels. These findings suggest that the number of replacement cells is not limited by the reduced thickness of the epithelium, and that recovery mechanisms may function to restore an optimum number of receptor cells.

Introduction

Chemoreceptor cells in both the olfactory and vomeronasal epithelium detect chemical signals in the environment, providing important information for the survival of most species. Many vertebrates rely chiefly on chemoreceptor input for locating food, social interactions, mating and reproduction, and detecting the presence of predators. Chemoreceptor neurons are unique in the vertebrate nervous system in that they undergo continuous replacement (for reviews see Graziadei and Monti-Graziadei, 1978; Halaz, 1990; Farbman, 1992; Morrison and Costanzo, 1992). Cell turnover is supported by a neurogenic population of cells at the base of the neuroepithelium. These cells maintain a neurogenic capacity throughout an animal's lifespan. Several studies in the olfactory system have shown that replacement of neurons occurs following experimentally induced degeneration of mature neurons (Graziadei, 1973; Graziadei and Monti-Graziadei, 1979, 1980; Monti-Graziadei and Graziadei, 1979). Quantitative studies of receptor cell replacement and functional recovery in the olfactory system have provided important information on the unique neurogenic capacity of this system (Costanzo and Graziadei, 1983; Samanen and Forbers, 1984; Costanzo, 1985).

The vomeronasal system, like the olfactory system, contains chemoreceptor cells. The vomeronasal system consists of the vomeronasal organ (VNO), the accessory olfactory bulb (AOB) and higher centers within the central nervous system. This chemosensory system is thought to play a critical role in the perception and processing of pheromones (Wysocki, 1979; Halpern, 1987; Halaz, 1990). The VNO is located at the base of the nasal cavity adjacent to the nasal septum. It is enclosed in a delicate capsule of overlapping bony plates and is a tubular structure opening rostrally into the nasal cavity and ending blindly at its caudal end. It consists primarily of a sensory epithelium, nonsensory epithelium, blood vessels and secretory glands (Figure 1). Receptor cells give rise to axons that project to the accessory olfactory bulb. Wilson and Raisman (1980) were the first to suggest that the VNO receptor cell population is replaced every 2–3 months in the mouse. Barber (1981a) used retrograde axonal transport of HRP combined with [^{3}H]thymidine labeling of dividing cells to demonstrate that axons of newly formed receptor cells reach their appropriate target, the AOB. Barber and co-workers (1978a,b, 1981b) also reported that replacement of neurons occurs following experimentally induced degeneration in

adult mice. In the present study immunocytochemistry and electron microscopy was used to confirm receptor cell replacement in the hamster vomeronasal organ. A quantitative analysis was made to determine the time course and degree of receptor cell replacement following unilateral transection of the vomeronasal nerves. Findings are compared to those reported for receptor cell replacement in the olfactory system. Preliminary results from this study have been reported in abstract form (Ichikawa *et al.*, 1995).

Materials and methods

Animals and surgery

Sexually mature male hamsters (*Mesocricetus auratus*), ranging in age from 2 to 4 months, were used in this study. Prior to surgery animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A small incision was made and the frontal bone covering the left olfactory bulb was then removed. A custom-made Teflon microdissection instrument (Teflon blade) was used to cut nerve fibers passing between the cribriform plate and the olfactory bulb. First the rectangular-shaped Teflon blade was positioned at the anterior-rostral tip between the olfactory bulb and cribriform plate. Then it was inserted downward along the contour of the cribriform plate until it reached the ventral-posterior end of the olfactory bulb. The blade was then moved from the anterior-rostral region across to the mediocaudal region, thereby transecting the vomeronasal nerve fibers at the point where they exit the cribriform plate to traverse along the medial surface of the bulb. Following the left vomeronasal nerve transection procedure the incision was sutured closed and animals were monitored until they fully recovered from the anesthesia, after which they were returned to their home cage.

Immunocytochemistry

Animals were anesthetized and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Vomeronasal tissue were cut into 20 μm sections using a freezing microtome. Sections were mounted onto gelatin-coated slides, rinsed with PBS and then incubated with 0.2% Triton X-100 and 5% horse serum for 1 h. Sections were then incubated overnight with mouse anti-neuron specific enolase antibody (CHEMICON) diluted 1:10 in PBS containing 1% bovine serum albumin at 4°C. After washing three times in PBS for at least 10 min each, sections were incubated with biotinylated secondary antibodies (1:100) in BSA–PBS for 1 h at room temperature. Sections were washed again in PBS, and then incubated with fluorescence-labeled streptavidin (1:100) in BSA–PBS for 1 h, followed by another PBS wash.

Electron microscopy

Animals were perfused transcardially with 0.9% saline followed by 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer. The VNO was immersed in the fixative at 4°C for a minimum of 12 h. The fixed VNO was then washed in the buffered solution and coronal slices (0.5–1.0 mm thick) were cut with a razor blade and postfixed with 1% osmium tetroxide in the buffer. The fixed slices were dehydrated and embedded in epoxy resin (Quetol 812). Sections 1 μm thick stained with toluisin blue were prepared to determine tissue orientation and for light microscopic observation. Ultrathin sections with a silver-gray interference color were cut and mounted on formval-coated, one-hole copper grids. After staining with uranyl acetate and lead citrate, the vomeronasal sensory epithelium was examined with a JEOL JEM 1200 EXII electron microscope.

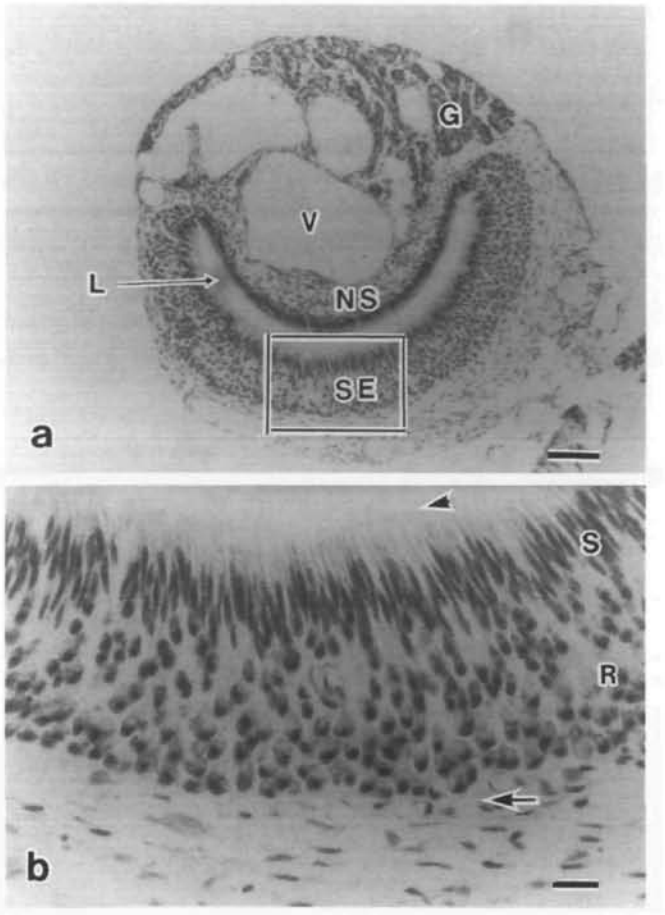

Figure 1 Histological section taken from the central region of the vomeronasal organ. **(a)** At low magnification a crescent-shaped lumen (L) is seen bordered by a sensory (SE) and nonsensory (NS) epithelium. Blood vessels (V) and glands (G) are also present. **(b)** Enlargement from a sample region of the sensory epithelium (SE). Supporting (S) cells are located near the luminal surface and receptor (R) cells are located in deeper layers of the epithelium. Arrowhead indicates the luminal surface, arrow marks the basement membrane. Bar: 100 μm in (a), 20 μm in (b).

Quantitative analysis

Forty-one experimental animals were included in this

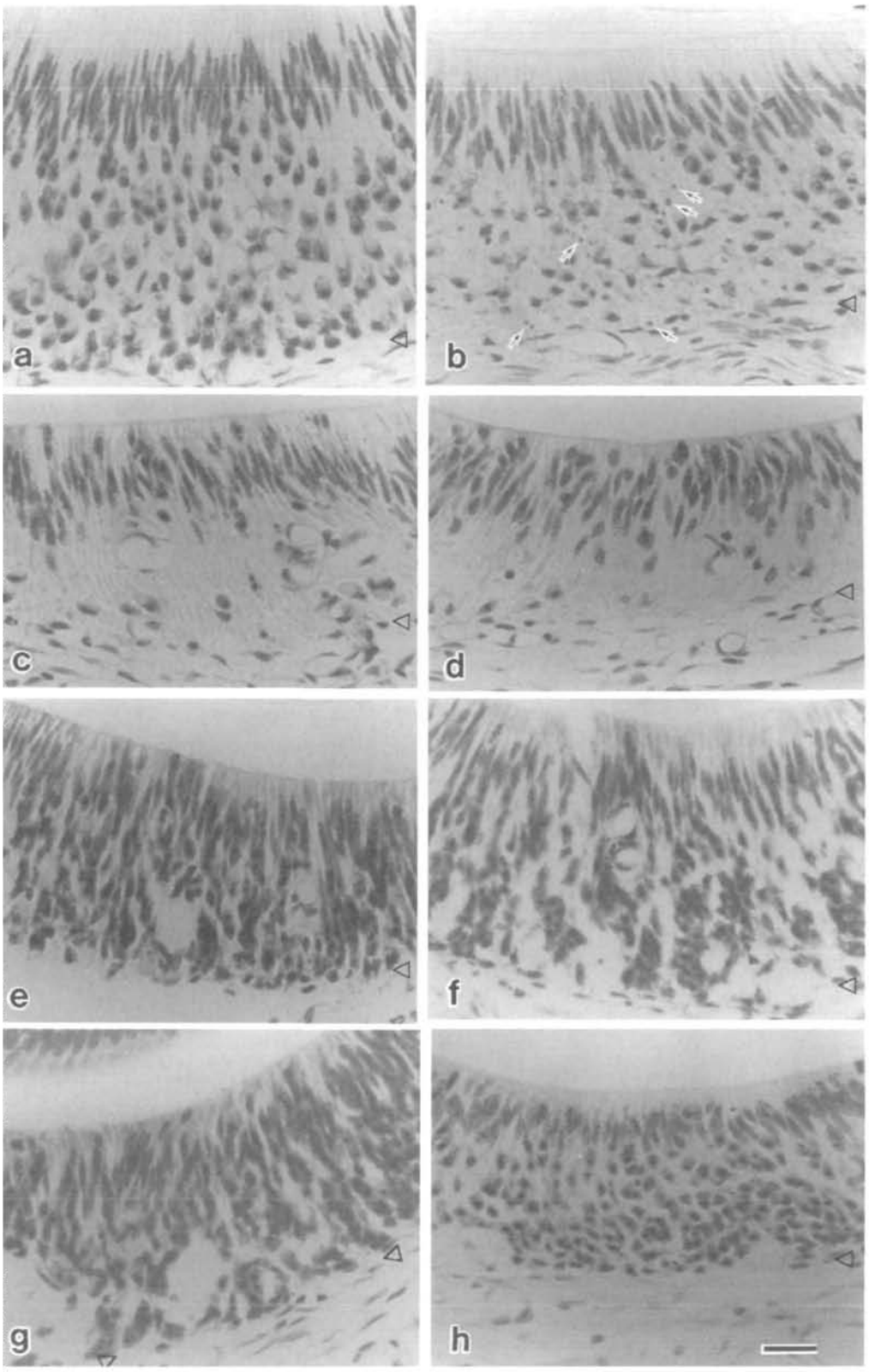

Figure 2 Sections of sensory epithelium illustrating stages of recovery following vomeronasal nerve transection. **(a)** Day 0, **(b)** day 2, **(c)** day 4, **(d)** day 6, **(e)** day 10, **(f)** day 15, **(g)** day 42, **(h)** day 60. Small arrows in (b) indicate picnotic vesicles. Triangles mark the basement membrane of the epithelium. Bar: 20 μm.

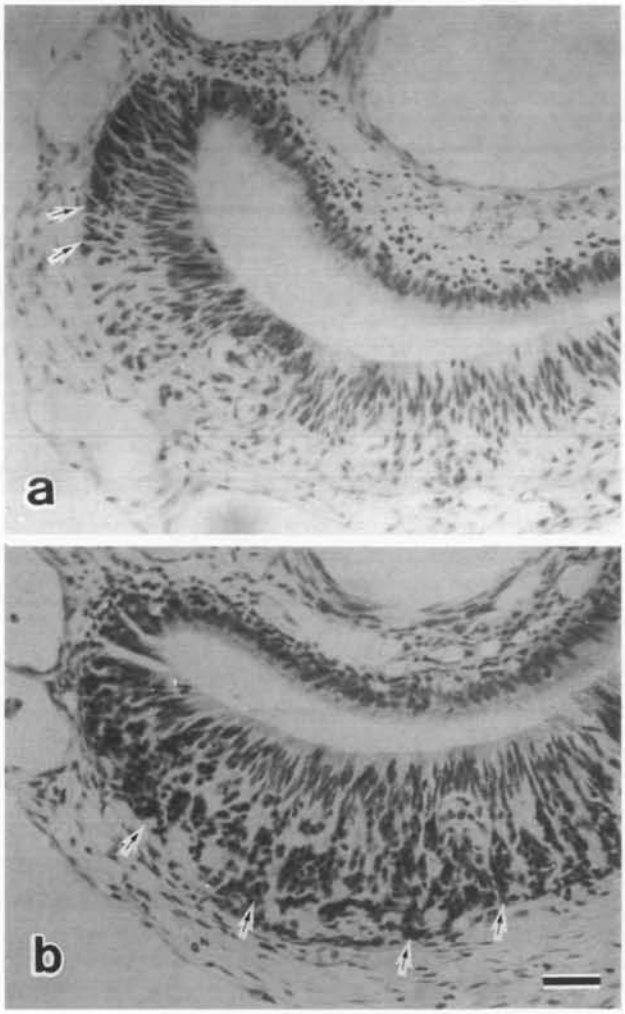

Figure 3 Photomicrographs illustrating the boundary region between the sensory and nonsensory epithelium at day 6 **(a)** and day 10 **(b)** following nerve transection. Arrows indicate clusters of darkly stained cells. Bar: 50 μm.

analysis; 2–5 animals were used for each postoperative recovery time of 0 (n = 4), 2 (4), 4 (4), 6 (5), 7 (2), 10 (4), 15 (5), 21(4), 25 (2), 42 (2) and 60 (5) days. After the assigned recovery period animals were anesthetized and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The vomeronasal organs and whole brains were dissected and embedded in paraffin. The VNO was cut serially into 10 μm cross sections. Every third section was stained with cresyl violet or hematoxylin–eosin.

Quantitative measurements were taken from three consecutive sections selected from the most central region of the vomeronasal organ (Figure 1) and averaged. Within each section, a 280 μm length of epithelium from the center (thicker) region of the sensory epithelium was selected for data measurement (see boxed area in Figure 1a). The sensory epithelium was photographed and epithelial

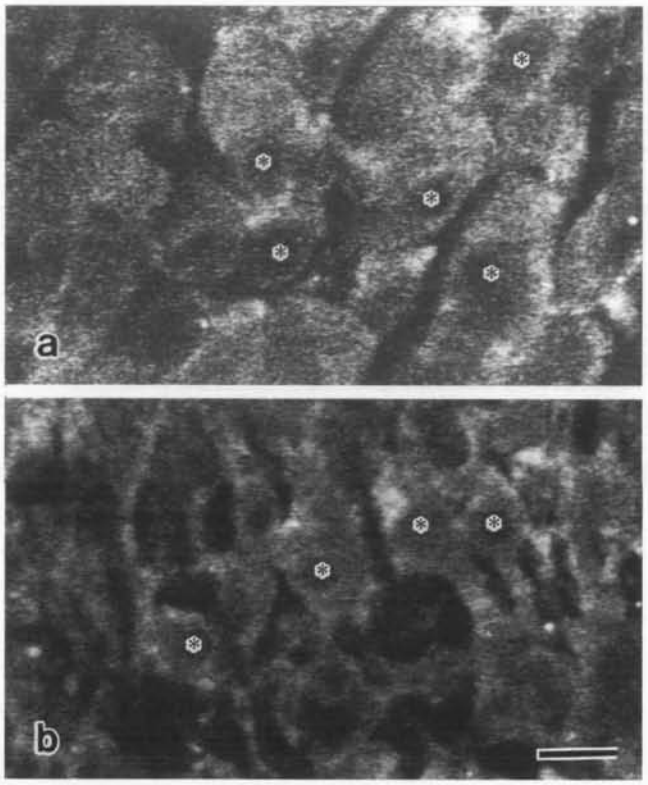

Figure 4 Confocal laser scan micrographs showing immunocytochemical labeling of neuron-specific enolase. **(a)** Control section of vomeronasal sensory epithelium. **(b)** Sensory epithelium at day 21 following nerve transection. Asterisks indicate staining of receptor cells. Bar: 5 μm.

measurements and cell counts were made from photographic prints magnified at 500×.

On the basis of morphological criteria, three layers were defined: (i) a superficial layer formed by cell processes extending from underlying cell types; (ii) a supporting cell layer consisting of supporting cells; and (iii) a receptor cell layer consisting of receptor cells. The two major cell types—receptor cells and supporting cells—can be seen in Figure 1(b). Near the luminal surface, supporting cells (S) have darkly stained nuclei and are elongated in shape. Receptor cells (R) have abundant cytoplasm and rounded nuclei with clear chromatin. For each tissue section, three parameters were measured within the 280 μm sample of sensory epithelium: (i) number of receptor cell nuclei; (ii) thickness of the receptor cell layer; and (iii) total thickness of the vomeronasal epithelium. Data from each of the three consecutive sections were averaged. To account for normal variability in both epithelial thickness and cell number, measurements from the contralateral VNO (control side) were used as an internal control. For each animal data measurements from the experimental side were expressed as a percentage of the control side (Figure 6).

Statistical comparisons of differences between recovery days were performed using Student's t-test.

Results

Histological observations

The time course of histological changes during recovery of the vomeronasal epithelium were examined following nerve transection. Immediately after nerve transection (3–4 h), the vomeronasal epithelium looked similar to the control sections (Figure 2a). Degenerative changes were first observed on day 2. At this time most receptor cell nuclei appeared darker and reduced in size. Numerous darkly stained granular deposits (picnotic vesicles) were also observed distributed throughout the epithelium (Figure 2b). On day 4, significant degeneration was observed and there was a marked reduction in the receptor cell number (Figure 2c). Supporting cell numbers did not change, but the arrangement of cells was somewhat irregular. There was also variability in the amount of degeneration observed among animals. On day 6, maximum degeneration had occurred. Only a few receptor cells were present at this time (Figure 2d). The arrangement of cells observed in the supporting cell layer was irregular when compared to the control side. The intercellular space between supporting cells was also increased. Clusters of dark cells were observed in the lower epithelial regions adjacent to the boundary region with nonsensory epithelium (Figure 3a). These dark cells have been previously identified as indicators of newly formed receptor cells (Barber and Raisman 1978a,b). On day 10, replacement cells were observed in the epithelium (Figure 2e). A considerable increase in the number of dark cells was observed in the deep areas of the receptor cell layer (Figure 3b). On day 15, dark cells were distributed unevenly in clusters throughout the receptor cell layer (Figure 2f). This uneven distribution was also observed on days 21 and 25. Some areas of the epithelium did not recover well and the number of receptor cells observed in these areas remained low. On days 42 and 60, the distribution of darkly stained cells continued to be unevenly distributed. Numerous acellular areas or poorly populated areas were observed within the receptor cell layer (Figure 2g,h).

Immunocytochemical observations

To confirm that the newly formed cells were receptor cells (e.g. neurons), a neuron-specific enolase imunocytochemical study was performed. We examined the vomeronasal epithelium on days 14 and 21, and observed that the antibody for neuron-specific enolase reacted positively with cells within the receptor cell layer. The immunopositive cells were stained more weakly on the recovery side than on the control side. The size of immunopositive cells was also smaller on the recovery side than the control side (Figure 4).

Electron microscopic observation

The fine structure of the newly formed epithelial cells was also examined to identify characteristics of vomeronasal receptor cells (Figure 5b). Most cells were oval shaped within the perikaryon, mitochondria, rER and Golgi complex, and free polysomes were observed. In the nucleus, a large nucleoli was present. Compared to receptor cells on the control side (Figure 5d), the structure of some organelles was not well developed. For example, the typical stacked smooth endoplasmic reticulum could not be observed in cells on the transected side (Figure 5b). The characteristics of the newly formed cells suggested that they are immature receptor cells developing within the vomeronasal epithelium (Garrosa and Coca, 1991; Garrosa *et al.*, 1992). The morphological characteristics of supporting cells observed on the transected side were no different to those on the control side (Figure 5a,c).

Quantitative analysis

Quantitative measurements of the number of receptor cells and epithelial thickness were obtained at different stages of recovery (Table 1, Figure 6). These data define the time course of cell recovery in the VNO.

Number of receptor cells (Figure 6)

Measurements of receptor cell number were obtained from standard 280 μm long samples of sensory epithelium. On the control side, cell number averaged 175 ± 5.1 cells. For each animal measurements from the experimental side were expressed as a percentage of the control side (Table 1). Two to three hours after nerve transection (day 0) the number of receptor cells measured on the experimental side was similar to that on the control side (93% of control). By day 2 there was a significant decrease in the number of receptor cells (50.5% of the control), and by day 4 additional degeneration had occurred, leading to a further reduction in cell number (20.5%). By day 6 the reduction in cell number reached a minimum (15.8%). During this same time period supporting cell numbers did not change and remained within 103% of control. On day 10, a significant replacement of receptor neurons was observed in the epithelium. The number of receptor cells increased to 81.0%, and by day 15 the number of replacement cells reached a maximum level of 118.4%. Although this number exceeded control levels, there was considerable variability and this increase was not statistically different from control levels ($P = 0.24$). By day 21, however, the number of replacement cells had decreased back to 68.8% of control. This decrease was statistically significant ($P < 0.05$). At longer recovery times (25–42 days) receptor cell number gradually increased, reaching 74% by day 25 and 88% by day 42. On day 60, the number of receptor cells returned to control levels (96.0% of control).

Thickness of epithelium

On the control side, the total thickness of the vomeronasal sensory epithelium measured in the central region averaged 130 ± 4.4 μm. The average thickness of the receptor cell layer was 83 ± 2.8 μm. The time course of recovery for both

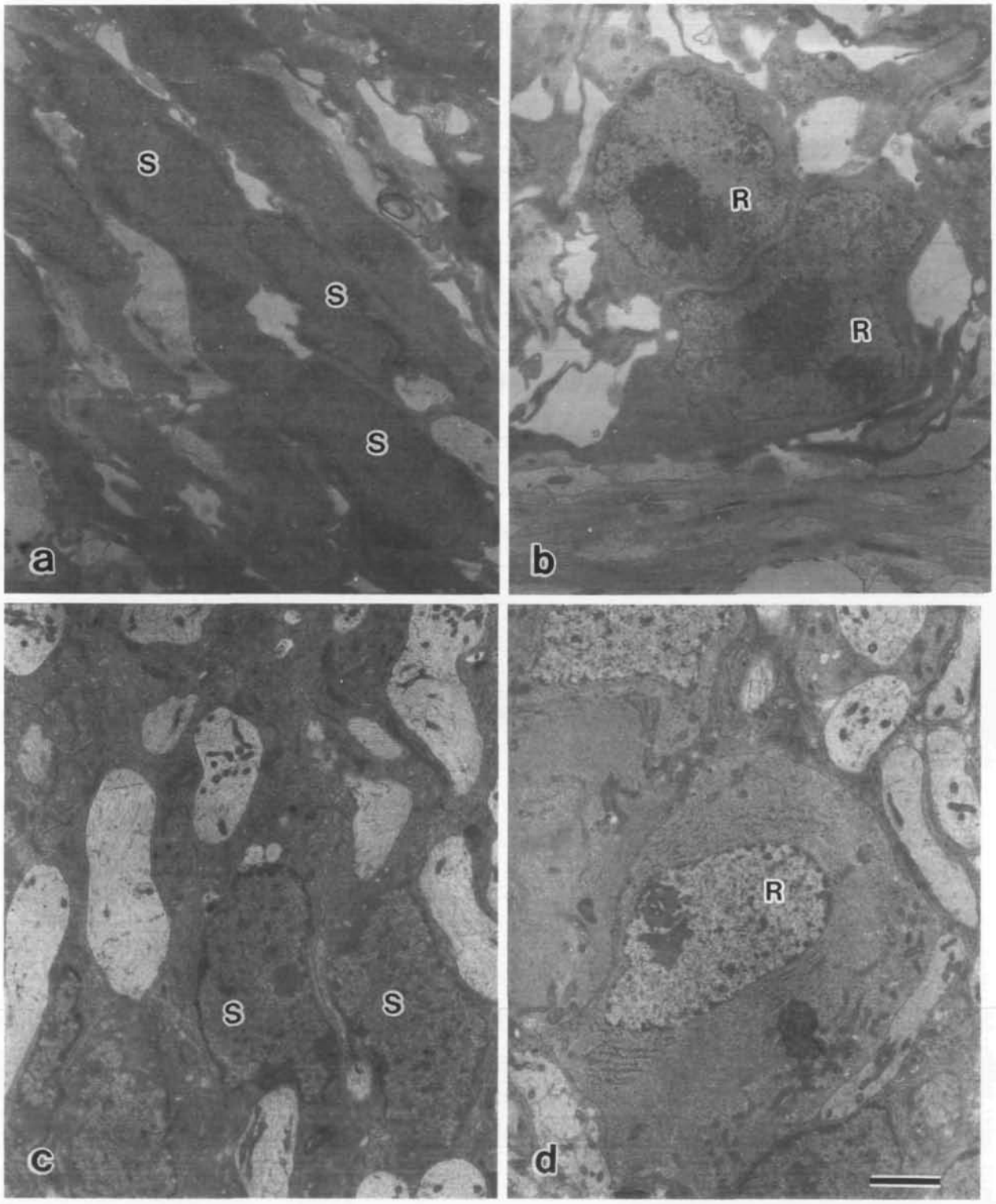

Figure 5 Electron micrographs showing supporting cells **(a)** and receptor cells **(b)** at day 21 following nerve transection. Secions from control epithelium are shown in **(c)** and **(d)**. R, receptor cell; S, supporting cell. Bar: 2 μm.

the total thickness and the thickness of the receptor cell layer was studied. On day 0 (3–4 h after transection), no changes were observed in either total thickness or thickness of the receptor cell layer. On day 2, there was a decrease in epithelial thickness (85.0 and 82.2% of control for total and receptor cell layer thickness respectively), and on day 4, the

Table 1 Changes in the number of receptor cells, and thickness (total and receptor cell layer) in the vomeronasal epithelium following vomeronasal nerve transection

Recovery day	Animals (*n*)	No. of cells (receptor cells)	Epithelial thickness (μm)	
			Total	Receptor cell layer
0	4	96.3 ± 3.0	94.5 ± 2.1	92.2 ± 1.0
2	4	50.5 ± 4.0	85.0 ± 3.5	82.2 ± 5.9
4	4	20.5 ± 10.3	72.5 ± 8.1	61.7 ± 9.3
6	5	15.8 ± 5.7	65.6 ± 6.1	55.2 ± 6.0
7	2	37.0	70.5	61.0
10	4	81.0 ± 5.2	57.0 ± 4.9	49.0 ± 3.7
15	5	118.4 ± 18.0	79.8 ± 5.9	70.6 ± 6.6
21	4	68.8 ± 3.9	59.5 ± 7.9	52.7 ± 6.6
25	2	74.0	70.5	64.5
42	2	88.0	50.4	51.5
60	5	96.0 ± 12.0	69.0 ± 10.0	72.0 ± 10.7

Data are expressed as a percentage of the control side (mean ± SEM).

thickness of epithelium continued to decrease (72.5 and 61.7%). On day 6, there was a further reduction in the thickness, and on day 10, the reduced thickness of epithelium remained low (57.0 and 49.0%). Interestingly, by this time the number of receptor cells had increased significantly. On day 15, the thickness of the epithelium increased a little but continued to remain below that of control (79.8 and 70.6 % of control, $P < 0.05$). On day 21, the thickness of epithelium decreased further and did not show any evidence of recovery. At longer recovery times (25–42 days) the thickness of the epithelium continued to remain below control levels, and by day 60 full recovery of epithelial thickness had still not occurred.

Discussion

Replacement of receptor cells in the VNO

Barber and Raisman (1978a,b) reported that the majority of receptor cells in the mouse vomeronasal epithelium were replaced after vomeronasal nerve section or removal of the accessory olfactory bulb. Wang and Halpern (1982a,b) also reported the regeneration of VNO receptor cells in garter snake after vomeronasal nerve transection. The present study confirms in hamster that vomeronasal receptor cells are capable of replacement following nerve transection. Changes in receptor cell number suggest recovery within 60 days (96% of control). The thickness of the sensory epithelium, however, did not recover, and by 60 days had only reached 69% of control. These findings have led to two hypotheses. The first is that the total number of replacement cells is not limited by a reduced thickness of the epithelium, and that recovery mechanisms function to replace an optimum number of receptor cells. The second is that the

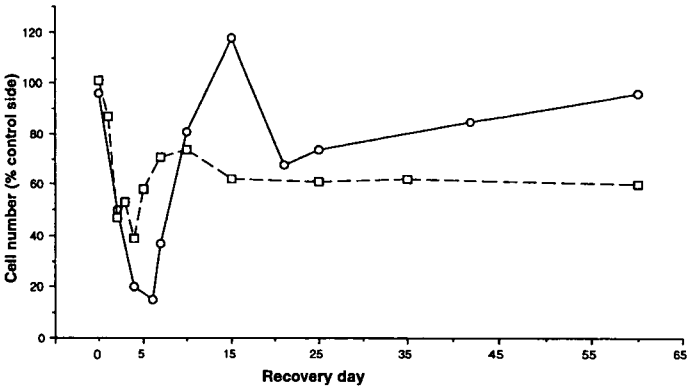

Figure 6 Changes in receptor cell number following nerve transection. Number of receptor cells measured on the experimental side are expressed as a percentage of cells on the control side (means ± SEM).

size of newly formed receptor cells are smaller than that of controls and that a larger number of receptor cells per unit volume can be packed into the epithelium even though the thickness is reduced. Consequently, the number of receptor cells can return to control levels even if the thickness of the epithelium does not recover.

The VNO in the rodent lacks a distinct basal cell layer such as that observed in the olfactory epithelium (Barber and Raisman, 1978a,b). Barber and Raisman (1978b) reported that the proliferation of receptor cells occurs at the boundary between the sensory epithelium and nonsensory epithelium, and that the newly formed receptor cells migrate to the center of the sensory epithelium. In the present study, immature receptor cells were observed at the boundary between the sensory and the nonsensory epithelium. However, clusters of immature receptor cells were also observed in the more central regions of the epithelium (Figure 3b). Thus a clear pattern of proliferation and migration to the center of the sensory epithelium was not observed in this study. It is possible that there are two mechanisms regulating neurogenesis in the vomeronasal epithelium. One that originates in the boundary regions and follows the pattern observed during normal development, and another that is activated in response to nerve transection or injury. The latter may not be restricted to boundary regions of the epithelium and may be similar to that observed in other sensory epithelia.

Comparison of recovery in the VNO and olfactory epithelium

This investigation represents the first quantitative study of receptor cell replacement in the VNO. In a similar quantitative study, the time course of receptor cell replacement was determined for the olfactory epithelium. Costanzo and Graziadei (1983) and Costanzo (1984) reported a quantitative analysis of changes in the olfactory epithelium following nerve transection in hamster. A comparison of data obtained from the VNO and olfactory epithelium is

given in Figure 6. In the olfactory system an immediate degeneration of receptor cells following nerve transection was observed, the number of cells decreasing to 47 and 39% within 2–4 days. During days 4–15 olfactory neurogenesis resulted in an increase in cell number, which was maintained at a level of 60–70% until day 194. In the present study, following transection of the vomeronasal nerve, there was gradual degeneration of receptor cells, the number decreasing to 50% by day 2 and to 16% by day 6. Maximum cell replacement was observed in the VNO during days 7–15, a longer time period than for olfactory cells. VNO cell number increased significantly and reached 118% of that on the control side by day 21. At longer recover times of 40–60 days cell number was restored to 96% of control. Several differences in the replacement of receptor cells in the olfactory and vomeronasal epithelia were noted. First the number of receptor cells in the olfactory epithelium failed to reach control levels, while in the vomeronasal epithelium the number of receptor cell recovered to control levels. A second difference noted was in the time course of recovery. Recovery of receptor cell number in the VNO occurred later than in the olfactory system, while the time course of degeneration appeared to be about the same (Figure 6). One possible explanation is that there is a different mechanism and/or capacity for receptor cell replacement for these two systems. In the olfactory epithelium, there is a layer of basal or stem cells positioned deep in the epithelium adjacent to the basement membrane. Newly formed cells migrate to the upper layers of the epithelium (Graziadei and Monti-Graziadei, 1979, 1980; Monti-Graziadei and Graziadei, 1979) and give rise to replacement receptor cells. In the vomeronasal epithelium, receptor cells are produced in the boundary regions between sensory and nonsensory epithelia (Barber and Raisman, 1978a,b). It has been reported that newly formed VNO cells migrate and traverse the epithelium horizontally. The mechanism of cell migration in the vomeronasal epithelium appears to take longer than the process observed in the olfactory epithelium. Another possibility is that there are differences in the number of stem cells. If the number of stem cells in the vomeronasal epithelium is less than that in the olfactory epithelium, then it would be reasonable to expect a longer time period for proliferation and replacement of receptor cells in the VNO.

Cell death in regeneration

In the present study, VNO receptor cell number reached a maximum and exceeded control levels on day 15. Subsequently, cell numbers decreased and finally returned to control levels. This decrease is thought to occur because of cell death. It is known that a significant number of neurons die during normal development in central nervous system (Jacobson, 1991; Kandel *et al.*, 1991). For example, loss of ~50% of retinal ganglion cells occurs in the rat during the 2 weeks after birth (Lam *et al.*, 1982; Potts *et al.*, 1982). A similar loss (118% of control at day 15 to 68% at day 21) was observed in the present study. The mechanisms of cell death during development have not been determined. Cell death observed during the replacement of vomeronasal receptor cells could represent a response to an overproduction of replacement cells. Signals that initiate the generation of replacement cells in response to nerve injury may be independent of mechanisms that maintain a steady-state population of receptor cells within the epithelium. Mechanisms that regulate the number of receptor cells and the underlying supporting matrix are not fully understood. It is possible that receptor cell replacement in the vomeronasal system may provide a useful model for the study of the cell death that occurs during normal development and maintenance of a receptor cell population.

Functional recovery of chemosensory cells

In the olfactory system, it has been shown that following nerve transection olfactory neurons are capable of re-establishing connections with the olfactory bulb and that these connections make functional synapses capable of transmitting receptor information to second-order cells in the olfactory bulb (Costanzo, 1985). Morrison and Costanzo (1995) confirmed by electron microscopy that the regenerating axons of olfactory neurons make synaptic contacts with second-order olfactory bulb neurons that have the same glomerular structure as the main olfactory bulb. Recently, Yee and Costanzo (1995) reported that the ability to perform odor-detection and odor-discrimination tasks was lost after olfactory nerve transection but returned with recovery. The recovery of olfactory function was consistent with the time course of replacement of receptor axon connections with the olfactory bulb. In the present study, the replacement of vomeronasal receptor cells was observed in the vomeronasal epithelium following nerve transection. However, it is not known whether the axons of replacement receptor cells reach the AOB or make synaptic contact with mitral/tufted cells in the AOB. Assessment of recovery of function after transection of the vomeronasal nerve has not been studied. However, the amount of VNO receptor cell replacement observed in this study suggests that functional recovery may be possible. Experiments are planned to examine the projections and synapse formation of axons from replacement receptor cells. If VNO axons are capable of re-establishing connections to targets in the accessory or main olfactory bulb, then behavioral testing could be employed to determine the extent of functional recovery in the vomeronasal system.

Acknowledgements

The authors express their thanks to Ms N. Iwasaki for technical assistance. This work was supported in part by grants-in-aid for scientific research 07304055 and 08640852 from the Ministry of Education, Science, Sports and Culture of Japan and by a grant from the National Institute on Deafness and Other

Communication Disorders, NIDCD (DC00165) awarded to R.M.C.

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Accepted on November 27, 1997