Dispersion of growing axons within the optic nerve of the embryonic monkey

(retinotopic projection/growth cone/retinal ganglion cell axon/primate embryo)

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ABSTRACT To determine whether individual optic fibers grow along constant sets of neighboring fibers, a group of 160 axons and 25 axonal growth cones were traced through a set of 500 serial electron micrographs of an optic nerve taken from a 39-day-old monkey embryo (*Macaca mulatta*). In single transverse sections, growth cones contact an average of 7.9 fibers, whereas axons contact 5.3 other fibers. The particular set of fibers in contact with one another changed rapidly, and, on average, growth cones and axons lost half of their original neighbors over a distance of only 8–10 μm . Between the first and last sections of the series, 92% of all initial contacts were lost. Individual axons moved freely between fiber fascicles, and the distance separating initial neighbors increased progressively. Most remarkably, the sets of fibers touched by the tips and the shanks of growth cones had no common neighbors in 17 out of 25 cases. These results demonstrate that, in primates, fibers in the optic nerve do not retain a particular set of immediate neighbors during their outgrowth.

The mechanisms that give rise to topographic connections between distant groups of neurons in the central nervous system remain an enigma despite several decades of intense research. One simple hypothesis to explain the formation of topographic connections is that axons of neighboring neurons keep in constant contact with each other as they grow toward their targets (1–5). By growing in tight formation from start to finish the axons could establish a congruent set of connections. In support of this idea, several studies have shown a remarkable degree of topographic order along the entire pathway linking the eye and the brain in a variety of species (2, 5, 6).

Results in other species, however, appear inconsistent with this simple explanation. For instance, in adult cats and monkeys, axons that originate from adjacent retinal ganglion cells are often scattered widely in the optic nerve and tract, and topographic order is either lost or seriously degraded (7–11). However, because these studies were based on adult animals, it is possible that the scatter of axons—particularly of those axons that originate from the older, central part of the retina—occurs only after the projections have formed. The intrusion of vascular channels, the proliferation of oligodendrocytes, or the process of myelination may split apart retinotopically related fibers and account for the meandering paths taken by numerous axons in mature animals.

In the present study we have determined whether the precise point-to-point connections between the retina and the brain—undoubtedly, an important determinant of the extraordinary visual capacity of primates—depends on highly ordered patterns of axonal growth. To do this at the level of individual axons and growth cones has required an analysis

of consecutive electron micrographs from serial sections through a segment of the optic nerve.

METHODS

Tissue Preparation. This study is based on an analysis of an optic nerve taken from a rhesus monkey (*Macaca mulatta*) on the 39th day of the 165-day gestational period. The embryo had a crown-to-rump length of 19 mm. The embryo was removed by cesarean section and perfused through the heart with 1.0% glutaraldehyde/1.25% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. A series of 500 consecutive transverse ultrathin sections was cut from a 50- μm segment of the stalk located 0.25–0.30 mm from the back of the eye. Fifteen sections were lost during sectioning, and the largest single gap in the series was 6 sections. Mean section thickness was estimated to be 0.1 μm .

Tracing and Analysis of Fibers. Electron micrographs were taken of several interweaving fascicles of fibers located near a prominent landmark—the embryonic fissure (Fig. 1). Each micrograph covered a field 12.6 $\mu\text{m} \times 15.4 \mu\text{m}$ at $\times 15,500$. All axons and growth cones were arbitrarily assigned a number in the first micrograph of the series (Figs. 2 and 3A) and were traced sequentially through the series at intervals of 0.4–0.5 μm . Approximately 24,000 fiber profiles were labeled on a set of 115 micrographs with fine-tipped marking pens. In total, 189 axons and 40 growth cones were traced in this manner. Of this sample, we were able to analyze the neighbor relationships of 160 axons and 25 growth cones that were confined to the field covered by the set of micrographs and for which all neighbors were identified. Neighbor relationships were assessed in detail at 9 equidistant points through the series. To accomplish this, approximately 10,000 contacts between adjacent fibers were recorded and compared. Contacts between fibers and processes of glial precursors were examined and recorded but were otherwise not included in the analysis. The perimeter and area of every fiber in the nerve ($n = 8200$) were measured by using a digitizing tablet connected to a computer. The groups of fibers that were examined in detail did not differ in size or ultrastructure from the entire population.

Even a small number of errors in the identification of fibers would have serious cumulative effects and would degrade the validity of the results. To correctly identify fibers we examined several of their characteristics and double-checked the results. The position of a fiber was an important identifying feature. Because the average diameter of axons (0.52 μm) was 5 times the section thickness, their positions changed almost imperceptibly between adjacent sections. In fact, over distances of up to 1 μm , fibers could usually be identified reliably by position alone. The cross-sectional shape and relative size of fibers were also stable over a distance of at least 0.5 μm , and these features were also used to identify fibers. Finally, the arrangement and density of organelles,

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particularly microtubules and microfilaments, were also useful in confirming a fiber's identity. Those fibers that could not be traced confidently or for which some neighbors were not identified were not included in the analysis ($n = 29$).

RESULTS

Optic Stalk. On the 39th day of gestation, the precursor of the optic nerve—the optic stalk—is composed of a 40- to 60- μm -thick wall of glial precursors and retinal ganglion cell axons (Fig. 1). The lumen of the stalk is still continuous with the optic recess of the third cerebral ventricle. The sections of the optic stalk that we examined contained a total of 8200 axons and growth cones, located almost exclusively in the ventral half of the stalk (Fig. 1). This is only 0.25% of the peak of nearly 3 million fibers reached at midgestation (12).

Fascicles. There were 125 fascicles in the stalk. They contained an average of 66 fibers. Neighboring fascicles were separated from one another by thin pale processes of glial precursor cells (Fig. 2). The frequency with which fascicles merged and split was remarkable. Groups of axons and even isolated fibers split off from fascicles, passed between glial processes, and merged with fibers in neighboring fascicles. Even over a distance of merely 10 μm , the size, shape, and fiber composition of most fascicles changed radically.

All fascicles at this age contained growth cones. From this observation alone we conclude that in primates the number of fibers increases in all fascicles rather than just in a peripheral subset as appears to be the case in birds (13). However, fascicles close to the edge of the nerve usually contained 2 to 4 times more growth cones than did most fascicles located closer to the center of the stalk.

Growth Cones. Growing retinal ganglion cell axons have elaborate growth cones at their tips. Complete three-dimensional reconstructions of growth cones in the primate optic stalk (14) have shown that most are 30–50 μm long and have several broad but very thin lamellipodia (Fig. 2).

FIG. 1. Drawing of the optic stalk—the precursor of the optic nerve—of a monkey embryo on the 39th day of gestation. Fascicles of fibers are drawn in solid black and most of these are located in the ventral half of the stalk. The vertical arrow just to the right of the embryonic fissure (EF) points out the region we analyzed in detail and the location of the micrograph reproduced in Fig. 2. Several neuroepithelial cells are drawn in the upper right quadrant. They extend from the luminal to the external surface of the stalk. Other cell nuclei are simply outlined. L, the lumen of the optic stalk; nec, necrotic cell; mit, a mitotic cell; EF, the embryonic (or choroid) fissure of the optic stalk.

FIG. 2. Electron micrograph of axons and growth cones in a fascicle close to the embryonic fissure. Glial processes have a very light cytoplasm. Axons have a darker cytoplasm. Growth cones sectioned through their lamellipodia (arrowheads) have dark cytoplasm and elaborate shapes. Several growth cones are situated between fascicles and glial processes. A schematic drawing of this field of fibers, in which all growth cones and growth cone shanks have been identified, is shown in the central part of Fig. 4A. The ventral surface of the optic stalk, covered by a basement membrane, is located at the bottom of the figure. (Bar represents 1 μm .)

Growth cones had larger perimeters and generally contained a higher density of microfilaments than did axons. Most growth cones were situated at the margins of fascicles, between glial processes and other fibers (Fig. 2). It has been suggested that growth cones of retinal ganglion cells may adhere preferentially to the basement membrane as they grow out of the eye and through the nerve (reviewed in ref. 15). In the rhesus monkey we have found no evidence of such a pathway preference. Not a single growth cone grew next to the basement membrane in either the retina (16) or the optic stalk.

Size of Fibers in Relation to Number of Neighbors. The perimeters of axons ranged from 0.7 to 3.2 μm , with an average of 1.5 μm . In comparison, the perimeters of growth cones averaged slightly more than 3 times as much (4.7 μm). Axons had an average of 5.3 immediate neighboring fibers in single transverse sections, while, despite their greater perim-

eters, growth cones had a mean of only 7.9 neighbors per section—merely 50% more than the smallest axons. This small increment in the number of neighbors, despite a 3-fold increase in surface area, is due to the fact that growth cones tend to cluster at the margins of fascicles. As a result, approximately two-thirds of their surface is apposed to glial cells and the flat surfaces of other growth cones.

Instability of Neighbor Relationships. Growth cones and axons typically lost half of their initial set of neighbors over a distance of only 8–10 μm . This loss was progressive (Fig. 3). The tips and shanks of 17 out of 25 growth cones did not have any neighbors in common, even though these regions were often separated by less than 30 μm . Likewise, out of 465 pairs of contacts between neighboring axons identified at the start of the series, 425 (92%) were lost by the end of the series (compare Fig. 4A and B). Most remarkably, 56% of the axons lost contact with all of their initial neighbors. It is therefore evident that the lack of tight coupling between young axons is due to the nonselective trajectories taken by individual growth cones and is not due to an incidental disruption of neighbor relationships that may occur only after growth cones have chosen their paths.

Dispersion of Initial Neighbors. Not only did growth cones and axons fail to remain in contact with their initial neighbors but also the distance separating initial neighbors increased as they advanced through the stalk. The separation between axons averaged 1.38 μm at 25 μm from the start of the series and averaged nearly twice as much, 2.54 μm , at the end of the series. This dispersion can be clearly appreciated by comparing the distribution of groups of neighboring fibers in Fig. 4A and B. The ultimate scatter of fibers that were once neighbors is likely to be considerably greater along the entire length of the optic pathway.

FIG. 3. Loss of neighbors by growth cones ($\blacktriangle$) and axons ($\diamond$). On the x-axis we have plotted the distance in μm separating sections in the series. On the y-axis we have plotted the number of neighbors that are retained between different levels of the series. For instance, growth cones had a mean of 7.9 neighbors per section. This fact is represented by the left-most $\blacktriangle$. Over a distance of 8–10 μm the average number of neighbors that are retained drops to less than half the original value. The standard error of the mean is, with the exception of the two right-most triangles, approximately equal to the vertical height of the symbols.

DISCUSSION

This study provides evidence that individual retinal ganglion cell axons and their growth cones do not retain a stable group of neighboring fibers as they grow through the optic nerve. Even over a relatively short distance (50 μm , or roughly 4% of the length of the nerve), 56% of all fibers traced through the set of serial electron micrographs failed to retain a single neighbor. The progressive loss of neighbors is the consequence of the nonselective behavior of growing axons and is not due to the disruption of initially well-ordered relationships among fibers by events such as cell proliferation or the ingrowth of glial processes.

Axon Order and Axon Disorder. In a variety of species, groups of axons that originate from neighboring cells remain together within the optic pathway (2, 5, 6, 17). By tracing bundles of axons labeled with horseradish peroxidase, usually in adult animals, it has been shown that order among axons in the optic nerve is related to the retinal coordinates of ganglion cell bodies and the age at which ganglion cells are generated. In some animals, particularly perciform fish, it is possible to predict the position of an axon at any point along the pathway simply by knowing the site or time of origin of the ganglion cell. Such findings led to the proposition that a coherent array of optic axons simply imprints an image of the retina onto the sheet of target neurons during development, and that additional cues, either recognition molecules or synchronous activity, are not needed to explain highly ordered visual connections (3–5).

The results of our study, however, demonstrate that, in the developing monkey, growth cones change their neighbors rapidly and disperse away from one another as they extend through the optic stalk. We saw no evidence that the loss of neighbors might have been related to systematic rearrangements of fiber relationships. Rather, the changes most likely result from the unpredictable trajectories taken by individual growth cones through a complex web of fascicles and glial processes. Given this result, it is likely that the erratic trajectories of numerous axons in the more mature nerves of species as diverse as catfish (18), mudpuppy (19), *Xenopus* (20, 21), cat (9), and monkey (7, 8) reflect the dispersion of fibers during their outgrowth.

Factors Limiting Axon Dispersion. It is known that coarse topography is retained within the optic pathway of primates, and the majority of axons that enter a particular quadrant of the nerve remain in that quadrant (8, 22). Thus, it is clear that the dispersion of axons does not completely scramble retinotopy in the optic pathway. The extent to which order is perturbed during axon growth probably depends upon several factors that differ between ages, between species, and even between different parts of the optic pathway. The size of the nerve, for example, is likely to affect the magnitude of dispersion. If the rate at which fibers changed neighbors were constant, then as the distance between cell body and target cells increased so would dispersion. Dispersion may be greater at the end of the optic pathway in species with large and long optic nerves, such as primates, than in species with small and short nerves.

The integrity and size of fascicles may also influence the degree of dispersion. Provided that fascicles do not fuse or split, the identity of the fibers in a fascicle will remain unchanged, and dispersion will be limited by the size of the potential pool of neighbors in the fascicle. However, in monkeys, fascicles exchange fibers, and may furthermore eventually contain up to 5000 fibers each, thereby providing conditions for a high degree of dispersion.

The strength of adhesive interactions between neighboring fibers may also affect dispersion. High affinity between growth cones and the surfaces along which they grow may minimize dispersion (23). If, however, bonds between sur-

FIG. 4. Dispersion of fibers. *A* and *B* are schematic drawings of the first and last sections of the series of 500 electron micrographs in which all fibers have been identified. Growth cones or their shanks have been drawn in black; axons are outlined. The dispersion of axons can be visualized by marking groups of axons that were initially neighbors—for instance, axons a1 to a9 in *A*—in section 501. These axons will usually be widely scattered.

faces are weak, then dispersion should be greater. In support, it has been reported that when an antibody directed against a neural cell adhesion molecule is injected into the eye of chick embryos, axons that originate from neighboring ganglion cells spread out farther than normal as they grow (24).

The responsiveness of growing fibers to cytochemical cues may also influence the magnitude of dispersion. If subpopulations of growing axons respond differently to particular molecules that are either present on their own surfaces or distributed in the tissue (1, 25), then this might keep related axons together and unrelated axons apart. Furthermore, if the expression of such markers were modified as these axons grew, then this could account for often dramatic large-scale rearrangements among optic axons (21, 26–33).

Conclusion. Our results demonstrate that in the optic nerve of the monkey embryo, growing fibers do not maintain precise neighbor relationships. In spite of this, topographic maps, such as that in the dorsal lateral geniculate nucleus of primates, develop with a high level of precision. Clearly, the coarse topography within the nerve and tract is not a sufficient explanation for the remarkably precise connections between neurons in the retina and brain. The degree of retinotopic order in the optic pathway is probably not a decisive factor in the formation of topographic connections.

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