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Rapid Remodeling of Sensory Endings in the Corneas of Living Mice

Lewis W. Harris and Dale Purves

Departments of Anatomy and Neurobiology and Neurological Surgery, Washington University School of Medicine, St. Louis, Missouri 63110

The terminals of trigeminal neurons were followed over time in the corneas of living mice by repeated staining with a nontoxic fluorescent dye. The purpose of these observations was to evaluate remodeling of sensory nerve endings in an adult mammal. Video images of topically stained nerve endings within particular corneal regions were recorded initially, and then again after intervals ranging from 4 hr to 30 d. Comparison of the 2 sets of images showed that sensory endings in the corneal epithelium undergo continual rearrangement under normal circumstances. Substantial changes in terminal configuration occurred over periods as brief as a day.

The degree to which neurons and their processes are subject to ongoing remodeling in the mature nervous system is a question that bears on a wide variety of issues including the response to neural injury, the malleability of neural maps, and the basis of learning. Several technical advances—including the use of nontoxic vital dyes, the development of low-light level video cameras, and the availability of digital image processing—have recently allowed identified neural elements to be followed over time in living animals (Purves and Voyvodic, 1987). Studies using this approach have demonstrated substantial changes in the architecture of pre- and postsynaptic elements over periods of weeks to several months in the peripheral visceral and somatic motor systems of adult mammals (Purves et al., 1986, 1987; Herrera and Banner, 1987; Lichtman et al., 1987; Wigston, 1987, 1989). In the present work, we have used these techniques to follow the normal plasticity of another class of peripheral innervation, namely the sensory innervation of surface epithelia. Such epithelia are especially interesting because their constituent cells are continually sloughed and replenished. The cornea was chosen because of its transparency and accessibility. The results reported here indicate a surprisingly rapid rate of nerve terminal remodeling at this site.

Some of this work has been reported in abstract form (Harris, 1987).

Materials and Methods

Adult male mice (CF1 stain; 25–30 gm) were anesthetized by intraperitoneal injection of chloral hydrate (0.6 gm/kg). The animals were placed in a specially designed head-holder and transferred to the stage of a compound microscope. The holder allowed positioning of the head in a standard orientation, which made it easier to locate the same corneal region at the time of later observations. The animals were ventilated by positive pressure and were kept warm by a small electrical heating pad.

The external surface of the left cornea was rinsed with sterile lactated Ringer's solution (Travenol; corrected to pH 7.4, the normal pH of tears), and carefully inspected with a low power objective (4×). Animals with any sign of corneal inflammation or abrasion were excluded from further study. The nerves innervating of the cornea were stained topically with 4-(4-diethyl-aminostyryl)-N-methyl-pyridinium iodide (4-Di-2-ASP, Molecular Probes; Magrassi et al., 1987). A drop of 4-Di-2-ASP (5 μM in lactated Ringer's; pH 7.4) was applied to the cornea and left in place for 7 min. Following the staining period, the eye was washed with Ringer's. A 12 mm circular coverslip cemented to a flexible rod was used to flatten the cornea slightly, thus bringing the superficial sensory terminals into a single focal plane. Epi-illumination was provided by a 75-W xenon lamp and a Leitz I 2/3 filter block (excitation 450–490 nm; the peak absorption of 4-Di-2-ASP in water is 475 nm). Images were obtained with a silicon intensifier target (SIT) camera (General Electric model 4TE57), the output of which was fed to an image processor (Trapix 5500, Recognition Concepts) coupled to a computer (MicroVAX II, DEC) and to a video monitor. Images were manipulated by the IMAGR program (Voyvodic, 1986; Purves and Voyvodic, 1987) and photographed with a videoprinter (Polaroid Model 48).

A corneal region that showed a particularly distinctive set of superficial endings was selected for observation with a higher power water immersion objective (50×; NA 1.0). Only a single site was studied in each cornea. Video images were digitized, averaged (×16), and stored in computer disk files. The animals were then removed from the head-holder and allowed to recover. In preliminary experiments it was found that staining began to fade within 20 min after application of the dye, and was no longer detectable after 1–2 hr. Therefore, the entire staining procedure could be repeated as soon as a few hours later, or after an arbitrarily long interval. To analyze the patterns of nerve terminal staining, stored disk images were retrieved and enhanced using digital image processing techniques (Purves and Voyvodic, 1987). By comparing the pattern of staining at different intervals, changes in the arrangement of sensory endings over time could be assessed.

Exposure of the cornea to the exciting wavelengths was minimized at both the initial and final imaging sessions by controlling the intensity and duration of illumination. A graded neutral density filter (Rolyn Optics) was used to adjust the light intensity to the minimum needed to acquire a satisfactory image with the SIT camera. To control the exposure time, a shutter that opened only in conjunction with image acquisition was placed in the light path just behind the field diaphragm. The total exposure time for each corneal region studied was measured by a timing program coupled to the shutter and was typically 10–15 sec. Light intensities in these experiments were measured by a light meter and ranged from 10,000 to 20,000 lux. Safe levels of illumination were determined by observing the effects of various exposure times and intensities on stained corneas in a series of control animals. Continuous exposure of the mouse cornea to intense light for long periods (total exposures in the range of 10 million lux-sec) produced obvious damage (swelling and disruption) of corneal nerve endings within several hours. Exposures in the range of 2 million lux-sec did not cause any obvious damage to the nerve terminals, but caused the corneal epithelial cells to stain when the procedure was repeated after 2–24 hr, indicating some epithelial damage (these cells do not normally take up dye). At shorter

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Correspondence should be addressed to Lewis Harris, M.D., Department of Neurological Surgery, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110.

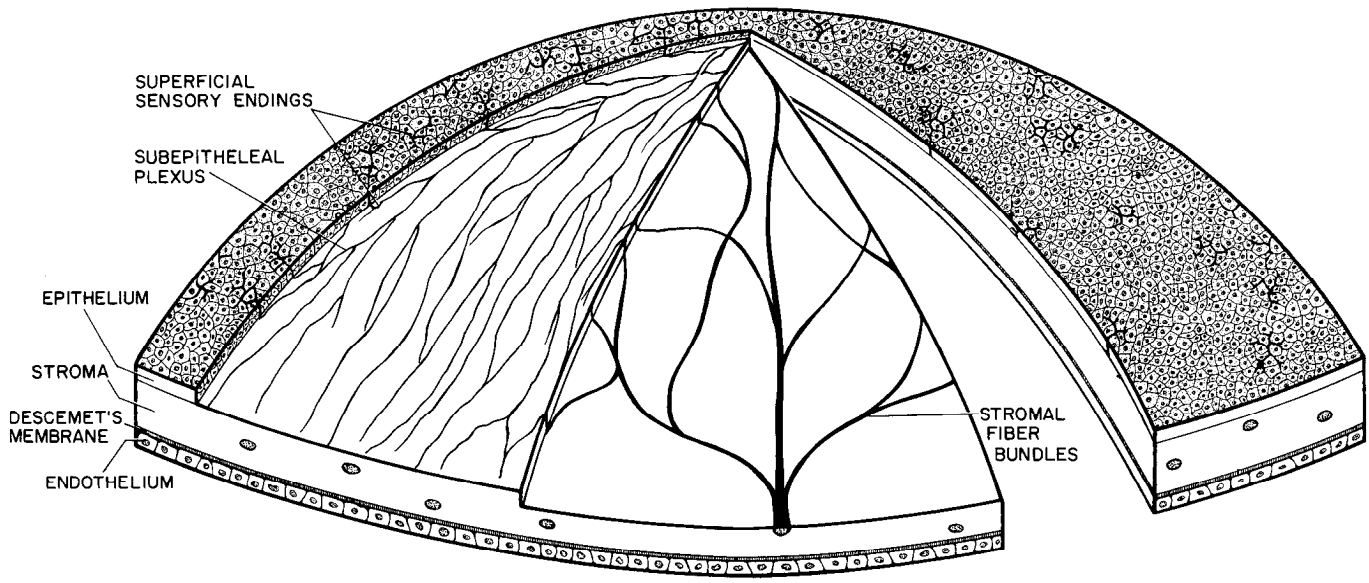

Figure 1. The mouse cornea has 4 layers: endothelium, Descemet's membrane, stroma, and epithelium. Trigeminal nerve fibers enter the corneal stroma in large bundles at its periphery and ramify radially within the stroma. The stromal nerve bundles give rise to smaller subepithelial fiber bundles, which are situated just beneath the basal layer of the epithelium. Processes from these subepithelial fibers arborize in the epithelium and eventually terminate in free sensory nerve endings near the corneal surface. These endings are typically tortuous and studded with varicosities (see Figs. 2–4). The anatomical constancy of the deep (stromal) fiber bundles provides a means of reliably returning to exactly the same region of the corneal surface.

exposure times there was no evidence of nerve terminal or epithelial damage. Two million lux-sec was therefore the minimal exposure that caused appreciable corneal damage by the criteria of vital staining. Light exposures in the experiments reported here were limited to less than $1/_{10}$ of this value.

Results

The innervation of the cornea can be subdivided into 3 categories: deep fiber bundles, subepithelial fibers, and superficial sensory terminals (Fig. 1). The vast majority of these processes arise from sensory neurons whose cell bodies are located in the ipsilateral trigeminal ganglion (Arvidson, 1977; Tervo et al., 1979; Marfurt and Huseman, 1986; Marfurt, 1988). In particular, nerve fibers in the corneal surface appear to arise entirely from this source (Marfurt and Huseman, 1986; Marfurt, 1988). Each of these categories of nerve fibers is transiently stained by topical application of 4-Di-2-ASP, which appears to label the corneal innervation as completely as conventional stains (e.g., methylene blue or gold chloride; Zander and Weddell, 1951; Whitear, 1960; Schimmelpfennig, 1982). The trigeminal innervation can therefore be visualized *in situ* from its entry in radially oriented bundles at the corneal periphery to its termination within the superficial epithelium. In preliminary experiments it was apparent that deep nerve fiber bundles maintained a constant position and configuration in the cornea for at least 4 months (5 animals). The branching patterns of these deep bundles could therefore be used as intrinsic markers to identify precisely an overlying region of the corneal surface at the two different times.

Observations in living mice were made at 4 different intervals: 4 hours, 1 d, 1 week, and 1 month; 12–20 animals were studied at each time point. To observe the cornea after an interval of 4 hr, the animals were re-anesthetized, intubated, and placed once again in the head-holder. Since the initial staining had already faded by the time of the second session, the staining procedure was repeated. Comparison of images obtained initially and after several hours showed no apparent change in the configuration of the same sensory endings in any of the 12 animals examined over this interval (Fig. 2). The similarity of terminal configuration at an interval of a few hours established the consistency of the staining, localization, and imaging techniques, and thus served as a control for any changes observed over longer intervals. These observations also confirmed the apparently innocuous nature of the initial staining and imaging procedures since when damaging levels of illumination were intentionally used (see Materials and Methods), disruption of nerve terminals was apparent within several hours.

In each of the 15 animals in which observations were separated by a day, superficial nerve endings retained their relationship to the underlying deep nerve fiber bundles and were always present at sites where they had been seen initially. However, examination of relationships within the terminal arborizations themselves showed appreciable changes in configuration (Fig. 3). Indeed, individual terminal processes could not always be recognized as derivatives of a branch that was originally present.

When the imaging sessions were separated by a week (18 animals), only an approximate correspondence of the overall location of some terminal clusters was still apparent, and at this interval the configurations of particular terminal arbors bore little resemblance to those seen initially (Fig. 4). Moreover, sites at which terminal clusters had initially been seen were sometimes free of endings 1 week later, whereas terminals were newly present at other locations. Such discrepancies were never seen in the corneas of the 27 animals examined at intervals of a day or less.

After 1 month (20 animals) the rearrangement of superficial terminals was so extensive that even the rough correspondence of terminal cluster location evident after 1 week was no longer discernable (Fig. 5). Neither the location of terminal clusters

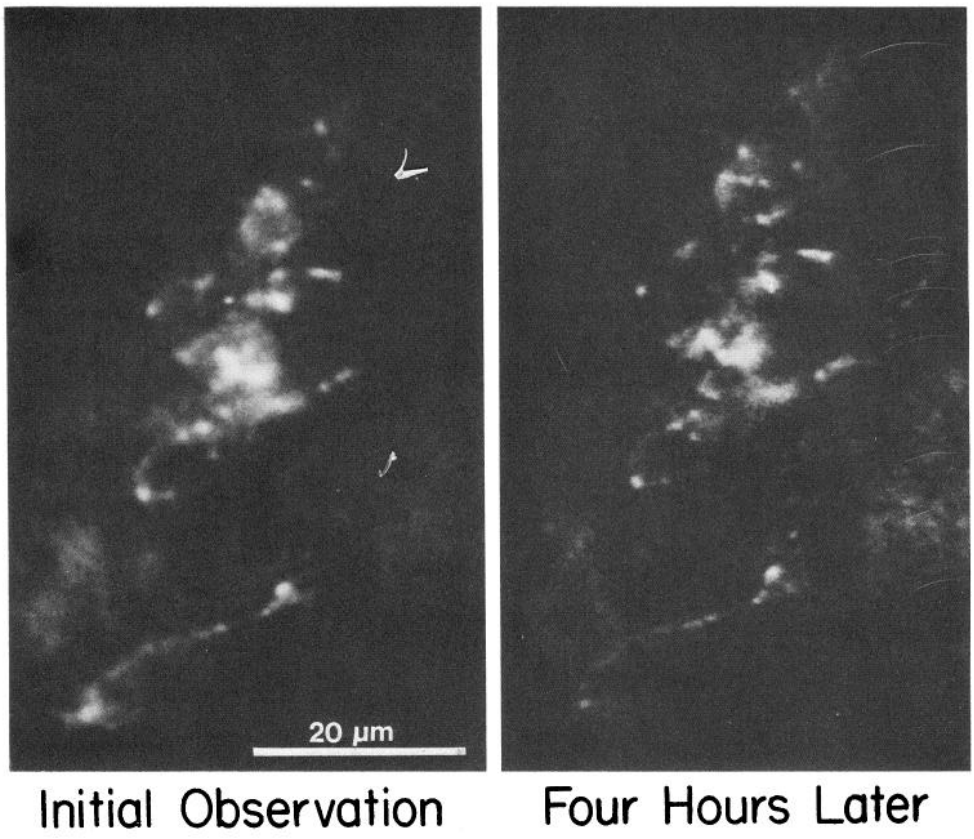

Figure 2. A representative cluster of sensory endings at an identified corneal region observed initially and 4 hr later. No definite changes in the configuration of such terminals were seen over intervals of a few hours.

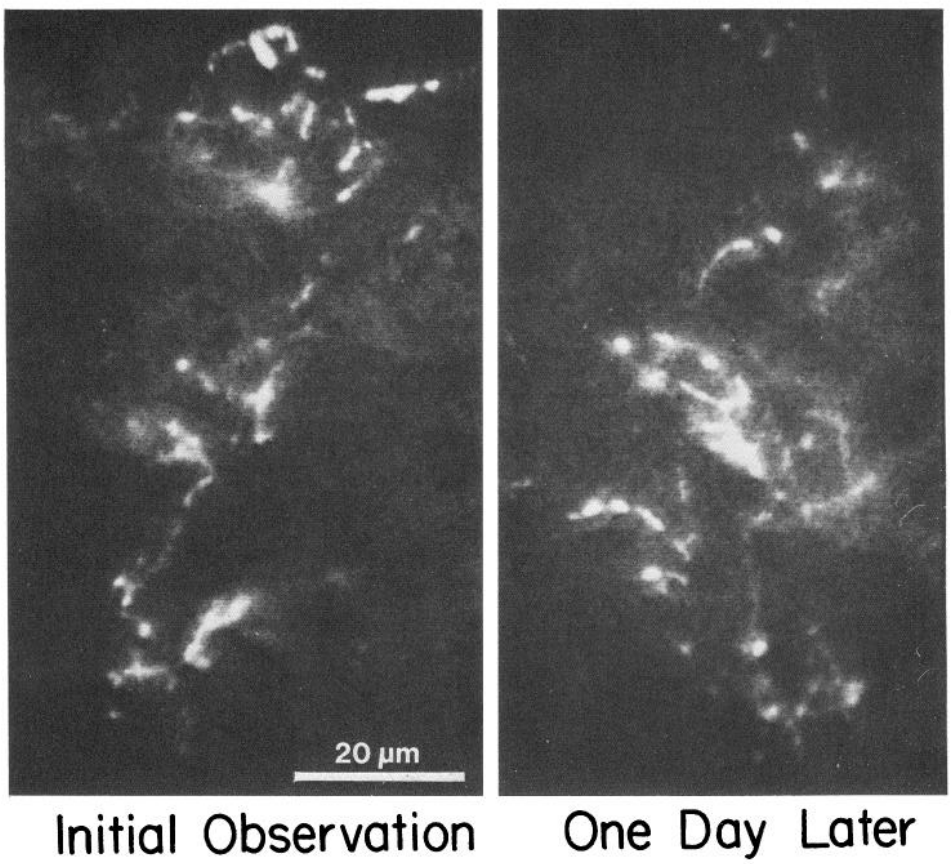

Figure 3. A representative cluster of sensory endings at an identified corneal region observed initially and 1 d later. After an interval of a day, terminal clusters were always seen at the sites where they had initially been present. However, the arrangement of the terminals within each cluster was appreciably different, as is apparent here.

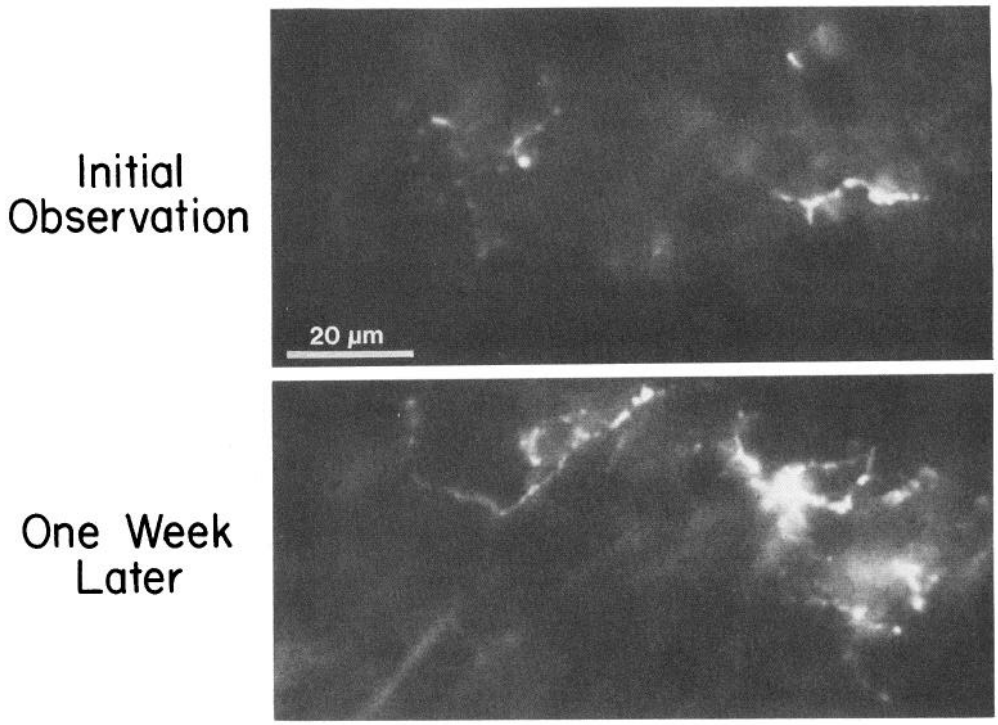

Figure 4. Representative sensory endings observed at an identified corneal region initially and 1 week later. After a week, marked changes were always evident in the structure of the terminal clusters.

nor their detailed structure after 1 month bore any resemblance to the initial pattern of sensory endings observed within the same corneal region.

Discussion

The configuration of identified sensory terminals in the adult mouse cornea shows little or no change over a few hours, but is appreciably altered after a day. After a week, much more extensive structural rearrangement within terminal clusters is observed, and some terminal clusters are evident at novel locations, whereas other clusters have disappeared. After 1 month, the arrangement of sensory endings within the same corneal region is altogether different.

One explanation for these changes might be that they are

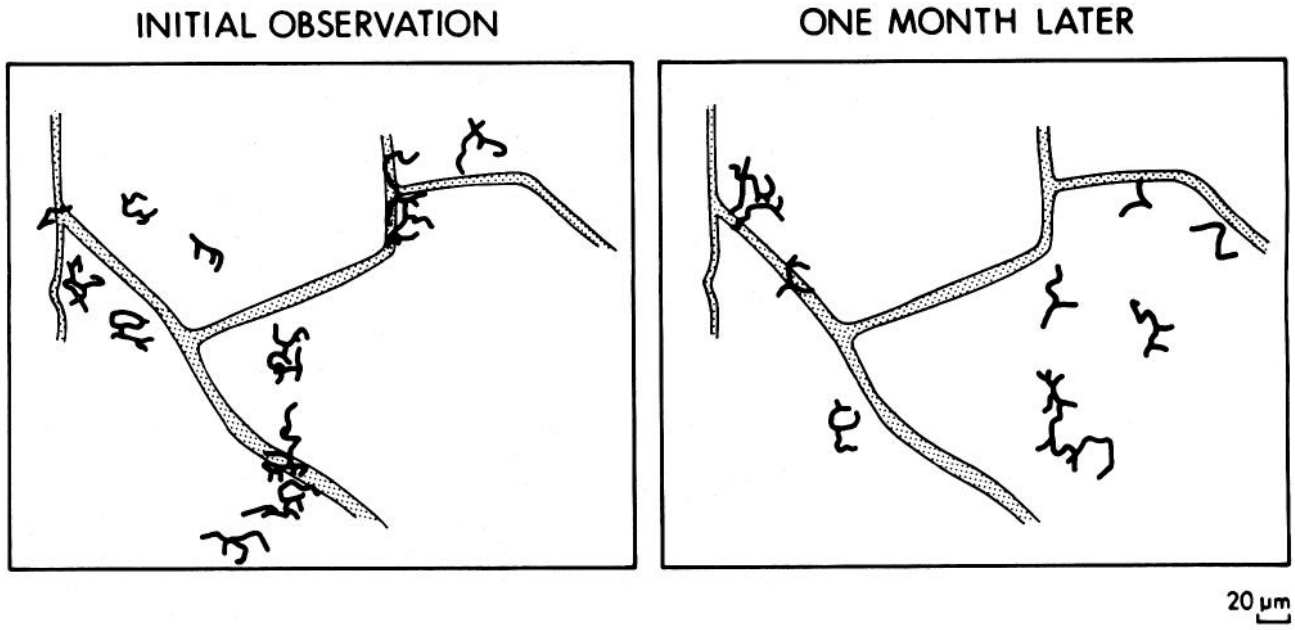

Figure 5. Overall arrangement of sensory endings within a larger corneal region observed initially and 1 month later. In order to show the location of terminal clusters over a substantial portion of the corneal surface, terminal clusters and deep (stromal) fiber bundles (stipple) have been traced at lower magnification from a series of video images. The tracings of terminal clusters are intended to show location only, not the detailed configuration evident in the preceding figures. After a month there is little or no correspondence between the initial and final position of sensory terminal clusters.

induced by the staining and imaging procedures. Despite efforts to eliminate any disturbance of the normal state of the cornea, it is difficult to rule out this possibility. Several factors, however, argue against this interpretation. First, the changes reported here have a different time course and pattern than the changes seen after experimental injury to the corneal epithelium (Beuerman and Kupke, 1982; Rozsa et al., 1983). Surgically induced trauma of the cornea elicits neural degeneration within hours after injury, which is followed by the proliferation of entirely new processes within 24 hr. In our experiments no changes were observed in the configuration of terminals over periods of a few hours, and, although changes were apparent after 24 hr, the original terminal arbors were still recognizable (i.e., the terminals were not replaced, as occurs after injury). Second, other experiments using styryl pyridinium dyes for vital fluorescent staining under similar conditions have shown them to be nontoxic to skeletal motor (Lichtman et al., 1987; Magrassi et al., 1987) and preganglionic nerve terminals (Purves et al., 1987). Finally, acute damage to sensory endings would not be expected to cause ongoing rearrangement several weeks later, as was observed in the present experiments. Indeed, the marked degree of rearrangement apparent after one to a few weeks implies that substantial remodeling should be evident over a day or two. Nonetheless, the possibility of some initial damage cannot be entirely eliminated.

The remodeling of sensory endings reported here is almost certainly related to the fact that these nerve terminals are situated in an epithelium that is continually renewed. The epithelial cells of the rodent cornea (see Fig. 1) are generated in the basal layer and migrate to the surface where they are sloughed approximately 4–7 d later (Hanna and O'Brien, 1960). Indeed, some minor changes in terminal configuration could represent the passive movement of the nerve endings as a result of the outward migration of epithelial cells. Passive rearrangement could not, however, account for the appearance of new terminals in locations where no terminals were previously present, or for the disappearance of terminals previously observed. These changes, which occur over the course of a few days, indicate active and ongoing extension and retraction of sensory nerve processes.

These observations add to a growing body of evidence which has demonstrated various degrees of ongoing rearrangement of axonal and dendritic branches in the peripheral nervous system of mature mammals. Thus, sympathetic ganglion cell dendrites in the mouse are remodeled over periods of several weeks by means of extension and retraction of processes (Purves et al., 1986), and changes in the configuration of synaptic terminals on the surface of parasympathetic ganglion cell bodies are appreciable over 1–3 weeks (Purves et al., 1987). Neuromuscular junctions also undergo substantial remodeling in frogs (Wernig and Herrera, 1986; Herrera and Banner, 1987), and to a lesser extent in mammalian muscles (Lichtman et al., 1987; Wigston, 1987, 1989). A particularly important facet of the present work is the demonstration that terminal rearrangement can occur over intervals as short as a day. Clearly, it would be of interest to know if remodeling at this rate is unique to epithelial innervation or whether it can occur elsewhere in the nervous system.

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