Development of the optic nerve in

**Xenopus laevis**

I. Early development and organization

BY CHARLES CIMA\(^1\) AND PHILIP GRANT\(^2\)

From the Department of Biology, University of Oregon

**SUMMARY**

Development of the *Xenopus laevis* optic nerve was studied by light and electron microscopy from embryonic stage 26, before the retina has formed, to juveniles, 8 months post-metamorphic. Low-power EM photographs of sections through the retinal optic nerve (RON), middle optic nerve (MON) and chiasmatic optic nerve (CON) were prepared at different stages and the areas containing large axons (0.5 \(\mu\)m) were traced in optic nerve reconstructions. Ordering of fibre size along a dorsoventral axis was noted in the embryonic nerve, and this pattern persisted throughout development. Most large fibres, myelinated and unmyelinated, occupy an eccentric dorsocentral position in the MON while small axons are seen in a ventral peripheral crescent. In the CON, the dorsal one third to one half is occupied by large fibres while the ventral CON contains small fibres exclusively. If, as assumed, large axons are older than small axons (0.1–0.3 \(\mu\)m), then patterns of large and small axons along the nerve might reveal a chronotopic fibre ordering. Chronotopic ordering was confirmed by autoradiographic analysis of the distribution of old, labelled fibres and young, unlabelled newly arriving fibres in optic nerves between stage 51 and 57. The young-old labelling pattern corresponds to the small and large axon patterns respectively, in all sections of the optic nerve. Chronotopic ordering of fibres in the developing optic nerve can be explained, in part, by the dorsoventral asymmetric marginal growth of the developing retina and the phenomenon of fibre following as ganglion cell axons join near neighbour fascicles in the retina, converge at the optic disc and grow through the optic nerve.

**INTRODUCTION**

The visual system of fish and amphibia has been used extensively to study the ordering of retinotectal connexions (see reviews of Hunt & Jacobson, 1974; Gaze, 1978; Jacobson, 1978; Horder & Martin, 1978; Fraser & Hunt, 1980; Conway, Feiock & Hunt, 1980). The chemoSpecificity hypothesis proposed to explain such ordering (Sperry, 1943, 1945, 1965), though supported by many observations, has been cast into some doubt by more recent observations of neuronal plasticity in the visual system (see above reviews). Apparent lability of retinotectal connexions has been interpreted by some as evidence in support

\(^1\) Deceased June 1980.

\(^2\) Author’s address: Department of Biology, University of Oregon, Eugene, Oregon 97403, U.S.A.
of alternative models which propose that optic axons reach visual centres as a direct consequence of contact guidance along so-called pioneering fibres (Horder & Martin, 1978). Horder and co-workers have proposed that simple mechanical guidance during development can account for the formation of continuously ordered patterns of connectivity in the visual system of fish and amphibia. One consequence of this hypothesis is that optic fibres maintain specific spatial relationships throughout their passage to visual centres, a view not fundamentally different from one proposed much earlier by Ströer (1940) for Triturus and other lower vertebrates. Both retinotopic and chronotopic ordering of fibres have been claimed for the optic nerves of several species of fish and amphibians (Bunt & Horder, 1978; Scholes, 1979; Rusoff & Easter, 1980; Easter, Rusoff & Kish, 1981; Fawcett, 1981), although retinotopic ordering has not been found in the cat (Horton, Greenwood & Hubel, 1979).

In our studies of retinal embryogenesis in Xenopus we have found that optic fibres appear hours before the retina completes invagination, and fasciculate as they arise to form bundles in the retina (Grant, Rubin & Cima, 1980; Grant & Rubin, 1980; Cima & Grant, 1980). If axons always retain nearest neighbour relationships as they enter the developing optic nerve (as they do in the zebra fish, Bodick & Levinthal, 1980), then pathways taken by pioneering fibres as they enter the optic stalk may determine the final organization of the optic nerve and may, in turn, order fibre projection to the tectum. Thus, studying early assembly of axons into an optic nerve may shed some light on what rules govern retinotectal connectivity.

In this ultrastructural analysis we describe how the optic nerve arises in the early Xenopus embryo as the first fibres (pioneers) enter the optic stalk, and examine how fibre organization changes in later development through metamorphosis. The effect of gliogenesis, myelination and metamorphosis on fibre organization is described in a following paper (Cima & Grant, 1982). In a final paper of this series, we will describe the retinotopic organization of the developing optic nerve based on HRP tracing techniques (Grant & Cima, 1982).

What emerges from the analysis in this first paper is that the optic nerve becomes chronotopically ordered in the early embryo, as the primary fascicle is established in the optic stalk, with older fibres in dorsal regions and younger fibres in ventral sectors. This ordering is seen in most of the nerve throughout development even during the periods of gliogenesis and myelination, and as the nerve shortens during metamorphosis.

MATERIALS AND METHODS

Embryos were obtained from Xenopus females by gonadotrophin stimulation and mating (Gurdon, 1967). They were sorted and staged according to Nieuwkoop & Faber’s (1956) external criteria and reared in 10% Steinberg’s solution in a temperature-controlled room (22 °C). After hatching, tadpoles were placed
Xenopus optic nerve development

in dechlorinated aged water (5 tadpoles/21), changed twice weekly and fed tadpole chow (Nasco) or powdered nettle leaves.

Although the initial staging of embryos and tadpoles was made according to Niewkoop Faber criteria (NF), the final staging of histological material in many instances was based on the staging series for the retina (Grant et al. 1980). In all cases, both stagings will be shown to facilitate identification for those more familiar with NF staging criteria.

Electron microscopy

Three specimens each of NF stages 26, 28, 30/31, 32, 33/34, 37/38, 42, 46/47, 48/49, 50, 53, 57, 61, 4 weeks postmetamorphic, 8 months postmetamorphic and one adult were collected and prepared for electron microscopy. Embryos were stripped of their jelly coats with fine forceps, beheaded and placed in fixative. Larval and postmetamorphic animals were perfused with fixative through the ventricle of the heart after first cutting the inferior vena cava. Initial fixation for late embryos, tadpoles and juveniles, was with (1) 2.5% glutaraldehyde, 1% formaldehyde prepared from paraformaldehyde, 2.5% dimethylsulphoxide in 0.1 m-cacodylate buffer at pH 7.4, while (2) 2% osmium tetroxide in 0.1 m-cacodylate buffer pH 7.0 with 8 mM-CaCl₂ seemed better for early embryos. Late embryos, larvae and tadpoles were immersed in the fixative overnight, washed in buffer and post-fixed in 1% osmium tetroxide for 4 h, dehydrated and embedded in Epon/Araldite. Early embryos were immersed in the first fixative for 1 h, washed, dehydrated and embedded as above. Animals were serially sectioned at 2.5 μm with a glass knife in the parasagittal plane. Sections were stained with toluidine blue-o and examined with the light microscope. Selected sections were re-embedded according to the procedure of Schabtach & Parkening (1974) and sectioned with either a glass or diamond knife (Dupont) on a Reichert ultramicrotome. Sections, 60–80 nm thick, were collected and mounted on Formvar-coated single-hole grids, double stained with uranyl acetate and lead citrate, carbon coated and examined with a Phillips 300 electron microscope.

Optic nerve reconstructions

Low-magnification electron micrographs (×900–3000) of the entire cross-sectional area of at least three regions along the optic nerve were made for two to three animals at each stage. Sections were traced on transparent paper and all axons larger than 0.5 μm in diameter were drawn in. The dorsoventral and nasotemporal orientation of each section was noted where possible. This was easiest to do in sections of the retinal optic nerve (RON) and chiasmatic optic nerve (CON) but less reliable in sections of the middle optic nerve (MON) because planes of sections were often not normal to the long axis of the nerve, or the nerve, which is quite long in tadpoles, undergoes some twisting between the retina and the brain. The tracings were photographed, mounted in proper
orientation to make a three-dimensional reconstruction. In some cases, the photographs themselves rather than the tracings were included in the reconstruction. Though axon diameter may vary from section to section because of different magnifications, the important feature in these reconstructions is the relationship between areas of the nerve with large axons and sectors containing few or no large axons. Most axons in the latter region are small, ranging between 0.1 and 0.3 μm in diameter. It should be stressed that areas with large axons also contain many small axons. The distinction between a large axon area and a small one is that the latter contains no or very few large axons.

**Autoradiography**

Stage-51 tadpoles were given a single intraocular injection of 1–5 μCi of tritiated proline ([³H]proline) obtained from New England Nuclear Corporation (specific activity 27 Ci/mmol). Animals were sacrificed 6–10 h after injection or were allowed to survive for longer periods (details given in results). Animals were fixed and processed as described for electron microscopy. Sagittal sections (with respect to body axis, but transverse sections with respect to long axis of the optic nerve) 2.5 μm thick were mounted on glass slides, dipped in Kodak NTB-2 emulsion and exposed for two weeks. Autoradiographs were developed in Kodak D-19 developer and fixed. Slides were counterstained with 0.01 % toluidine blue-o and mounted. Darkfield micrographs were made with a Zeiss Universal microscope. Silver grains were counted in enlarged light micrographs of sections. A transparent polar coordinate grid was placed over the sections and grains were counted in seven separate annuli, 13 mm thick from periphery to centre of the section. Total number of grains in each annulus was counted and the area measured with a digitizer attached to a computer. The density of grains (grains/cm²) was calculated for each annulus. It was not necessary to convert the density of grains on the photos to the actual grain density in the section since we were simply comparing the relative densities; absolute densities are not important. Seven to ten sections from RON to CON were analysed for each of two optic nerves. Grain density in the CON was compared in dorsal and ventral sectors.

**Regions of optic nerve studied**

Sections of the optic nerve examined in this study are the RON, defined as that point between the optic nerve head in the retina and the limits of the pigmented epithelium outside the retina, the proximal optic nerve (PON), a section from the pigmented region of the optic nerve just beyond the edge of the globe, the MON, a section halfway between the distal extent of the globe and the optic chiasm, and the CON, a section immediately prior to optic nerve entry into the ventral diencephalon. Sections of the MON in the cranial cavity, the intracranial MON (ICMON) were taken in some instances.
RESULTS

Establishment of primary fascicle

When the optic vesicle begins to invaginate at stage III (Grant et al. 1980, NF 26), the optic stalk is a thickened cylinder of pseudostratified epithelium whose walls are continuous with both the presumptive neural and pigmented retinas. The cytoplasm of stalk cells consists of ribosomes, glycogen granules, yolk platelets, lipid droplets, mitochondria and a sparse endoplasmic reticulum. Extracellular spaces are seen as large gaps between adjacent ventral stalk cells (Fig. 1). Spaces are also seen between cells in ventral retina and often extend throughout the entire width of the cross-sectional area of the stalk, terminating between basal- and apical-end feet from stalk cells. No extensive necrosis is seen anywhere in the stalk or retina at these early stages.

From stage IV to stage VIII (NF 28–33) the cytology of stalk cells remains unchanged. It is uncertain whether the intercellular gaps, seen throughout the optic stalk and retina, form continuous channels through the stalk as described in the mouse (Silver & Robb, 1979; Silver & Sidman, 1980). Various-sized intercellular spaces are randomly arranged in the Xenopus stalk with no greater numbers in one region than in another.
Retinal ganglion cell axons are found in the proximal ventral optic stalk as early as stage IV (Cima & Grant, 1980) before the eye cup has completed invagination. Fibres are seen in extracellular spaces between the basal ends of stalk cells, often loosely arranged in bundles, with some axons in contact with adjacent cytoplasmic processes from stalk cells (Fig. 2).

At stage VII (NF 32) axons have reached the chiasm and ascended into the contralateral diencephalon (Grant et al. 1980). Within a few hours, by stage VIII (NF 33/34), a loose collection of axons, putative growth-cone profiles (elongated, irregular profile filled with numerous glycogen granules, mitochondria but without aligned microtubules), and cytoplasmic processes can be seen in sections of the primary fascicle (Fig. 3 A, B). Presumably at these early
stages most axons are actively growing with enlarged, irregular growth cones, branching through intercellular spaces. This primary fascicle near the retina is found in the ventrotemporal region of the stalk consisting of approximately 40–70 small axons and many growth cone profiles. A single fascicle becomes the optic nerve with loosely packed axons separated by gaps of various sizes. Many more growth-cone profiles are seen in sections near the chiasm and diencephalon than near the retina. Optic stalk cells in contact with optic fibres extend long cytoplasmic processes which partially or completely surround the fascicle. These fine processes eventually become the limiting membrane of the optic nerve. Gradually, between stages 33 and 37/38, the axons in the fascicle become more compact and fewer growth-cone profiles are seen, except at the CON which continues to exhibit the more primitive loose arrangement of growing and branching axons.

Axon diameter and fibre age distribution

At stage X (NF 37/38), the future optic nerve consists of a single fascicle with approximately 700–1000 axons (875 ± 133 in three embryos) surrounded by processes from retinal and optic stalk cells forming a primitive limiting membrane (Fig. 4). Most axons in the bundle are small, (0·15–0·35 μm), densely packed, separated by spaces measuring approximately 11 nm. In sections along the
nerve, a single elongated stalk cell process may extend into the centre of the fibre bundle from squamous cells at the periphery.

A few fibres exceeding 0.5 μm in diameter are present, and are localized in the more dorsal parts of the CON (Fig. 4). This is the first stage at which large axons are seen in the optic nerve; in a short while their number increases. This becomes evident even in the small bundles segregating from the primary fascicle in the CON in more medial sections, where bundles of the optic tract are forming in the ventral diencephalon (Fig. 5A, B). The primary optic bundle subdivides into several smaller bundles, each separated by fine, finger-like cytoplasmic processes from adjacent ependymal cells. Axons in these small fascicles also display a fibre size gradient, with the largest in more dorsal regions of the fascicle (Fig. 5B). Large axons form a crescent-shaped arrangement extending from the

Fig. 5. Stage 40 CON. (A) Low-power micrograph showing large primary fascicle segregating into three smaller bundles in more medial sections of the CON. Bundles are separated by cytoplasmic processes from dorsally lying ependymal cells containing dense cytoplasm. Arrows point to the smallest bundle (shown enlarged in (B) showing gradient of axon size with the largest axons occupying more dorsal parts of the fascicle. Dorsal is at the top in both figures. Bars = 1 μm.
Figs. 6-11. Reconstructions of optic nerves at different stages showing sections through RON, MON and CON. A cartoon illustrates the orientation of the nerve and shows the relative proportion of the nerve cross section occupied by fibres from retinal quadrants nasal (n), dorsal (d), temporal (t), ventral (v). Large axons ($\geq 0.5 \mu m$) are shown in all cases. All clear areas in these sections are filled with small fibres ranging in diameter from 0.1 to 0.35 $\mu m$. Bars under section labels equal dimensions in microns as indicated for each stage.

Fig. 6. Stage 40. Note that there are virtually no ventral retinal fibres at this stage. Most large axons are seen in dorsal, nasal and temporal quadrants of the PON and occupy the dorsal sectors of the MON and CON. Bars = 5 $\mu m$.

dorsal periphery into nasal and temporal quadrants while the centre and ventral edges are free of large fibres. Each bundle displays this fibre size gradient.

If large fibres are older than small fibres, as seems to be the case for several vertebrate neurons including retinal ganglion cells (Ramon Cajal, 1892; Tennyson, 1965; Kimmel, 1972; Kalinina, 1974; Rager, 1976), then the distribution of large and small fibres in sections of the developing optic nerve might tell us whether fibres are chronotopically ordered, as they are in goldfish (Rusoff & Easter, 1980; Easter et al. 1981). Accordingly, our reconstructions of the developing optic nerve distinguish those regions containing a high proportion of large myelinated and unmyelinated fibres from regions containing only small fibres.

Optic nerve reconstructions at developmental stages 40–57 are shown in Figs. 6–9. Occasionally, large axon profiles were seen suggesting swelling during fixation (Fig. 7). This artifact, however, did not alter the relative positions of large and small axons in nerve cross sections; it may have exaggerated them, perhaps because large axons are more sensitive to fixation than small. Overall
Xenopus optic nerve development

Fig. 7. Stage 42. A few ventral fibres have assembled in the optic nerve at this stage but fibres from all other quadrants predominate. Large axons collect in the periphery of the RON. Centroventral regions contain a greater concentration of small axons. A distinct dorsoventral asymmetry of large- to small-axon-containing regions is established in the MON and CON at this stage. Bars = 5 μm.

patterns, rather than absolute sizes of axons are important. The earliest optic nerve reconstruction was prepared at stage 40 (Fig. 6). As shown by the cartoon accompanying the figure, few fibres are present from the definitive ventral retina; its development lags behind the rest of the retina (Grant et al. 1980; Holt, 1980). Except for the ventronasal sectors, large axons are seen over most of the PON section, usually in separate clusters extending to the periphery. Large axons are found in the dorsal portion of the MON and CON. As many more large axons accumulate the asymmetric pattern in the CON becomes more apparent, persisting throughout development until after metamorphosis. In all stages, large myelinated and unmyelinated fibres are always concentrated in the dorsal half to two thirds of the CON. The overall pattern along the embryonic nerve is relatively uniform with a dorsoventral asymmetry of large to small fibres from RON into the small bundles of the optic tract (see Fig. 5).
Twelve to fifteen hours later at stage 42, the same asymmetric pattern of large and small fibre regions is seen along the nerve (Fig. 7). Most large axons are seen in dorsotemporal quadrants of the MON and CON, while in the RON they occupy a peripheral, horseshoe-shaped zone in all quadrants except ventral and centre.

Between stages 42 and 47, an interval of approximately 2–3 days (Nieuwkoop & Faber, 1956), the fibre pattern does not change significantly although one to two thousand new axons have entered the optic nerve (Wilson, 1971). Glial cell bodies and a few myelinated large fibres first make their appearance in the centre of the MON at stage 46/47 (Cima & Grant, 1982). Thereafter, to the adult, glial cell bodies accumulate in the central core of the optic nerve accompanied by increasing myelination. These two processes of gliogenesis and myelination, secondarily contribute to the overall pattern of large and small fibre areas in the nerve (Cima & Grant, 1982).

Seven days after stage 47, the optic nerve at stage 49 illustrates the early effect of these processes (Fig. 8). It contains about 3500 new axons, or a total of 7000 (Wilson, 1971). Many more ventral retinal fibres have accumulated in the nerve.
during this period, though they still make up a small proportion of the total fibre population. Some changes in the pattern of large and small fibres can be noted at this stage.

In the centre of the RON, a few pigment cells (pigmented astrocyte precursor, Cima & Grant, 1982) have appeared. Large fibres are now distributed uniformly over the RON including the ventral quadrant. We assume that large ventral fibres have probably matured in the nerve after having entered as small fibres at an earlier stage.

The MON displays a pattern that persists through metamorphosis. Large myelinated and unmyelinated fibres occupy an eccentrically placed dorso-central region over most of the cross-sectional area. Ventrally, a narrow horseshoe-shaped peripheral band with no large fibres (all less than 0.3 μm), extends into adjacent nasal and temporal quadrants. Glial cell bodies occupy the central core of the nerve among a few large myelinated fibres.

Except for the many more large axons in the CON at this stage, the pattern is unchanged; most large axons occupy the dorsal half to two-thirds of the CON with no axons in the ventral portion larger than 0.3 μm.

The period from stage 50 to 57, approximately 16 days at 22–24 °C (Nieuwkoop & Faber, 1956) is marked by a sudden increase in ventral retinal growth
(Beach & Jacobson, 1979) with an accompanying increase in the number of ventral fibres entering the optic nerve (Fig. 9). The RON retains its homogeneous distribution of large and small axons but in the PON and MON, the segregation of small axons into an area in peripheral ventral crescent is quite distinct. Dorsoventral patterns in MON and CON become more evident as many more large fibres become myelinated.

Summarizing, a dorsoventral pattern of large- and small-axon-rich areas develops in the embryonic optic nerve, which persists with only minor modifications through larval development, at least to the beginning of metamorphic climax. If, as proposed, axon diameter correlates with axon age, then the pattern suggests that the optic nerve is chronotopically ordered during development with most old fibres in dorsal-central locations and most young fibres at the ventral periphery.

Metamorphic remodelling

A profound change occurs in the optic nerve during metamorphic climax (from stages 57 to 66, Fig. 10). Numerous abnormal myelin figures are seen in the MON reflecting metamorphic remodelling as the optic nerve shortens to approximately one third its original length (Cullen & Webster, 1979; Cima & Grant, 1982). In spite of the extensive remodelling changes taking place in the MON, the dorsal ventral asymmetry of large- and small-fibre areas is retained as in earlier sections. No metamorphic remodelling of myelin is seen in the RON and CON at this or any other stage (Cima & Grant, 1982) and the patterns of large and small axons are unchanged from earlier stages except for an increase in numbers of large fibres accompanied by myelination.

Fibre number more than doubles in the optic nerve between a young postmetamorphic juvenile and an adult (Wilson, 1971). At a late juvenile stage (8 months postmetamorphic) the pattern changes (Fig. 11). Many more fibres are myelinated in all sections of the optic nerve including the RON (Cima & Grant, 1982). In RON and PON, large fibres are distributed uniformly over the cross section but in the MON, a peripheral to central gradient of small to large myelinated fibres is seen over the entire cross section. A gradient of myelinated fibre size is also seen in the CON, with the largest at the dorsal sector grading off to smaller fibres ventrally. The many ventral fibres that have entered the optic nerve after stage 53 have matured, many have become myelinated and the previous asymmetry of fibre size has been replaced by graded patterns, peripheral–central in the MON and dorsoventral in the CON.

Autoradiographic analysis of chronotopic ordering

If fibres are indeed chronotopically ordered during development of the optic nerve then it should be possible to demonstrate such ordering by methods independent of fibre size. Radioactive proline ([3H]proline) will label optic axons for a long period (several weeks) after injection into the retina (Grafstein,
Fig. 10. Stage 61. An optic nerve in the midst of metamorphic climax showing abnormal myelin figures and degenerating myelin in the MON. The patterns of large and small axons are unchanged from earlier stages showing a uniform distribution of large axons in the RON with a dorsoventral gradient in the MON and CON. Bars = 10 μm.

Fig. 11. Juvenile (8 months postmetamorphic). In both RON and PON the large axons, many now myelinated, are distributed uniformly in the cross section. In the MON, peripheral-central gradient of small to large myelinated axons is seen while in the CON, a dorsoventral gradient of large to small myelinated axons is most apparent. Bars = 10 μm.
Fig. 12. Autoradiographs of stage-57 retina and optic nerve. (A) Transverse section of retina labelled with [3H]proline at stage 51. Central retina is heavily labelled between arrows. Unlabelled portion at the margins represents retinal growth between stages 51 and 57. (B) Dark-bright-field micrograph of cross sections of stage-57 MON, labelled as in (A) with [3H]proline. Myelin and silver grain concentrated in the central portion. Relatively grain-free area at the periphery. (C) Dark-bright-field micrograph of stage-57 CON. Labelled with [3H]proline as in (A). Optic nerve appears as an elliptical mass attached to the dark mass of brain tissue above. Note that most grains are concentrated in more dorsal portions of the CON with a crescent-shaped non-labelled band in the ventral one third. Clumps of grains (arrows) are fascicles of fibres which have already diverged into the optic tract. Bars = 50 μm.

Murray & Ingoglia, 1972; Grafstein & Laureno, 1973). Hence, a single intraocular injection of [3H]proline at an early tadpole stage followed by a long survival period should label only those fibres present at the time of injection (old) while all fibres entering the optic nerve subsequent to the injection would be unlabelled (young axons). If old and young fibres do organize into asymmetric patterns in the optic nerve then autoradiographs of optic nerve sections should display labelling patterns corresponding to axon size patterns.

Two stage-51 tadpoles were given a single intraocular injection of [3H]proline and allowed to survive to stage 57 (approximately 24 days at 20–21 °C). During
Fig. 13. A reconstruction of a stage-57 optic nerve with autoradiographs of sections to show distribution of silver grains. Note that most silver grains are localized over areas containing large myelinated and unmyelinated fibres. In RON, distribution of grains is uniform while in PON, MON and CON, a ventral peripheral crescent (marked off by solid line) contains fewer grains than dorsal central regions of large axons.
Table 1.

<table>
<thead>
<tr>
<th>Optic nerve section</th>
<th>Optic nerve I outer annulus</th>
<th>Optic nerve I inner annuli</th>
<th>Optic nerve II outer annulus</th>
<th>Optic nerve II inner annuli</th>
</tr>
</thead>
<tbody>
<tr>
<td>RON</td>
<td>4.3</td>
<td>4.7</td>
<td>3.5</td>
<td>3.9</td>
</tr>
<tr>
<td>RON+3</td>
<td>3.3</td>
<td>6.0</td>
<td>4.2</td>
<td>5.6</td>
</tr>
<tr>
<td>RON+5</td>
<td>4.5</td>
<td>5.4</td>
<td>2.6</td>
<td>4.2</td>
</tr>
<tr>
<td>RON+8† PON</td>
<td>3.5</td>
<td>6.0</td>
<td>2.6</td>
<td>4.8</td>
</tr>
<tr>
<td>RON+10 (MON)</td>
<td>3.3</td>
<td>6.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>RON+13 (MON)</td>
<td>—</td>
<td>—</td>
<td>3.1</td>
<td>5.3</td>
</tr>
<tr>
<td>RON+15 (MON)</td>
<td>2.8</td>
<td>5.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ICMON</td>
<td>4.0</td>
<td>6.7</td>
<td>2.4</td>
<td>5.1</td>
</tr>
<tr>
<td>CON</td>
<td>5.0*</td>
<td>7.3**</td>
<td>2.0*</td>
<td>6.6**</td>
</tr>
</tbody>
</table>

Autoradiographic analysis of silver grain density over sections of optic nerves at stage 57. Grain counts made from 8 × 10 enlargements of photos and calculated as grains per cm². All silver grains in circular rings (radius 13 mm) were counted from periphery to centre of section, except for CON where counts were made in ventral (*) and dorsal (**) areas. +, Number of 5 μm sections from RON. ICMON = intracranial MON. Four to five inner annuli were counted and averaged since all counts were relatively similar. Outer = the most peripheral annulus of the optic nerve cross-section.

this period the fibre population of the optic nerve doubles from approximately 9000 to nearly 20000 fibres (Wilson, 1971). Thus, we would expect approximately 45% of the 20000 fibres at stage 57 to be labelled. Autoradiographs of transverse sections of the retinas injected with [3H]proline at stage 51, reveal a labelled central portion and an unlabelled band around the ciliary margin (Fig. 12A). The bands of newly added unlabelled retinal cells at the margin represent the addition of new ganglion cells following the effective labelling period.

Autoradiographs of sections of the MON at stage 57 reveal a densely labelled area in the centre of the nerve (Fig. 12B) roughly corresponding to the original area of the nerve at the time of injection. Moreover, the overall grain density is greater over the dorsocentral region of large axons, with a ventral peripheral crescent band relatively free of grains. The distribution of grains in the CON also corresponds to the dorsoventral asymmetry of large and small axons seen at stage 57 (Fig. 12C). Grain density over the RON is relatively uniform, a pattern that also corresponds to the distribution of axon sizes. A reconstruction of one of these optic nerves reveals that patterns of dense and less dense grain areas correspond respectively to the dorsoventral patterns of large and small axons along the stage-57 optic nerve (see Fig. 13).

Calculations of grain densities from periphery to centre in sections of two optic nerves are shown in Table 1. Grain density over the RON is uniform but in the PON grain density in the periphery is significantly less (approximately one half) than the rest of the nerve. An identical pattern, seen in sections of the
Xenopus optic nerve development

MON, shifts to a distinct dorsoventral asymmetry in the intracranial MON and CON. In all sections except the RON, the region of lowest grain density corresponds to the most peripheral annulus of the cross section. The highest grain densities in the CON overlap the regions of large axons, even in the more medial sections where small dorsal bundles of large axons are in the optic tract. The autoradiographic data are in agreement with a chronotopic ordering of fibres in the optic nerve during development between stages 51 and 57.

DISCUSSION

Establishment of the primary fascicle

Retinal ganglion cell axons are first detected in the optic stalk before optic vesicle invagination is completed (Grant et al. 1980; Cima & Grant, 1980). In contrast to chick and mouse retinas (Rogers, 1957; Kahn, 1973; Silver & Robb, 1979), fibres appear in the stalk before the choroid fissure is fully developed (Grant et al. 1980; Holt, 1980). Since invagination is asymmetric, beginning as a dorsal crescent that sweeps ventrally (Grant et al. 1980), we believe the first fibres entering the optic stalk come from dorsal retina. They are found in spaces between basal ends of columnar cells of the ventral optic stalk, usually as a single bundle enveloped by fine cytoplasmic processes from neighbouring stalk cells. As reported by Silver & Sapiro (1981) only a single primary fascicle develops in the stalk.

Intercellular spaces are seen in ventral stalk and retina as early as stage III (NF 26), before any fibres have appeared in the retina. Pioneering fibres growing in spaces between stalk cells have also been reported in mammals (Silver & Robb, 1979; Kuwabara, 1975; Silver & Sidman, 1980). In mammals such spaces seem to arise from massive cellular necrosis in ventral retina and optic stalk prior to fibre ingrowth. We find no evidence of extensive cell death, however, in Xenopus retina or stalk before fibre emergence which is in agreement with earlier observations (Glücksman, 1940; Straznicky & Gaze, 1971). Fibres fill extracellular spaces in the ventral stalk because they are pathways of least resistance, a view of axon growth that was suggested as early as 1910 by Harrison. Our limited data prevent us from concluding whether spaces form continuous channels through the stalk as has been described for the mouse (Silver & Sidman, 1980).

The fact that retinal ganglion cell axons are usually seen in bundles (Cima & Grant, 1980; Grant & Rubin, 1980) suggests that the axon surface itself serves as a substratum for axon growth within the retina. Fibres also maintain intimate contact with each other in the optic stalk, indicating that fibre following continues as new fibres arrive and the optic nerve is assembled. Other surfaces are available for axon growth in the stalk, such as the surface of finger-like cytoplasmic projections from basal stalk cells that envelop fibre bundles, or the matrix materials and basement membranes lining intercellular spaces.
Asymmetric distribution of axon size and chronotopic ordering

Between NF stages 28 and 37/38, a period of about 20 h, approximately 1000 small, unmyelinated axons assemble in the primary fascicle. Subsequently, the rate of fibre addition is linear, about 500 fibres added per day (Wilson, 1971). Excluding some large pioneering axons seen in stage-28 retinas and stalks (Cima & Grant, 1980), large, unmyelinated axons (> 0.4 μm) are first detected in the primary fascicle at stage 37/38 and their number increases linearly in the developing optic nerve at a rate proportional to the rate of fibre addition (calculated from Gaze & Peters, 1961; Wilson, 1971). Large axons are first to be myelinated at stage 47 (Cima & Grant, 1982) and the proportion of total fibres that is myelinated gradually increases from 1% at stage 50 to 14% in the adult, with the greatest increase occurring after metamorphosis (Wilson, 1971).

Increase in numbers of large axons during development may result from ingrowth of axons from newly differentiated large ganglion cells and/or from previously differentiated small ganglion cells which gradually increase in size as they mature or become myelinated. If, indeed, large axons are derived from maturing small axons, then the former are chronologically older, although it is quite evident that not all small axons are young since approximately 70% of adult optic axons are less than 0.5 μm (Gaze & Peters, 1961). We have assumed in this analysis that large optic axons are generally older than small ones during development. Regions lacking large axons are presumed to contain a large proportion of newly entering or young axons. This assumption relating fibre size and age is based, in part, on the observation that most axons do not exceed 0.35 μm in diameter before stage 37/38; large axons appear only later. In chick optic nerves, for example, more than 96% of the optic fibres are less than 0.25 μm in diameter at 12 days of incubation but in subsequent development an increasing percentage of axons is large and myelinated (Rager, 1976). Most newly arriving fibres in the goldfish optic nerve are also small and unmyelinated (Easter et al. 1981). Finally, it is known that most large neurons (ventral motor neurons or Mauthner neurons) arise as small cells with small axons which gradually enlarge as cell bodies mature (Tennyson, 1965; Kimmel, 1972).

During development, the increasing numbers of large axons are not distributed uniformly over the nerve cross section but occupy an asymmetric dorso-central position, while areas of small axons occupy the ventral periphery. This pattern arises in all sections of the embryonic optic nerve, becoming more apparent in larval MON and CON at least through metamorphosis. It is unaffected by metamorphic remodelling although later in juvenile and adult the dorsoventral asymmetric size pattern becomes less evident. If, as we propose, axon size correlates with axon age, then regions with many large axons are older than regions containing only small axons. Accordingly, it appears that the optic nerve becomes chronotopically ordered early in development, with most newly arriving axons entering centroventral regions along the optic nerve to the chiasm.
Later, in the young tadpole, fibres enter the RON uniformly over the entire cross section, occupying a ventral peripheral crescent in the MON and growing distally where they accumulate in the ventral one third of the CON. In contrast to the RON and the MON, dorsoventral asymmetry of fibre size in the CON is relatively unchanged from the beginning of optic nerve development.

Autoradiographic evidence of the distribution of old (labelled) and young (unlabelled) fibre regions correlates precisely with the distribution of large- and small-axon-rich regions in optic nerve cross sections. Regions of less-dense labelling, those containing newly arriving fibres, comprise a horseshoe-shaped peripheral band extending from the ventral pole in the MON and make up the ventral one third of the CON. Both these regions match areas containing small axons exclusively.

Presumably, a similar autoradiographic pattern might result if [3H]proline is preferentially taken up and transported by large axons. This does not seem to be the case because (1) there is no clustering of grains over large myelinated axons in these sections and (2) small fibres transport label as effectively as large since [3H]proline injected into embryonic retinas is rapidly transported in embryonic axons when no myelinated or large axons are present (Grant & Ma, unpublished). Rapid transport of [3H]proline was also demonstrated in retinas of zebra-fish embryos before the appearance of large myelinated fibres (Schmatolla & Fisher, 1972). Axon diameter, at least at this time scale, does not appear to be a critical factor in transport of radioactive proline in the retina. Finally, retinas injected with [3H]proline at stage 51 and sacrificed 24 h later show labelling in all parts of the optic nerve and tract including those parts of the pathway containing only small axons (Grant & Ma, unpublished). These observations suggest that preferential labelling of large fibres cannot be responsible for the patterns seen, that most, if not all large and small optic fibres were labelled at stage 51 and retained this label 20 days until fixed and examined at stage 57. Unlabelled regions in these cross sections are presumed to contain newly arriving fibres.

The resolution of the autoradiographic procedure makes it impossible to rule out the arrival of small, young fibres in more central regions where large fibres are found. These regions do contain many small fibres, many of which may be unlabelled but cannot be distinguished against the background of labelling in the region.

Both lines of evidence support the conclusion that chronotopic ordering of axons begins early in the primary fascicle and persists with only minor changes throughout optic nerve development. Newly arriving fibres enter the RON in all sectors, possibly along quadrant-specific bundles but as they grow distally they tend to occupy a peripheral ventral crescent in the MON and eventually collect in the ventral one third of the CON. This is not to say that new fibres do not enter other parts of the MON and CON, but a greater proportion accumulate ventrally.

The dorsoventral chronotopic ordering of fibres in *Xenopus* optic nerve
resembles a similar ordering in the goldfish and zebrafish nerves (Easter et al. 1981; Bodick & Levinthal, 1980; Levinthal & Levinthal, 1982). Newly arriving fibres tend to collect in the more ventral portion of developing optic nerves. As in goldfish, all newly arriving fibres need not remain together in a bundle as they course through the nerve (Easter et al. 1981). Our results tell us only that many if not most newly arriving fibres collect at the ventral periphery as they grow in the nerve. In Xenopus, this could reflect the fact that most fibres entering the nerve between stages 51 and 57 come from the rapidly growing ventral retinal quadrant (Beach & Jacobson, 1979).

Chronotopic ordering becomes less evident in the late juvenile and adult optic nerves because retinal growth declines and fewer small axons enter the optic nerve. Moreover, many small axons already present in the nerve, including those in the ventral quadrant, increase in diameter and become myelinated and chronotopic order disappears.

Wilson (1971) noted a peripheral–central organization of large and small fibres in Xenopus and a dorsoventral pattern of large and small axons has been seen at the CON in the developing optic nerve in Ambystoma (Herrick, 1941). Asymmetric distribution of large and small optic fibres has been observed in the optic nerves of the cat (Hughes & Wassle, 1976), pigeon (Duff & Scott, 1979), and monkey (Potts et al. 1972). In the cat and monkey, small axons are in the centre with most large diameter fibres at the periphery, resembling the Xenopus optic nerve at embryonic stages. In the pigeon, large diameter axons are segregated from medium and smaller axons across the long axis of the cross-sectional area of the nerve and their position shifts along the nerve. In the rabbit, rat and hamster, however, axons of different sizes are distributed uniformly over the cross-sectional area of the nerve (Vaney & Hughes, 1976; Hughes, 1977; Rhoades, Hsu & Parfett, 1979). The significance of axon size patterns has not been evaluated in most species, but they may also reflect some form of chronotopic fibre ordering.

We suggest that two morphogenetic factors are principally responsible for chronotopic ordering in the Xenopus nerve, asymmetric growth of the retina coupled to the phenomenon of fibre following. Both factors are responsible for an orderly development of radial fascicles within the optic fibre layer of the retina (Grant & Rubin, 1980).

Of the two, marginal retinal growth seems more important; it is asymmetric along the dorsoventral axis with ventral retinal growth lagging behind dorsal retina in the embryo (Grant & Rubin, 1980; Holt, 1980). Accordingly, the proportion of fibres entering the optic nerve from ventral retina is much less than from dorsal retina at early stages. After stage 53, however, ventral retina grows more rapidly than dorsal retina, maintaining this higher growth rate until well after metamorphosis (Beach & Jacobson, 1979). In late tadpole and early juvenile retinas, approximately ten times more ventral than dorsal retinal fibres
enter the optic nerve. Some of the oldest fibres in late tadpole optic nerve are derived from the embryonic dorsal retina while most young fibres come from ventral retina. This disproportionate ingrowth of fibres from different retinal quadrants will affect the organization of fibres in the optic nerve, particularly if fibres from the same quadrant remain together as they grow through the nerve.

Fibre following insures that ganglion cell axons arising at the periphery of the growing retina are directed into the optic nerve by joining neighbouring fascicles (Grant & Rubin, 1980). We suggest that as they enter the optic nerve, they do so on fibres from neighbouring ganglion cells and for the most part continue to grow in the optic nerve along these fibres to the chiasma; they maintain near neighbour relationships, a pattern already demonstrated in the developing zebra fish (Bodick & Levinthal, 1980).

The model of optic nerve organization described above is entirely consistent with the morphogenetic model of visual pathway development proposed by Horder & Martin (1978). The rules governing development of optic fibre organization are established early in the embryo as pioneering fibres enter the optic stalk and grow to the chiasm. Except for some modifications of the optic nerve arising after metamorphosis, we suggest that the same two morphogenetic factors continue to guide fibre organization throughout development to the adult.

Work supported by grant EY 02642 awarded to P. Grant by the National Eye Institute, National Institutes of Health. The material comes from a thesis submitted by C. Cima in partial fulfilment of the requirements for the Ph.D. degree. We are grateful to H. Howard for photographic assistance.

REFERENCES


NIEUWKOOP, P. D. & FABER, J. (1956). *Normal Table of Xenopus laevis* (Daudin), Amsterdam: North Holland.


Xenopus optic nerve development


(Received 5 May 1982)