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How axons grow down the *Xenopus* optic nerve

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SUMMARY

Retinotectal fibres from different parts of the retina have been filled with Horse Radish Peroxidase (HRP). During their course down the nerve their ordering decays, but it is re-established at the chiasma, except for nasal fibres which remain relatively disordered. The consequences of this for development are discussed.

INTRODUCTION

In the vertebrate visual system information is transmitted from the retina to the brain by the axons of the retinal ganglion cells. In the frog many of these axons terminate directly on the main visual area of the brain, the optic tectum; there are no intervening synapses. The behaviour of this retinotectal projection, as defined by the positions of termination of the optic axons on the tectum, is now quite fully documented if not understood, (Gaze, 1978; Conway, Feiock & Hunt, 1980). We know rather less, however, about the actual behaviour of nerve fibres as they travel from the retina to their eventual site of termination on the tectum. In particular we do not know whether the orderly formation by optic fibres of a retinotopic map on the tectum is dependant upon the maintenance of a similar orderliness in the optic nerve and tract, as has been suggested in some recent reviews, (Horder & Martin, 1978), or whether the interactions between optic fibres and tectum and possibly optic tract are sufficient in themselves to accomplish this – as was originally suggested by Sperry (1950). Neither do we know what sort of interactions occur between optic fibres on their way to the tectum.

In this paper I will describe how fibres from different parts of the retina run in the normal optic nerve, and discuss the implications of this for axonal behaviour in the *Xenopus* visual system.

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METHODS

Laboratory-bred *Xenopus laevis* between 1 and 4 weeks after metamorphosis were used. Animals were anaesthetized in MS222 Sandoz (tricaine-methane sulphonate) 1:1500. The chosen part of the outside of the eye was exposed, and then a small region of sclera and choroid was torn away from the retina in this area, leaving most of the eye intact.

Horse Radish Peroxidase (HRP) application

HRP (Sigma type VI) was made up in approximately 40 % solution in water containing 0·05 % poly-l-ornithine (Sigma). Gelfoam was soaked in this, and allowed to dry out in air. Small pledgets 0·2 mm in diameter were broken off for application to the retina. The pledgets of gelfoam were then placed on the area of exposed retina, and wedged between this and the orbit. The animals were then allowed to recover from the anaesthetic.

Dissection and sectioning

Twenty-four hours after the HRP application the animals were anaesthetized with MS222, then perfused with 2·5 % glutaraldehyde in 0·1 M-phosphate buffer (pH 6 4) with 20 % sucrose. The eye, optic nerve and brain were dissected out in continuity, sutures being passed through eye and brain, so that the preparation could then be mounted on card with the optic nerve pulled straight. Fixation was continued for 45 min after which the preparations were washed in 0·1 M-phosphate buffer with 20 % sucrose for a further 45 min. They were then transferred to gelatin albumen (45 g albumen in 100 ml PO_4 buffer, 0·75 g of gelatin in 50 ml PO_4 buffer, +30 g sucrose) overnight. Next day the preparations were embedded in gelatin albumen, which was set by adding 1 part in 10 of 25 % glutaraldehyde. The blocks were then frozen on a freezing stage, and sectioned at 50 μm intervals on a sledge microtome so as to provide cross sections of the optic nerve. The sections were mounted on gelatin-coated slides and allowed to dry in air.

HRP reaction

The slides were processed according to the method of Mesulam (1976). They were rinsed briefly in distilled water and then soaked for 20 min in developing solution (100 mg Sodium nitroprusside, 65 ml distilled water, 5 ml 0·1 M acetate buffer at pH5, 50 mg benzidine dihydrochloride, and 30 ml absolute alcohol). At the end of this time 4 ml 0·3 % Hydrogen Peroxide per 100 ml of solution was added, and the reaction was allowed to proceed for a further 20 min. At the end of this they were counterstained in 0·1 % neutral red, dehydrated, cleared in Xylene and mounted in Xam.

Processing of results

The serial sections were drawn using a camera lucida, the position of each filled nerve fibre being marked with a dot. These drawings were fed into a computer via a graphics tablet (Green, Perkins, Piper & Stenning, 1979). The following manipulations and calculations were performed.

(1) The nerve was not always cut in exact cross section, so that it was sometimes oval rather than circular in outline. Such sections were converted back into circles by rotating them about their minor axis until major and minor axes were equal.

(2) For tabulation of the circumferential position of filled fibres the sections were divided up into 12 segments, using the intersection of the major and minor axes as the centre. The number of fibres in each segment was counted and also the proportion of the total number of fibres in each segment.

(3) The radial positions of filled fibres were calculated by dividing the sections up into 10 concentric circles. These were spaced at equal intervals from the centre. The number and proportion of fibres in each ring was calculated.

The following calculations were done to measure whether filled fibres were spreading apart or maintaining their relative positions as they passed down the nerve.

(4) The cross-sectional area of the nerve varies in a systematic way along its length, probably due to variations in the amount of glial material and myelin. In order to make it possible to compare the cohesiveness of filled fibres between sections and between preparations, the area of the sections was adjusted to a standard value. All further calculations were done on both standardized and non-standardized sections. The values produced from the standardized sections are in arbitrary units. Only the standardized values are used in this paper.

(5) The centre of the group of filled fibres was calculated by taking the mean x and y coordinates of all the points. The distance and the square of the distance of each fibre from this central point was measured, and then the mean of these values for each section was determined. The mean square distance of each fibre from the average fibre position gives an approximate measure of the area occupied by the filled fibres. After standardization, the area of each section is 1, therefore multiplying the mean square distance by π gives an estimate of the proportion of a section occupied by filled fibres. Thus, if the fibres were completely randomly arranged in the section, the mean square distance would be about $1/\pi$ or 0·32.

All variables were plotted against the distance of each section from the optic nerve head.

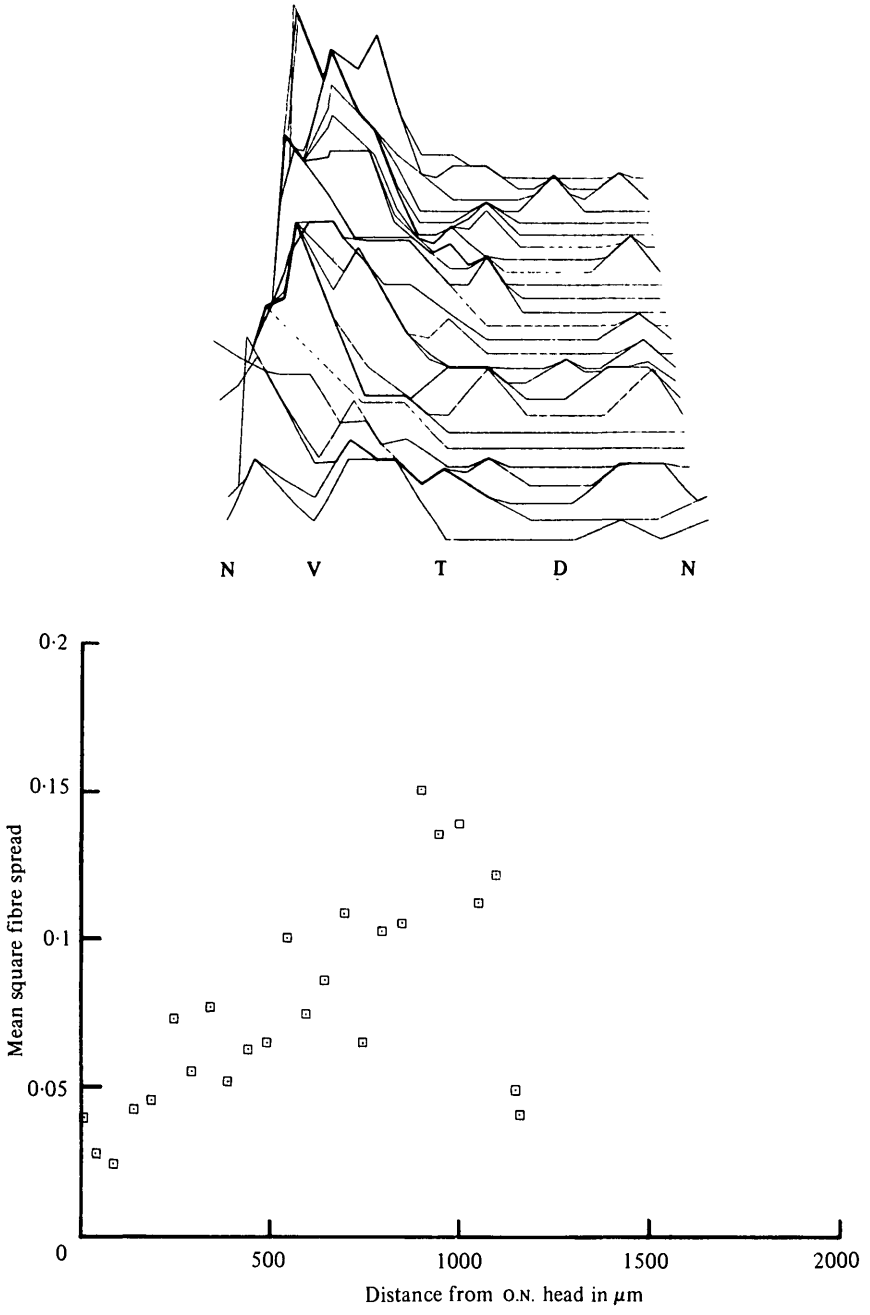

Fig. 1. The result of filling fibres from ventral retina. Each line in the diagram represents one 50 μm section, with the optic nerve head at the near end and the chiasma at the far end. The x axis represents the circumferential position of fibres within a section, and the y axis the proportion of fibres in each circumferential position. Fibres which start ventrally travel ventrally all the way to the chiasma. The lower diagram shows the fibre spread, calculated as described in the text, plotted against distance from the optic nerve head in microns. The fibres become gradually more dispersed until the final section, just before the chiasma, where they regain their original cohesiveness.

RESULTS

General anatomy of the optic nerve

The nerve is cylindrical for most of its length, including the optic nerve head, but tends to become slightly flattened dorsoventrally just before the chiasma. Its diameter is slightly less at the optic nerve head than elsewhere.

The optic nerve head

In most preparations it is possible to see filled fibres running across the surface of the retina and into the optic nerve head, although it is not usually possible to see the ganglion cells from which they come. These fibres always come directly from the site of HRP application. The fibres plunge into the optic nerve, always at the same point on the circumference from which the fibres came: thus fibres from ventral retina pass into the ventral optic nerve, fibres from nasal retina pass into the nasal optic nerve etc. Fibres are never seen to cross from one side of the nerve to the other (Fig. 5). Filled fibres only occupied a small proportion of the nerve section at this point. The average of the mean square fibre spreads from all the preparations was 0·04. Thus filled fibres occupied about 0·14 or 14 % of the area of the section at the optic nerve head.

The optic nerve

No major rotations or crossings over of axons were seen in the optic nerve, but there were more subtle changes in the positions of fibres relative to one another. Essentially fibres continue to occupy the same circumferential position within the nerve as they did at the optic nerve head, but they spread out slightly to occupy a larger proportion of the nerve than they did at that point. This spreading out happens circumferentially, but not radially. Fibres which start peripherally continue to run peripherally, despite the fact that they are spreading around the circumference of the nerve. Very few fibres were seen going to targets other than the optic tectum. The preparations contained between 10 and 200 visibly filled fibres.

There is some regrouping of fibres just before the chiasma. The extent to which this happens depends on the part of the retina from which the fibres come.

Axons from ventral retina

Axons from ventral retina occupy the ventral optic nerve throughout their course. They spread out relatively little. The mean fibre spread calculated after normalization of the area of the nerve sections increases by 0·11 (arbitrary units) and the mean square fibre spread by 0·067, averaged over five preparations. The maximum spread is found just before the chiasma. The fibre spread at the end

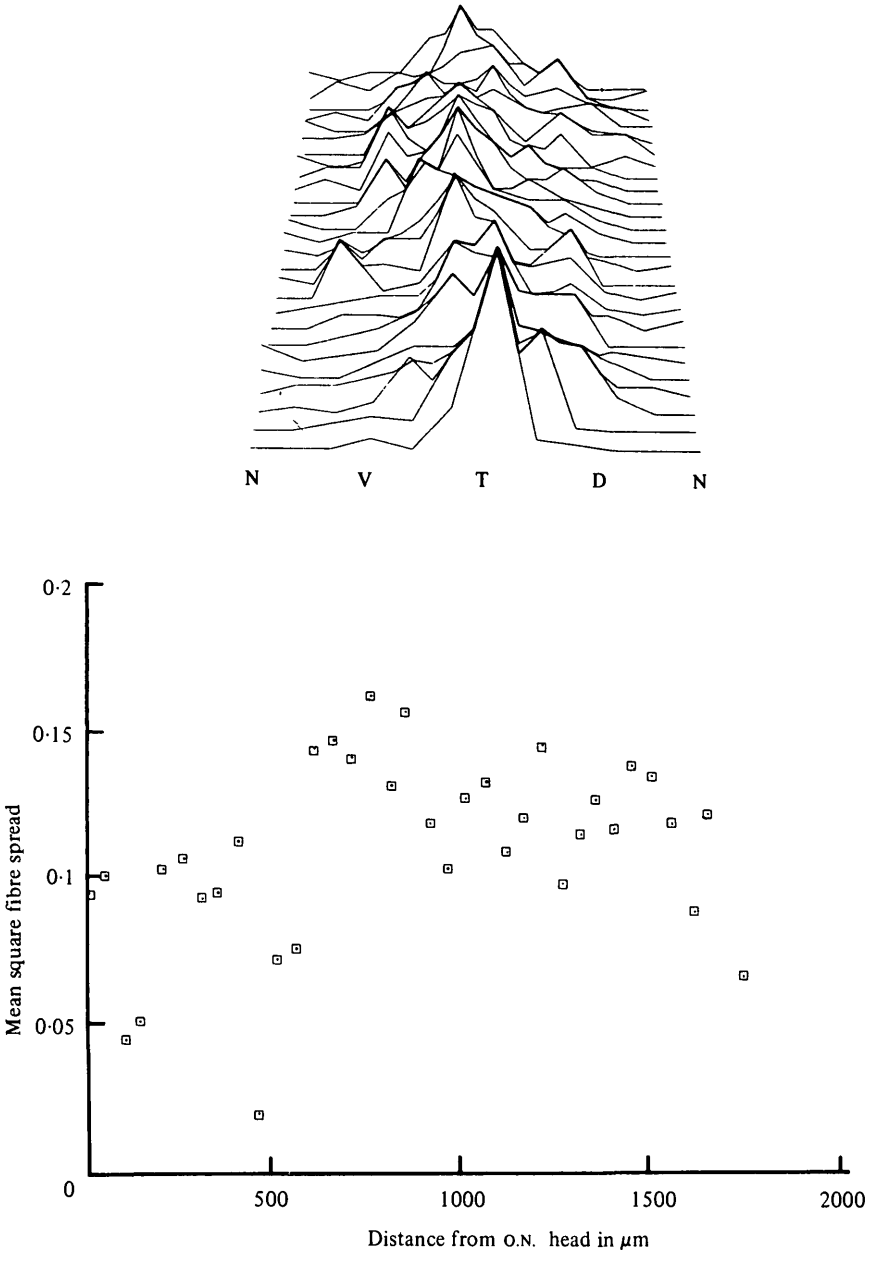

Fig. 2. The result of filling fibres from temporal retina. Filled fibres run temporally all the way down the nerve. Some degree of spreading out is seen, which is reflected in the fibre spread, which increases in the middle of the nerve, but returns to the same value as at the optic nerve head by the chiasma. Conventions as in Fig. 1.

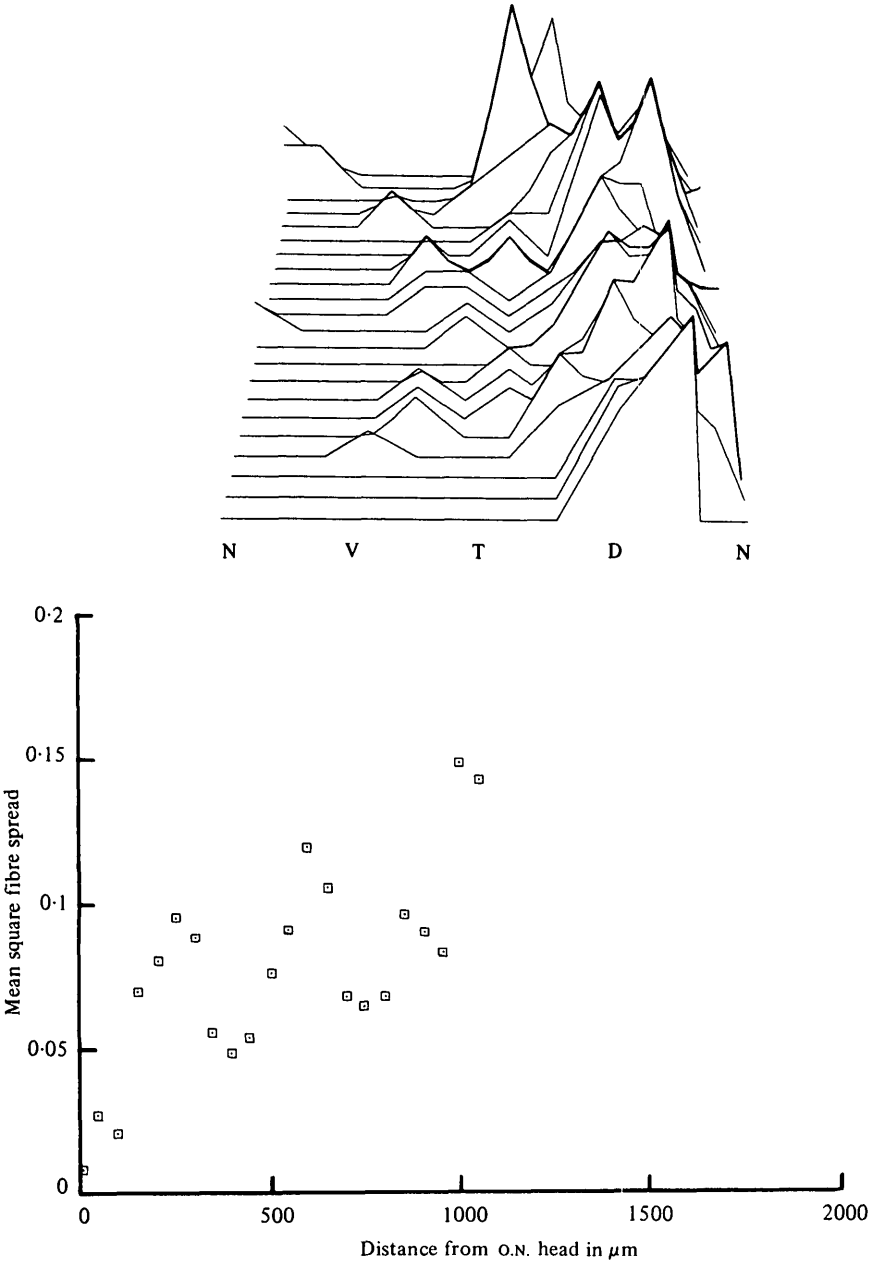

Fig. 3. The result of filling fibres from dorsal retina. Filled fibres run dorsally for most of the length of the nerve, but, just before the chiasma they rotate round to occupy a more temporal position. The fibre spread increases slightly as the brain is approached, and, in this example, does not decrease again. Conventions as in Fig. 1.

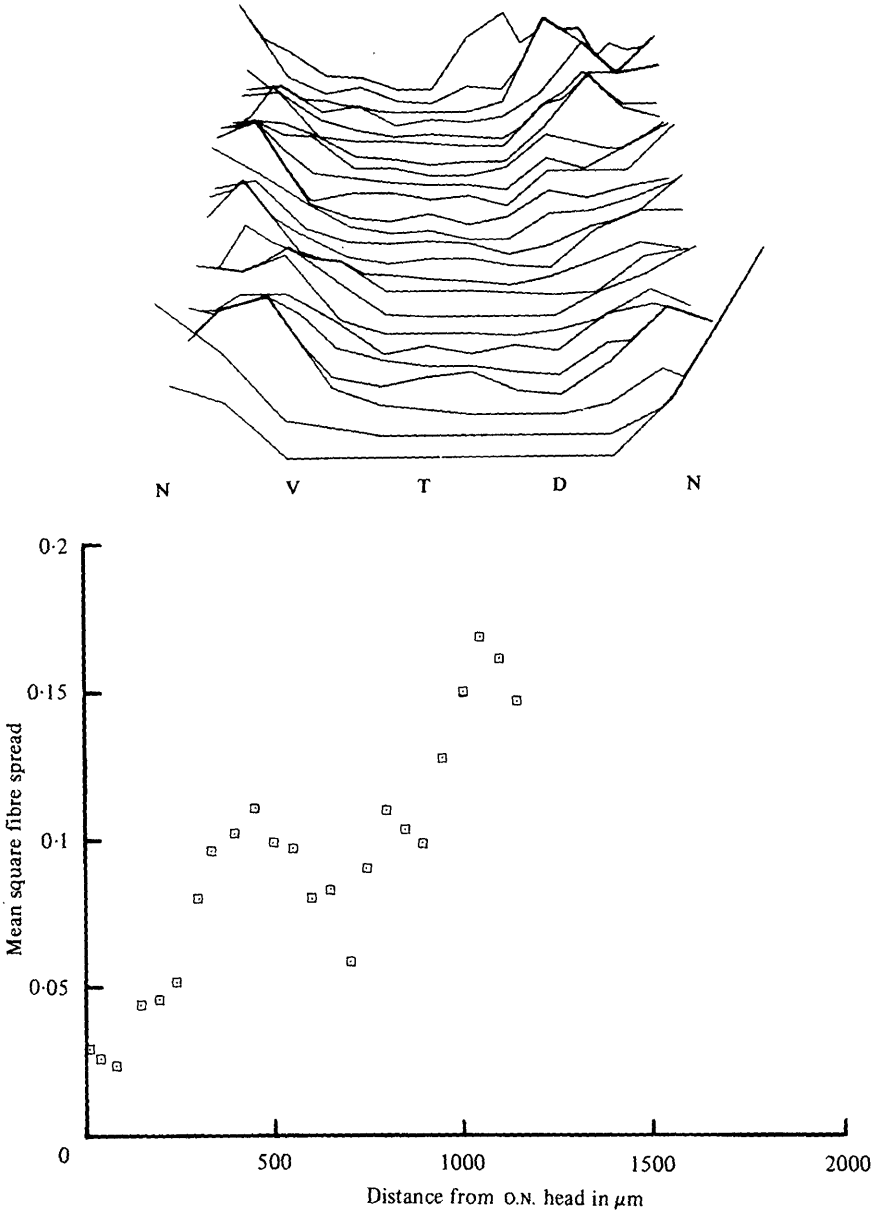

Fig. 4. The result of filling fibres from nasal retina. Filled fibres run nasally for most of the course of the nerve, but a group of fibres is seen to rotate to a dorsotemporal position just before the chiasma. The fibre spread is at its maximum at this point, and does not drop as fibres reach the brain. This is the same preparation as is illustrated in Fig. 5. Conventions as in Fig. 1.

of the optic nerve is not higher than at the optic nerve head – the fibre spread decreases just before the chiasma (see Fig. 1).

Axons from temporal retina

Axons from temporal retina occupy the temporal optic nerve throughout their course. They also spread out relatively little. The mean increase in mean fibre spread was 0·13 and in mean square fibre spread 0·096. Both these variables decrease just before the chiasma to the same value as at the optic nerve head (see Fig. 2).

Axons from dorsal retina

Axons from dorsal retina occupy the dorsal optic nerve for most of its length, but, just before the chiasma the fibres tend to move to occupy a more temporal part.

The mean increase in mean fibre spread for seven preparations was 0·14 and in mean square fibre spread was 0·096. In four of the seven preparations these values did not fall to the same values as at the optic nerve head by the chiasma, but in the other three the fibre spread was no higher at the chiasma than at the optic nerve head, (see Fig. 3).

Axons from nasal retina

Fibres from nasal retina occupy the nasal optic nerve for most of its length. However, just before the chiasma the fibres initially spread out dorsally and, in several preparations, then split into two groups, one nasal and one dorsal to temporal. The mean increase in mean fibre spread for seven preparations was 0·225 and in mean square fibre spread 0·13 (see Fig. 4). Both these values are higher than for fibres from other parts of the eye, and the increase in mean fibre spread is significantly greater for nasal fibres than for dorsal, temporal or ventral fibres ($P = < 0{\cdot}02$). The increase in mean square fibre spread is significantly greater for nasal fibres than for ventral fibres ($P = < 0{\cdot}02$), but the differences between nasal and dorsal and temporal values are not statistically significant.

In only one nasal preparation does the fibre spread decrease just before the chiasma, in the others the fibre spread is at its highest at this point.

DISCUSSION

In this paper I have examined the organization of fibres in the optic nerve of *Xenopus* at a certain period of its life. These fibres, once they have finished growing are effectively anchored at either end, so what we are looking at is the product of all the events that have happened in their past history. These are, first the growth of the axon along the optic nerve, secondly, gliogenesis,

thirdly, the shortening and remodelling of the nerve that occurs at metamorphosis, and fourthly, the subsequent growth of other axons.

The other important point is that the optic nerve is a structure that is continuously developing throughout the life of *Xenopus*. At the time that this experiment was performed, when the animals had just metamorphosed, the retina is still growing rapidly, and ganglion cell axons are continuing to make their way down the optic nerve to the brain.

The technique used in this study was to fill a small proportion of the axons in the nerve with Horse Radish Peroxidase (HRP), and to follow their course down the nerve in serial transverse sections. These sections were then fed into a computer via a graphics tablet (Green, *et al.* 1979), as described in the Methods section.

This provides a visual presentation of the position of filled fibres within the nerve, and an estimate of the extent to which a group of fibres maintains its cohesiveness.

There are two features of optic nerve fibre growth that one wishes to understand; first one needs to know in which part of the cross section of the nerve growing fibres are found, and secondly, which circumferential position fibres from different parts of the circumference of the eye occupy. This paper mainly addresses the second problem, but before discussing that it is necessary to give a brief review of present knowledge on the first. Throughout most of the development there is an unequal size distribution of fibres within the optic nerve, as has been shown by Cima (1980) and also in this laboratory. The smallest diameter fibres are found to be running either centrally in or spread throughout the cross section of the nerve at the optic nerve head, but from shortly behind the optic nerve head until just before the chiasma the smallest fibres run all around the outer circumference of the nerve. Just before the chiasma the smallest fibres concentrate ventrally, leaving only larger fibres in the dorsal part of the nerve. That these small fibres represent the youngest ones is suggested by autoradiographic evidence from Cima and Grant (personal communication). They injected tadpole eyes with [^{3}H]proline so as to fill all the optic nerve fibres present at that time. The animals were then allowed to grow for a few weeks, during which time many further axons grew down the optic nerve. The animals were then killed and autoradiographed. The proline filled axons were found peripherally at the optic nerve head, but centrally in the remainder of the nerve. In the experiments described in this paper HRP was always applied to the retina peripherally, and the resultant filled fibres were found peripherally in the optic nerve. The fact that young fibres are found only ventrally in the optic nerve at the chiasma fits well with our knowledge of the anatomy of the optic tract in which new fibres grow up the outside of the brain, and in which fibres from nasal retina occupy the anterior and posterior margins of the tract when looked at in cross section (Fig. 6). Against this background it is now possible to discuss the results presented in this paper.

The optic nerve head

At the optic nerve head fibres from ventral retina occupy the ventral optic nerve; nasal fibres run nasally, dorsal fibres run dorsally and temporal fibres run temporally. There is no crossing over of fibres from one side to the other. There is, however, another transformation taking place at this point; the youngest fibres from peripheral retina which start off running centrally in the nerve have to pass to the outer surface of it, along which they grow the rest of the way to the brain.

Behaviour of fibres in the optic nerve

Axons from all parts of the retina become partially disorganized in the optic nerve (Fawcett, 1980). Fibres that occupy a small area at the optic nerve head spread out as they pass down the nerve, while occupying the same basic position within it, i.e. ventral fibres remain ventral, temporal fibres temporal etc. This spreading does not bring fibres that started peripherally at the nerve head into the centre of the nerve; instead they spread out round the circumference (Fig. 5). If, as is suggested by the experiments of Cima and Grant discussed earlier, fibres growing at the same time occupy successive rings in the nerve, like the rings of a tree, it looks as if fibres are only able to intermingle with those which are growing at the same time. Similar observations have been made on preparations filled from the tectum (Fawcett, 1980).

Apart from the general observation that the ordering of fibres decays somewhat during their passage, there is a definite difference in behaviour between fibres from nasal retina and those from the rest of the eye. Fibres from other than nasal retina become disorganized in the nerve but regain much of their order just before the chiasma, as is shown by the measures of fibre spread returning at this point to the same value as at the optic nerve head. This does not happen to nasal fibres which spread out dorsally as they approach the chiasma, in some cases splitting into two groups, nasal and dorsotemporal. This probably reflects the reorganization of the nerve into the optic tract in which fibres from nasal retina occupy the anterior and posterior edges (Fig. 6). The tendency of fibres from dorsal retina to move temporally at the same point can be explained in the same way. In about half the dorsally filled preparations the fibre spread did not return to optic nerve head values at the chiasma. This is probably due to the fact that the nerve 'splits' nasodorsally as fibres travel to the margins of the optic tract (Scalia & Fite, 1974) so some fibres from a dorsal fill may be sufficiently nasal to be included in the more anterior group.

The increase in fibre spread from its lowest value at the optic nerve head to its highest value at, or just before, the chiasma is higher for fibres from nasal retina than for the rest the increase in mean fibre spread is significantly greater for nasal fibres than for the three other groups, but the only significant difference in mean square fibre spread is between nasal and ventral.

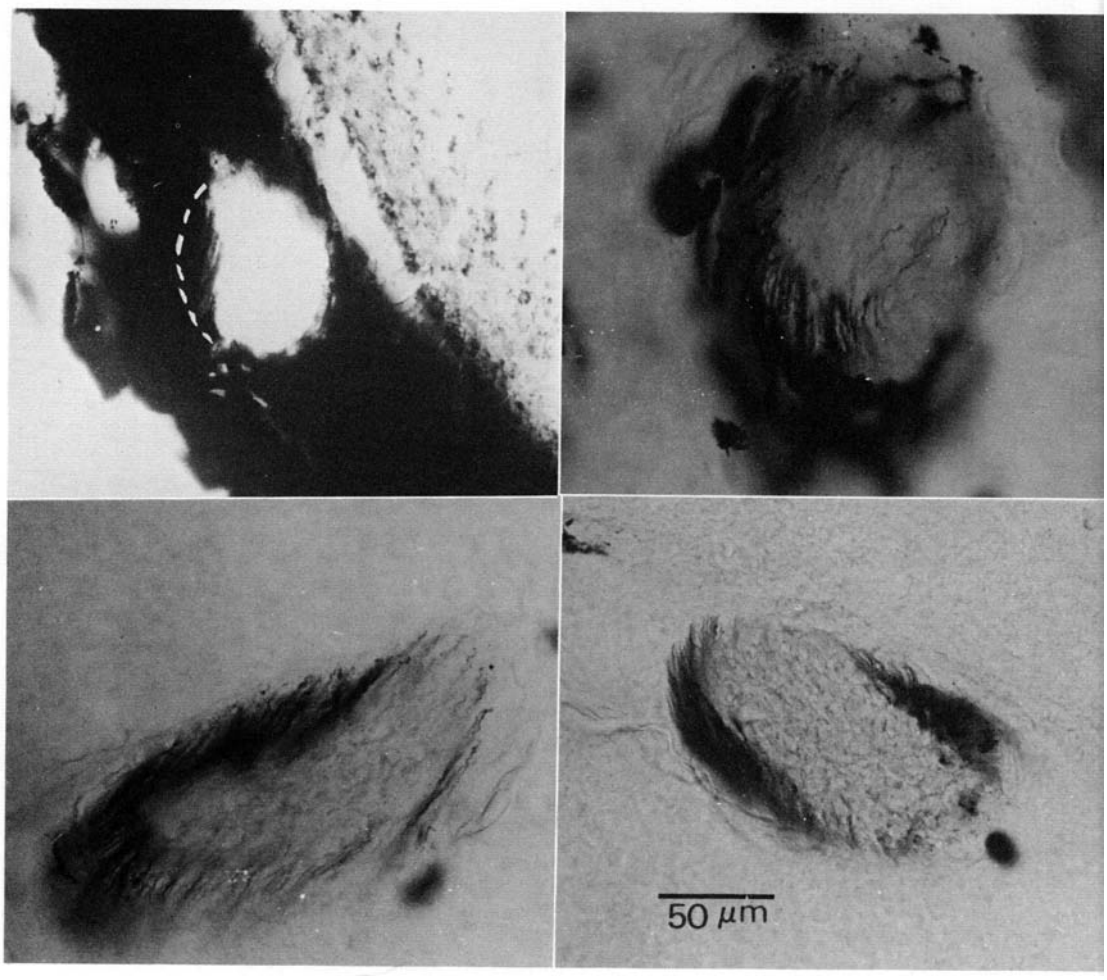

Fig. 5. A preparation filled from nasal retina. Nasal is to the left, temporal to the right, dorsal to the top and ventral to the bottom. Top left. The optic nerve head. Filled fibres are restricted to the nasal side. Top right. 150 μm further on. Fibres have started to spread round the nerve peripheri. Bottom left. 200 μm before the chiasma. The majority of fibres are still found in the nasal nerve, but many have spread out around it. Bottom right. Just before the chiasma. The fibres have split into two groups.

There are two possible explanations for this difference. The first is that fibres from nasal retina may be less able to maintain their cohesiveness than fibres from other parts of the retina; in other words a sort of differential adhesiveness. The second possibility is that there could be specific targets for fibres from each part of the retina at the chiasma. There would have to be two target areas for nasal fibres since they must 'split' so as to occupy the anterior and posterior margins of the optic tract. Their apparently greater spread could merely be a reflection of the fact that they are gradually splitting into two groups. The observation in this paper that for most groups of fibres cohesiveness is restored

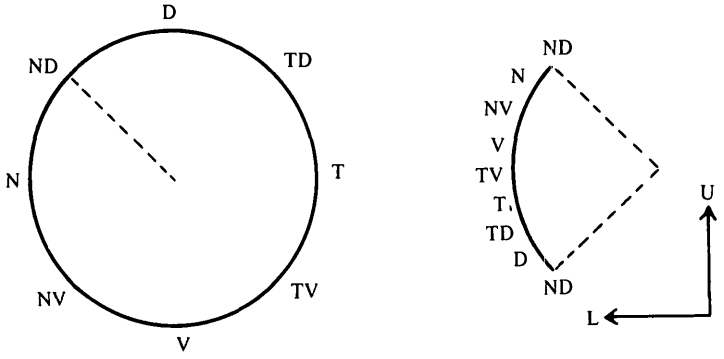

Fig. 6. The transformation which occurs at the chiasma. Left: plan of the retina, and hence the retinotopic order in the optic nerve. Right: section through the optic tract. The centre of the retina is now represented deep in the brain, and the peripheri superficially. From Gaze & Grant, 1978.

at the chiasma would tend to support this second view. However the observation by Steedman and Gaze (personal communication) that nasal fibres are found throughout the anterior–posterior extent of the optic tract rather than exclusively at the anterior and posterior margins of it would be consistent with the first possibility. If nasal fibres are less able to 'stick together' than fibres from the rest of the retina it could provide a mechanism whereby they are swept to the back of the tectum, whereas temporal fibres occupy its more mature rostral pole – the end at which the optic tract delivers fibres to the tectum.

The other main conclusion to be drawn from these experiments is that it seems unlikely that simple mechanical factors are sufficient to guide fibres to a termination on the correct area of the tectum. At the optic nerve head the new fibres from peripheral retina have to pass from the centre to the outside of the nerve. Fibres arrive at the optic nerve head organized in fascicles, and this rearrangement could only be made with a full preservation of neighbour relations by rotation of whole fascicles by 180°; this would in itself create a disordered optic nerve which would require resorting later (Fig. 7). It seems more likely that individual fibres or groups of fibres interleave their way through the existing ones.

It is possible that part of the disordering of fibres seen in the optic nerve of animals of the age examined in these experiments is due to factors other than those influencing fibre growth – namely gliogenesis and remodelling of the nerve at metamorphosis. However fibres still have to grow down the nerve as it now is, and, during their growth they will become neighbours with fibres from somewhat different parts of the retina due to this disordering. If one is to invoke fibre following as the sole means of guiding axons to their correct targets, one must assume firstly that disorder is introduced solely by gliogenesis or remodelling and secondly that an axon which starts to follow a fibre from a neighbouring ganglion cell in the retina is able to recognize this fibre so as not

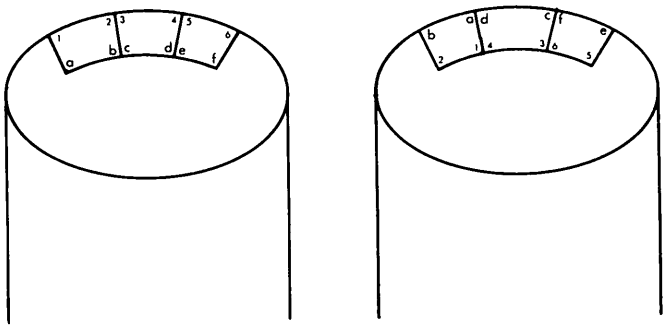

Fig. 7. The disorganization of the optic nerve which would result from the rotation of whole fascicles of fibres so as to bring the fibres from peripheral retina (a–f) from the interior of the nerve, where they are found at the optic nerve head, to the outside, where they are found for the rest of its length, while preserving rigid retinotopicity.

to be influenced by the fibres from different parts of the retina with which it will come into contact during its growth down the optic nerve. In other words if the fibre following is to work as the sole means of getting fibres to their correct targets it must be accompanied by either an enormously selective mechanism whereby a fibre will only follow its immediate neighbour, or by an accurate fibre–fibre recognition process. It was just these sorts of complications that 'fibre following' hypotheses were originally designed to avoid.

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