Changes in the retinotectal projection pattern during growth and optic nerve regeneration in the goldfish

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PhD Thesis

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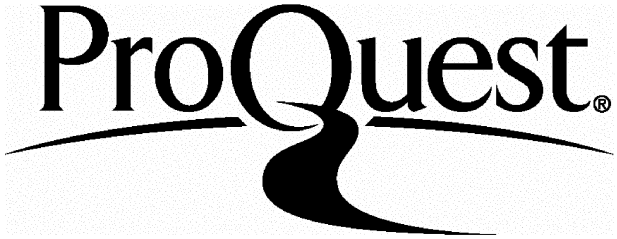

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ABSTRACT

In the goldfish, retinal ganglion cells (RGC) send their axons through the optic nerve and tract to the contralateral optic tectum where their terminal arborisations form a precise retinotopic map. The cut optic nerve regenerates to reestablish this map.

Retina and tectum grow throughout life but with different modes of growth. Nevertheless, the map remains retinotopic. A caudal shift of RGC terminal arbors was proposed to allow for the different modes of growth: the hypothesis of shifting connections. The retinotectal projection of the normal goldfish was studied using the neuronal tracer horseradish peroxidase (HRP) and was shown to be consistent with this hypothesis.

It had been proposed that, in regeneration, RGC can utilise the similar firing patterns of their near-neighbours to recognise them and to form terminal arborisations next to them on the tectum. An anatomical method to measure map precision was developed to test this hypothesis. This method, using the retrograde transport of wheatgerm agglutinin conjugated to HRP (WGA-HRP), is described. When WGA-HRP is used appropriately, its uptake can be restricted to terminal arborisations. Measurement of nearest-neighbour distances of RGC so labelled gives an estimate of map precision.

In regeneration in standard laboratory conditions, RGC labelled after injection of WGA-HRP at a standard tectal site were initially scattered. As regeneration proceeded, a retinotopic cluster of labelled RGC was seen and scattered cells were eliminated.

Fish with optic nerve cut and lens ablation on one side were maintained in tanks in diurnal, continuous or stroboscopic light. Stroboscopic light obliterates local similarities in firing patterns. Fish in stroboscopic light had less precise maps after optic nerve regeneration than controls. This result is consistent with the hypothesis that locally similar firing patterns are important in establishing precision in the regenerating retinotectal map .

ABBREVIATIONS

ACh	acetylcholine
APV	2-amino-5-phosphonovaleric acid
D	dorsal retinal pole (on tracings of retinal wholemounts)
EPSP	excitatory post-synaptic potential
GFAP	glial fibrillary acidic protein
HRP	horseradish peroxidase
ION	isthmooptic nucleus
LGN	lateral geniculate nucleus
MURF	multi-unit receptive field
N	nasal retinal pole (on tracings of retinal wholemounts)
NI	*nucleus isthmi*
NMDA	*N*-methyl-D-aspartate
RGC	retinal ganglion cells
SFGS	*stratum fibrosum et griseum superficiale*
SO	*stratum opticum*
SURF	single unit receptive field
T	temporal retinal pole (on tracings of retinal wholemounts)
TTX	tetrodotoxin
V	ventral retinal pole (on tracings of retinal wholemounts)
WGA-HRP	wheatgerm agglutinin bound to horseradish peroxidase

CONTENTS

* Chapter 5 comprises the discussion mainly in the light of work carried out up to 1985

FIGURES AND TABLES

Chapter 1: INTRODUCTION*

1:1: Questions addressed in this thesis

In the studies described in this thesis, I have used the goldfish retinotectal system as an experimental model. It is an example of a continuous map. I sought to determine or deduce some of the "rules" that govern the setting up of the map and that maintain its continuity during development.

It had been established that the retina and tectum of fish and amphibia continue to grow throughout life, though the modes of growth of the retina and the tectum are different. Using horseradish peroxidase (HRP) tracing techniques, I sought to demonstrate a pattern of optic axons on the tectum which would be consistent with a shift of terminal arborisations having taken place in order to maintain the integrity of the map during growth.

Given the hypothesis that electrical activity between neighbouring retinal ganglion cells is important in establishing a precise map, I sought to develop a technique which resolved the map to an adequate extent and yet did not rely on electrophysiological techniques. By attaching a lectin, wheatgerm agglutinin (WGA), to the neuronal tracer HRP, the uptake of HRP can be limited to the terminal arborisations where adsorption and internalisation of the tracer can occur. I showed that the size of the resulting cluster of labelled cells in the retina could be used as a measure of the precision of the retinotectal map. The method was therefore anatomical and quantitative.

Using this method, I compared the resolution of the map after regeneration of the retinotectal projection under standard and experimental lighting conditions. If local similarities in the patterns of electrical activity in the retina are important for the terminal arborisations of regenerating retinal ganglion cells (RGC) to determine the relative neighbourliness of other RGC terminal arborisations on the tectum, obliteration or alteration of these local similarities should allow an assessment of their contribution.

The relevance of the regenerating retinotectal system to the developing animal is not known and will be discussed.

* The Introduction deals mainly with work published before 1985

1:2: Beginnings

Interest in the regeneration of severed nerves led to the discovery that the amphibian optic nerve will regenerate to restore vision after optic nerve cut (Matthey, 1926). Since then, the lower vertebrate visual system has been used as an experimental model in order to determine the "rules" which govern the formation of a topographic map.

In a series of experiments on fish and amphibia, Sperry (1943, 1944, 1948) showed that in animals in which the eye was rotated, with and without optic nerve cut, the visual field assessed by behavioural methods remained rotated. The animals oriented themselves and struck in the opposite direction in response to a lure. These findings were interpreted as indicating that the retinal and tectal cells had re-established their original connections in spite of the functional disability that resulted. In addition, tectal lesions combined with optic nerve cut in adult anuran species showed appropriately placed scotomata after optic nerve regeneration, again using behavioural responses to assess the map (Sperry, 1944).

Sperry proposed the theory of chemospecificity (Sperry, 1963). According to this theory, synaptic connections would be formed selectively, independently of the functional outcome. Synapse formation would be regulated by highly specific cytochemical affinities between retinal and tectal cells which differentiated the neurons from one another. The theory also included the presence of two or more 'morphogenetic gradients' which were thought to be at right angles to each other, giving each neuron a chemical co-ordinate by which it could establish its place in the map. Such chemical interactions were thought to take place in the optic pathways too. It was recognised that such a theory would require a large number of chemical labels.

The chemospecificity theory was valuable in that it was readily testable within the model of the lower vertebrate retinotectal map. Thus while it is now known that the theory and some of the experimental work that it generated are flawed, the stimulus to experimentation was substantial.

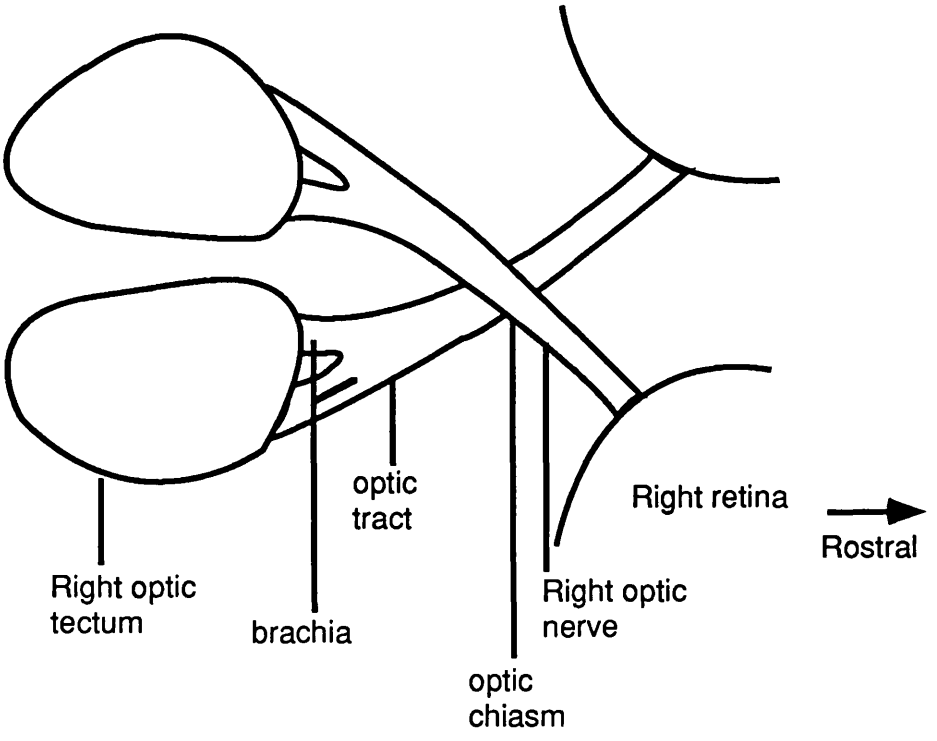

Figure 1: Schematic representation of the goldfish visual system, viewed from above

1:3: Anatomy of the experimental model

The lower vertebrate visual system has been extensively studied as a model for topographic map formation. Specifically, in the teleost, the retinal ganglion cells (RGC) from each eye project topographically to the contralateral optic tectum, a midbrain structure, which is the largest single visual projection target (Figure 1). The axons of the RGC unite at the optic nerve head to form the optic nerve which then passes caudally and medially. The optic nerves cross completely at the optic chiasm and continue towards the tecta as the optic tracts. As each optic tract approaches the tectum, it divides into two brachia, medial and lateral, which innervate the dorsal and ventrolateral halves of the tectum respectively. Axons from the ventral retina travel in the medial brachium and those from the dorsal retina in the lateral brachium (Attardi and Sperry, 1963; Sharma, 1972). Fascicles of axons form an array over the optic tectum, within the *stratum opticum* (SO; Attardi and Sperry, 1963; Murray, 1976). Axons leave their fascicle to pass towards their termination site in the deeper *stratum fibrosum et griseum superficiale* (SFGS; Attardi and Sperry, 1963). In the rostral tectum, a few axon terminals can also be found in the deepest layer, the *stratum fibrosum periventriculare* (Sharma, 1972). Temporal retina projects to rostral tectum, nasal retina to caudal tectum, ventral retina to medial tectum and dorsal retina to lateral tectum (Jacobson and Gaze, 1964). The projection is uniform since the goldfish retina has no specialised *area centralis*.

In this thesis, some of the assumptions which had been made about the retinotectal projection, and particularly about the tectal paths of the RGC axons, will be challenged, and a pattern of tectal axons consistent with shifting connections will be described (Cook, Rankin and Stevens, 1983).

1:4: Methods of mapping the retinotectal projection

The advantages and disadvantages of mapping methods are discussed in detail in Chapter 3, section 3:1. The following are brief descriptions.

1:4:1: Behavioural methods

The early experiments in the 1940s were limited by the behavioural methods which were then available. The results of experimental manipulation were determined either by the animal's response to small visual stimuli or by its compensatory head movements in response to rotation of the visual field (the optokinetic response). Although these methods led to important discoveries, they required complex responses and yielded little information about the anatomy of the map. Resolution of the map was limited.

1:4:2: Histological methods

Before the use of specific neuronal tracers, the selective silver impregnation of anterograde and retrograde degeneration was the major method for tracing neural efferents and afferents. This method was limited by the size of the lesion and relied on the staining properties of damaged cells.

Histological methods have been used to map the projection of the regenerating optic nerve in the goldfish (Attardi and Sperry, 1963). The lesions were large (half-retinae) therefore the degree of map resolution was low. Subsequently, the results of these experiments have been found to be inaccurate, again raising doubts about these histological methods.

1:4:3: Autoradiography

Autoradiographic tracing relies on the ability of axons to take up tritiated amino acids which are then incorporated in newly synthesised proteins and transported to nerve terminals by the process of axonal transport (Neale, Neale and Agranoff, 1972). Resolution of the map is limited by the size of the lesion.

1:4:4: Electrophysiological mapping

The topographic projection can be mapped by recording action potentials in the optic tectum in response to a stimulating light source or reflective probe in the visual field of the contralateral eye (Gaze, 1958; 1959). Gaze demonstrated retinotopic maps in frogs with normal and

regenerated projections.

Electrophysiological mapping is a relatively sensitive method. Gaze (1959) compared histological, behavioural and electrophysiological methods in the regenerated projection in *Xenopus laevis*. When behavioural responses were not detectable, electrophysiological responses could be mapped.

There has long been uncertainty about the anatomical basis of the multi-unit receptive field recordings. This question is addressed in section 3:1.

1:4:5: Neuronal tracing with horseradish peroxidase (HRP)

Given the limitations of the methods described above, the attractions of a neuronal tracer which can be transported anterogradely and retrogradely, and which can be readily detected after being taken up at small lesion sites, are evident.

Peroxidase activity is widespread in nature and horseradish root is a rich source (Mesulam, 1982). The peroxidase-peroxide complex can oxidise a wide variety of chromogens. Once it has been oxidised, the ideal chromogen should be easily visible and form polymers which remain at the site of oxidation. HRP has a molecular weight of approximately 40kd. It is taken up by axon terminals by endocytosis (Holtzman and Peterson, 1969) and also enters cut axons (Halperin and LaVail, 1975). It is transported antero- and retrogradely thereby delineating axonal paths when it is revealed by the oxidation of the chromogen. In general it is not taken up by intact axons of passage (LaVail and LaVail, 1974).

The hypothesis that locally correlated neural activity has a role in establishing the retinotopic projection (Willshaw and von der Malsburg, 1976) identified a need for a method of determining map precision which is non-electrophysiological (though axonal transport and activity are related) and of high resolution. The method should be non-electrophysiological because tests of the hypothesis must involve disruption of the impulse

pattern. Since the hypothesis states that neuronal activity is important for fine tuning of the map rather than for establishing gross order, the method must also be of high resolution.

Using HRP and WGA-HRP, I was able to examine the results of uptake by either axons of passage and axon terminals or axon terminals alone, respectively. Electrophysiological mapping yields information about an axon's origin and termination (or possibly its subsequent synapses) but does not give information about the axon's route. In addition, anatomical maps can be obtained at an earlier stage of regeneration than electrophysiological maps.

1:5: Orientation of the map and chemical markers

1:5:1: The contribution of Roger Sperry

Sperry's experiments in the 1940s (see section 1:2) led to the hypothesis that precise chemospecific labels exist on retinal and tectal cells so that retinal cell axons can recognise their tectal targets (Sperry, 1963). They also raised the possibility that factors operating in regeneration might be operating in development also. Further manipulation of the goldfish retinotectal projection seemed to support the specificity theory (Attardi and Sperry, 1963). Removal of a half-retina and division of the optic nerve were reported to result in orderly regeneration of fibres within the appropriate brachium as well as to the appropriate region of the tectum. Attardi and Sperry (1963) proposed a chemospecific basis to route selection as well as to selection of termination site. However, their findings have been challenged. For example, retinal ablation experiments have shown that although regeneration of the optic nerve to its appropriate tectal region occurs initially, later there is expansion of the projection over the whole tectal surface (Schmidt, Cicerone and Easter, 1978). Sperry himself realised that a body of experimental evidence had built up which contradicted some of the tenets of the original chemospecificity theory (Meyer and Sperry, 1976).

1:5:2: Acquisition of polarity

At what stage in development would the putative markers become established in order to achieve a correctly oriented map? Experiments involving rotation of the embryonic eye cup at various stages of development (Stone, 1944; Jacobson, 1968) and mapping by behavioural and electrophysiological methods at adult stages showed that polarity is apparently established early in development. In *Xenopus* (Jacobson, 1968), if the eye cup was rotated before stage 30 (Nieuwkoop and Faber, 1956), connections were formed as if no rotation had taken place. After this time, one or both axes were inverted in the mapped projection indicating that the RGC were in some way specified. Later, the results of temporary transplantation of the developing eye to the flank (Hunt and Jacobson, 1972) were taken as evidence that polarising information is also present on the side of the body. For instance, when a stage 28 donor's left eye was transplanted to the right flank of an intermediate host, thus producing anteroposterior inversion, transplantation to a final carrier without rotation yielded a normal map. This suggests that the eye gained positional information from the intermediate host rather than the donor. And if the eye was transplanted in its original orientation to an intermediate host, then inverted when transplanted to a final carrier the map was reversed. Again, this suggests that the eye acquired its positional information on the host flank.

1:5:3: Experiments questioning Jacobson's work

However, these results have been questioned and, indeed, have not been confirmed. Sharma and Hollyfield (1980) rotated eye primordia in *Xenopus laevis* at stages 24-42. In all cases, the map was rotated to the same extent as the angle of rotation of the choroidal fissure, a marker of the ventral pole. The results indicated that from the earliest stages of eye development the eye carried positional information which could not be overridden by extraocular influences.

Munro and Beazley (1982) carried out further 'eye-to-body-transplant' experiments. Eye primordia of *Xenopus laevis* at stages 22-26 were transplanted, 180^0 rotated, into the flank of embryos of similar stage and

later into the orbits of final hosts at stages 32-36, either directly or with 180^0 rotation. Electrophysiological mapping showed that in the former case the map was rotated and in the latter that the map was normal. This is further evidence that the polarity cues are already specified at stage 22, the earliest stage of the experimental animals.

The formation of compound eyes in *Xenopus* embryos at stage 32-33 provides further tests of polarity. They are formed in *Xenopus* by the surgical apposition of two half eyes in one orbit at an embryonic stage. Fawcett and Gaze (1982) used HRP techniques to demonstrate fibre pathways from double nasal (NN), double temporal (TT) and double ventral (VV) eyes. Fibres from VV eyes all entered the tectum via the medial brachium. In the normal eye ventral fibres lie in the medial brachium. Similarly, fibres from TT eyes entered the tectum directly at the rostral pole just as they do in the normal eye. At the time of formation of the compound eye, RGC have acquired information about their origin which is 'expressed' in the paths which they take.

Cooke and Gaze (1983) carried out similar experiments in which sectors of the eye primordium were replaced with sectors grafted from the opposite position in the rudiment on the same side of the head of the donor. Grafts were performed before stage 28. In these experiments, the polarity of the final projection was assessed by electrophysiological mapping. The maps were often compound, again suggesting that the eye primordium had received positional cues before the grafts were formed. Interestingly, the handedness of the graft's map tended to be reversed. These results were explained by the authors in terms of intercalation of positional cues which could be affected by cell death. Cell death at the graft margin would lead to 'shortening' of the 'distances' between the positional cues of neighbouring cells and therefore there would be a degree of intercalation rather than complete autonomy.

These sector graft experiments also demonstrated that the set of positional cues in the eye primordium appear to be continuous rather than to follow any geometric rules. Application of geometric rules to a biological

system is a relatively crude way of establishing rules of map formation. There is little reason why the eye primordium should be governed by such geometric principles. Interpretation of results should include an assessment of the artificiality of the experimental situation. Geometric aspects of the map may include the choroid fissure ventrally in the eye and the division of brachial routes taken by axons of dorsal and ventral retinal origin.

How then did these differences in experimental results arise? Holt (1980) proposed that the basis of the change from labile to determined polarity, as found in Jacobson's experiments at stage 28-31 in *Xenopus,* lay in cell movements in the optic stalk leading to formation of the ventral fissure. She showed, using ^{3}H-thymidine labelling, that cells in the ventral retina, where the choroid fissure forms, move into position from the optic stalk during eye cup formation. Thus, depending on the timing and depth of the surgical procedure, cells destined to form ventral retina may or may not be included in the rotated eye cup. If migration of the cells has been complete, the ventral fissure will be situated dorsally and the map will be rotated. These findings again stress the importance of taking into account what we know about cells when considering mechanisms of pattern formation. Polarity may be established by cell movements rather than by 'factors' in the tissues surrounding the eye either in the orbit or on the flank.

1:5:4: Compound eyes

While evidence exists for the concept of polarity being important in map formation, and probably established in part by cell movements, what evidence is there for chemospecificity? Again, the surgically formed 'compound' eye can be used as an experimental model; I have already argued that it can be used as a means of testing for dorso-ventral polarity. Straznicky, Gaze and Keating (1981) showed that initially double temporal eyes confined their projection to rostrolateral tectum, and double nasal eyes had more extensive projections but with reduced autoradiographic labelling rostrally. In the normal eye temporal fibres project rostrally and nasal eyes caudally. These results suggest a degree of specificity in the formation of initial synapses.

Also, there is evidence of specificity in the paths taken by RGC axons from compound eyes to the tectum. All fibres from ventral compound eyes passed into the medial brachium (Straznicky, Gaze and Horder, 1979). In this case the specificity is at the level of the brachium. It is possible that an increase in interaxonal adhesion might effectively abolish the lateral brachium, if this biological property were the overriding factor.

1:5:5: Half-eyes

If the nasal or temporal half of an eye in a *Xenopus* embryo of stage 32 is removed, the residual half-eye which is surgically rounded up eventually produces in most cases what appears to be a full size normal eye with a normal retinotectal projection (Feldman and Gaze, 1975). In some cases, mirror-image duplicated maps were seen. When dorsal or ventral half-eyes were rounded up only normal projections were produced (Feldman, 1978). In the case of the dorsal half-eyes the half-eye remained smaller than the normal eye, which may be a reflection of the lower capacity for growth in the dorsal retina. In effect this too demonstrates a feature of cellular determination.

These and subsequent experiments provided further evidence against Jacobson's theories on the time of determination of polarity since they demonstrated that the embryonic half eye could regulate after damage at stage 32 (Feldman and Gaze, 1975; Feldman, 1978; Straznicky, Gaze and Keating, 1980). Jacobson's experiments had suggested that polarity had already been determined by this stage.

1:5:6: 'Pie-slice' grafts

Wedges or 'pie-slices' of embryonic eye tissue transposed to incorrect regions of the eye were found to project autonomously to the optic tectum regardless of the projection of their neighbours (Cooke and Gaze, 1983; Willshaw, Fawcett and Gaze, 1983). It therefore appeared that the transposed retinal ganglion cells were not respecified in their new position, even before stage 32. This is in contrast to the experiments described above, for example, in which regulation of half eyes occurred in most cases to form a complete projection.

These findings were interpreted as demonstrating the existence of positional markers which are specified early and which are used in the formation of the retinotectal map.

1:6: Tectal markers

1:6:1: Rotation and translocation of tectal implants

At the level of the tectum, changes in the orientation of portions of the tectum in *Xenopus* yielded equivalent changes in the orientation of the electrophysiological map (Levine and Jacobson, 1974). Yoon (1973) carried out a series of experiments in adult goldfish in which rotated tectal implants were shown to retain their original polarity even in a compressed projection (Yoon, 1977; see below). Graft translocation without rotation (Yoon, 1980; Gaze and Hope, 1983) demonstrated that translocation of the corresponding part of the visuotectal map occurred. Thus there is some evidence for both markers of polarity and/or affinity labels on the tectum. At much earlier stages of development in the amphibian brain, Chung and Cooke (1975) found normal maps in spite of rotated tectal tissue. However, when the diencephalon developed behind the tectum rather than in front, the map was reversed. The authors proposed that the diencephalic cells determined polarity rather than the tectal cells themselves.

1:6:2: Compression experiments

Experiments which involved removal of a half-tectum demonstrated that the projection could be compressed onto the tectal remnant. This could occur with and without regeneration (Yoon, 1976; Cook, 1979). Optic axons regenerating to a rostral half-tectum that had previously received a compressed projection initially formed a projection restricted to the appropriate part of the retina (Cook, 1979). This argues against induction and maintenance of tectal labels. However, these experiments did provide evidence for specificity in the early stages of map formation in regeneration.

1:6:3: Re-labelling experiments of Schmidt

Evidence implying that retinal fibres might alter tectal markers came from experiments by Schmidt (1978). However, these experiments are

controversial and have not been confirmed. The nasal half-retina was removed and an expanded projection from the remaining temporal half-retina was formed. Subsequently the optic nerve was crushed and the resulting projection mapped to determine whether an expanded or restricted map was formed initially. If expanded, this might imply that fibres from the half-retina had induced a change in tectal markers, such that on regeneration the fibres would go straight to their recent sites of termination. Numbers of experimental animals which were mapped were small (two). The autoradiograph of the projection from the half-retina, while not completely confined to the rostral half-tectum, showed a distribution of label which was far from uniform. The expanded maps were detected after 46 and 50 days of regeneration, and there were no earlier studies to exclude an intermediate stage with a more restricted map.

1:6:4: The 'arrow' model

The experiments described above, in particular those involving compression, expansion and the rotation of tectal grafts, led to the elucidation of the 'arrow' model by Hope, Hammond and Gaze (1976). This was put forward partly in answer to the chemospecificity hypothesis of Sperry. Sperry's theory had required modification following the compression/expansion experiments and its biological basis had never been made clear. Hope, Hammond and Gaze showed that the experimental findings could be explained without the need to invoke tectal specificities. They referred to 'systems matching', a process by which axons could organise themselves on the tectum after obtaining a small amount of information such as the directions of the rostral and medial poles of the tectum. This information could be encoded in two concentration gradients, one for each axis. In this respect the arrow model is similar to Sperry's hypothesis where he describes morphogenetic gradients: the difference lies in the lack of differential affinities between retinal and tectal cells. The authors stated that the tectal gradient did not have to consist of the concentration of a chemical substance, but could be encoded for example by the direction of electrical activity. This is an interesting attempt to use the biology of the system to obtain directional information and is reminiscent of the model of Willshaw and von der Malsburg published in the same year

(1976). In the latter model, the pattern of electrical activity is invoked to encode positional information but at a local level rather than as a gradient. The 'simple arrow' model of Hope, Hammond and Gaze did not require a true gradient at all; left/right differences, such as structural asymmetries, between tectal cells would suffice.

Tectal graft rotation experiments were considered not to be a true test of the presence of tectal locus specificities since they did not exclude systems matching. The tectal graft would have directional information within it, and a rotated map within the graft would form whether the tectal graft were specified or not (Hope, Hammond and Gaze, 1976). Reciprocal translocation of tectal grafts would lead to a normal map if the simple arrow model applied since there would be no gradient and the directional information within the grafts would be no different. If there were true gradients within the tectum, translocation would lead to reversals of the gradients at the junctions of the grafts and lead to the alteration of positional information. The experiments were technically difficult and only 4 out of 21 animals survived to be mapped. Interestingly, two of the maps were normal and two showed translocation of the map corresponding to the surgical manipulation. Thus the matter had not been resolved. However, this model made progress from the chemospecificity theory which had dominated experimental work up to this point. The lack of an absolute need to invoke retinotectal differential affinities and the possibility of self-ordering within neuronal populations had been established.

1:7: Growth of the retinotectal system

Any theory that seeks to explain the formation of organised neuronal maps in lower vertebrates must take into account the disparate modes of growth of the retina and the tectum. New cells are added to the retina and tectum throughout adult life. In spite of this, the animal maintains its vision, and an organised map can be demonstrated at all stages of development.

1:7:1: Evidence in *Xenopus*

The different modes of growth were first demonstrated in the visual system of *Xenopus* (Straznicky and Gaze, 1971; 1972). Using autoradiography with tritiated thymidine, the authors showed that the retina adds cells in annuli at its periphery and that this growth continues into post-metamorphic life. The youngest cells are therefore at the periphery of the retina. In the tectum, however, they showed that the dividing cells are at the caudomedial edge. Therefore in order to maintain an ordered map, the need for shifting connections throughout development and into post-metamorphic life was deduced. For instance, the oldest (central) retinal cells would have to shift their terminal arbors caudally into relatively younger areas of tectum in order to maintain the map.

Evidence that shifting of terminal arbors does occur came from double labelling autoradiographic techniques, using both tritiated thymidine and proline. Scott and Lazar (1976) showed that, in *Xenopus,* proline labelled retinal cells projected caudal to a band of thymidine labelled tectal cells, implying a shift of terminal arborisations beyond the band of cells which had been generated earlier. Jacobson (1977), using a similar technique in older animals, showed that optic axons did not extend into the region caudal to the thymidine labelled tectal cells. He preferred the idea that retinal and tectal growth were matched from the time of synapse formation due to asymmetric retinal growth (Jacobson, 1976); though if this idea were extended to other neuronal maps, it would be difficult to envisage how the growth of all maps could be similarly co-ordinated during development. While growth of the retina in *Xenopus* is indeed asymmetric, further studies (Gaze, Keating, Ostberg and Chung, 1979) confirmed that retinal growth is in general annular. Therefore these results confirmed the need for a shift in terminal arbors, and Jacobson's results showing a lack of dividing cells at one pole of the *Xenopus* retina were not confirmed.

1:7:2: Evidence in goldfish

Similar experiments on the goldfish visual system showed that the retina and tectum grow throughout adult life (Meyer, 1978). Cell counts of all classes of cells within the retina, including retinal ganglion cells, were

performed at different stages of development, and related to the overall retinal area (Johns and Easter, 1977). Cells were shown to be distributed nearly homogeneously and the density of RGC fell during development, though that of rods remained constant. They therefore confirmed that the adult goldfish retina continues to grow, but that a component of the retinal growth is cellular hypertrophy as well as the increase in neuronal cell number. The relative contributions of neuronal number and hypertrophy to the total increase in retinal area have been estimated to be 20% and 80% respectively (Johns, 1977). Initially it was thought that the density of rods was maintained and the distribution kept uniform by a process of "shearing" whereby connections between cells were broken and new ones formed (Johns, 1977). However, it now appears that new rods are intercalated, and old connections need not be broken (see below).

Not all neurogenesis in the teleost retina is confined to the circumferential germinal zone. Using ^{3}H-thymidine autoradiography, it has been shown that mitotic figures exist within the outer nuclear layer of the growing goldfish retina (Johns and Fernald, 1981). Rods are found within this layer; it is a neural layer which does not contain the nuclei of glial cells or vascular structures. It was deduced that these dividing cells differentiate into rods, on the basis of their morphological characteristics. The zone of rod neurogenesis in the outer nuclear layer has been further characterised (Fernald and Scholes, 1985). Using ^{3}H-thymidine labelling and reconstruction of retinal autoradiographs, it has been shown that the rod precursors are concentrated in a circular front 100μm inside the retinal margin. They move outward from older to newer retinal tissue, augmenting the rod population.

The rod precursors themselves appeared to arise from clusters of undifferentiated cells in the inner nuclear layer. At early stages of development, labelled cells were found in the inner nuclear layer (Johns, 1982). It was presumed that rod precursors arose from this population of cells and migrated into the outer nuclear layer before dividing to form rods.

In this way a uniform density of rods can be maintained throughout

development. It has been proposed that this serves to maintain visual sensitivity (reviewed in Raymond, 1985).

Measurements of cell number and overall tectal size have also been made (Raymond and Easter, 1983). Again, spreading apart of existing tectal cells contributed to overall growth more than the increase in cell number (80% versus 20%), and again cell density was uniform throughout the tectum. Is it possible that terminal arbors need not shift during development, since the component of growth due to stretch may simply displace the terminals passively, still attached to the underlying tectal cells? This is unlikely. Passive stretch would carry the arbors of peripherally terminating RGC outwards, away from the tectal location appropriate to their position in the retinotectal map.

Therefore the initial findings in the *Xenopus* visual system have been confirmed and extended in the goldfish. A body of evidence suggesting a requirement for shifting connections during development of the goldfish retinotectal system was being established at the time of the studies to be described here. The plasticity of neuronal connections shown in expansion and compression experiments appears to have its counterpart in the developing system. In its strictest form, Sperry's chemospecificity theory had been well tested and found to be untenable. It was unable to explain all experimental results in any form.

Using horseradish peroxidase neuronal tracing methods, I sought to provide further evidence, with an anatomical basis, for the hypothesis of shifting connections. Although such a method provides static evidence for a dynamic process, it could provide evidence of a pattern of axons consistent with a shift having taken place. In addition, retrograde and anterograde tracing methods could be used to complement each other and provide confirmatory evidence.

1:8: Paths of axons

1:8:1: Retinal paths of ganglion cell axons

Light and electron microscopy, with and without tracer methods, have been used to examine the cellular structure of axon paths within the goldfish retina (Easter, Bratton and Scherer, 1984). In general, fibres were shown to run in fascicles. Non-myelinated fibres ran superficially and were taken to represent the axons of young, that is, peripheral, RGC. These axons were the only ones present in peripheral retina, and peripheral retinal lesions resulted in degeneration of axons within this superficial layer. The other class of axons were deeper in the retina, larger and myelinated. However, the arrangement of myelin differed from the compact myelin sheaths seen in the optic nerve in that it was looser. The origin of the cellular processes surrounding these axons was assumed to be glial. The myelinated fibres were assumed to be older because of their morphology as described above, and because of their relationship to the non-myelinated fibres the origins of which had been extensively investigated. It was argued that an age- related order of RGC axons is established within the retina, with older axons from more central retinal origins situated deep in the retina and newer axons situated progressively more vitread.

Further evidence for the most superficial axons being the youngest came from the reconstruction of growth cones within this layer. They were not seen in deeper layers. Also in this layer were the glial end feet which arise from Muller (glial) cells. The role of glial cells in growth cone guidance is not known, but they may in addition have a role in maintaining potassium balance locally (Oakley, Katz, Xu and Zheng, 1992). The end feet may be 'pushed aside' by the advancing growth cone.

The retinae of other species have been similarly examined (chick, Krayanek and Goldberg, 1981; monkey, Ogden, 1983). In the chick embryo, extracellular spaces were seen to precede the outgrowth of the optic axons, and new optic axons were seen to run on the vitread surface in a pattern similar to that seen in the adult goldfish. Again the filopodia of the growth cones were seen to be in close contact with the glial endfeet. The

organisation of the retinal paths of RGC axons in the monkey is more complex, but the general pattern of longer axons of more peripheral origin running vitread to the shorter fibres is maintained.

Although the pattern described above for the adult goldfish may be the case for the majority of RGC axons, Easter, Bratton and Scherer (1984) described some 'local wandering'. Some axons ran obliquely to join an adjacent fascicle, and some formed hairpin loops initially running centrifugally. Using retrograde HRP labelling, Cook (1982) has shown that some axons run parallel to the retinal margin before turning centrally. Small groups of axons were labelled through lesions in the tecta of normal fish. The cell bodies of RGC were labelled in an arc on the flat-mounted retina. Though these cells were labelled both via axons of passage and via terminals, errant axons were seen emerging from labelled RGC which corresponded to labelling via the terminals. Therefore although these axons take abnormal retinal routes, they do appear to terminate retinotopically.

Cook's findings on these errant axons were confirmed by Springer and co-workers (Springer, Morel, Grobman and Wilson, 1989). They labelled RGC axons with cobalt via lesions in the goldfish optic nerve. Although the authors did not demonstrate the retinotopic termination sites of these axons, the optic nerve lesions confirmed that these errant axons arose from cell bodies in the ventrotemporal and ventronasal retinal quadrants. The axons' turning points at the nasal and temporal poles were also confirmed. The significance of the sites of the turning points is not known. Thus, although in general there is usually a retinotopic organisation of retinal fibres, spatial relationships during growth cannot account entirely for retinotopy.

1:8:2: Optic nerve and tract

Experiments in normal adult fish involving HRP labelling and mapping by axon degeneration have shown that the newest, unmyelinated axons cluster together ventrally in the optic nerve adjacent to the artery (Dawnay, 1979; Easter, Rusoff and Kish, 1981; Bunt, 1982). The oldest fibres, from central retina, lie dorsally. Fibres from ventral retina are on each

edge of the optic nerve, dorsal fibres are central and nasal and temporal fibres are in between. Insertion of a pin coated with HRP into the goldfish optic nerve results in an annular pattern of labelled RGC bodies: the retina is known to grow by the addition of annuli at the retinal periphery therefore an annulus represents a generation of RGC (Rusoff and Easter, 1980). Scholes (1979) demonstrated age-related order in the ribbon-shaped optic nerve of cichlid fish by examining the pattern of fibre degeneration in cross-sections of the optic nerve following lesions of retinal sectors. Such lesions resulted in sheet-like degeneration patterns within the optic nerve, with the oldest fibres on one side of the cross-section of the ribbon and the new, unmyelinated fibres at the other. In the other axis of the optic nerve, the organisation is circumferential with respect to the retina, with the break occurring at the choroid fissure, the ventral-most point of the retina. Thus the cichlid optic nerve is organised both retinotopically and chronotopically.

A complex rearrangement occurs at the level of the brachia when the optic tract divides around the *nucleus rotundus** . Axons from ventral retina pass into the medial brachium while those from dorsal retina pass into the lateral brachium (Scholes, 1979; Bunt, 1982). In addition, axons from temporal retina keep towards the *nucleus rotundus* and those from nasal retina remain outermost. The axons of RGC of temporal origin pass to rostral tectum and those of nasal origin to caudal tectum, thus the rearrangement within the brachia appears to give axons an appropriate trajectory as they approach the tectum. While axons running with axons of similar age can account for the order within the optic nerve, more complex interactions must occur at the level of the tract and brachia for the re-ordering to occur. Again, these are static descriptions of order within the optic pathways.

1:8:3: Tectum

Optic axons form fascicles on the tectum (Attardi and Sperry, 1963; Murray, 1976). They lie in the *stratum opticum.* Using anterograde and retrograde HRP labelling, these fascicles will be demonstrated to contain age-related axons. In view of the disparate modes of growth of retina and tectum described above, it was proposed that axon terminals must shift

* Renamed *nucleus pretectalis superficialis pars magnocellularis* (Northcutt and Braford, 1984) to denote lack of homology with *nucleus rotundus* of birds or mammals.

during normal development. Axon paths consistent with such a shift will be described (Chapter 4).

1:8:4: Paths in regeneration

Attardi and Sperry (1963) claimed that in goldfish with half-retinal lesions, regenerating axons took appropriate routes back to the tectum. Their histological methods were limited in their resolution and subsequent studies have suggested that there is widespread 'misrouting' of regenerated optic axons in fish and amphibians (Horder, 1974; Fujisawa, 1981; Dawnay, 1982; Cook, 1983).

In the goldfish, regeneration has been shown by electrophysiological methods to yield a retinotopic map from as early as 22 days after optic nerve cut in animals kept at room temperature (Horder, 1971). Although a retinotopic map is established early in regeneration, misrouted axons are seen in the optic tract by HRP labelling (Dawnay, 1982; Fujisawa, Tani, Watanabe and Ibata, 1982) and in the tectum by electrophysiology (Horder, 1974), autoradiography (Meyer, 1980) and HRP labelling (Fujisawa 1981, Cook 1983). Thus, despite the refinement of the retinotectal map after regeneration the axonal paths remain disordered.

More recent evidence concerning axon order in the optic tract during regeneration will be considered in Chapter 6.

1:9: Activity dependent sharpening of the retinotectal map

Hebb (1949) proposed that the co-existence of pre- and post-synaptic activity on repeated stimulation of an axon would lead to changes in the synapse which would lead to an increase in that synapse's stability. The proposal was an attempt to explain memory and learning but has been extended to other neural systems. Hebb's theory has been incorporated into theories of map refinement in the goldfish retinotectal system and in other neural networks (Willshaw and von der Malsburg, 1976; Changeux and Danchin, 1976; Whitelaw and Cowan, 1981). According to these theories, the RGC can gain positional information from the correlated firing patterns which exist between near-neighbours. Therefore axons which fire

together can terminate next to one another. Willshaw and von der Malsburg (1976) proposed that the orientation of the map could be established by a weak form of neural specificity and that local correlations of firing patterns could operate within these boundaries. They predicted the overlap of pre- and post-synaptic clusters of cells, which is of interest given some of the findings described in this thesis (Chapter 4). Whitelaw and Cowan (1981) placed more emphasis on retinal and tectal markers and their gradients which could explain compressed, expanded and rotated maps, but they also invoked the idea of modifiable synapses.

Evidence exists for the correlated firing of neighbouring RGC and the anti-correlated firing of non-neighbouring RGC in the goldfish in the absence of visual stimulation (Arnett, 1978). Similar findings have been reported in other species (rabbit, Arnett and Spraker, 1981; cat, Mastronarde, 1983).

The hypothesis that neural activity contributes to the sharpening of the retinotectal map has been tested in experiments using tetrodotoxin (TTX). TTX is a neurotoxin which blocks action potentials by blocking voltage-dependent sodium channels. Repeated injections into the goldfish eye block nerve conduction.

In an elegant experiment, Harris (1980) grafted an embryonic eye from a TTX-sensitive species, the Mexican axolotl, onto a Californian newt which is insensitive to TTX and which 'manufactures' the toxin. The grafted eye would therefore be exposed to TTX and would be affected by it while the host newt remained insensitive to TTX. Harris demonstrated that axons from the transplanted eye made synapses in the host tectum, in spite of the lack of neural activity. However, the neuronal tracing methods used (^{3}H-proline and HRP) were insufficient to demonstrate the degree of retinotopy.

During optic nerve regeneration, it has been shown that repeated injections of TTX into the goldfish eye (Schmidt and Edwards, 1983; Meyer, 1983; Schmidt, Edwards and Stuermer, 1983) cause enlargement of multi-unit receptive fields (MURFs) but do not affect the orientation of the map. Using ^{3}H-proline, Meyer showed the same lack of map refinement in fish

treated with TTX.

Is there a critical period in which TTX has its effects on the regenerated map? Schmidt and Edwards (1983) showed that lack of activity during the first 2 weeks did not cause enlargement of MURFs, whereas during days 14-34 of regeneration, at the time of synapse formation and maturation, it did. Meyer (1983) found that TTX need only be present for days 42-81 of regeneration to achieve loss of map refinement.

The methods used in these experiments are open to criticism. In order to record MURFs in fish which have been exposed to TTX, the TTX must be allowed to wear off. It has been suggested that once the retinotectal system has been released from TTX blockade, further map refinement can take place (Meyer, 1983). TTX abolishes all neural activity in the treated eye rather than just altering the correlated nature of the firing pattern. The fact that re-crush of the optic nerve previously treated with TTX and now in the absence of TTX leads to a normal projection argues against a permanent toxic effect of TTX (Schmidt and Edwards, 1983). However, it has been shown that intraocular injections of TTX reduce fast axonal transport of amino acids (Edwards and Grafstein, 1983). It cannot be assumed that the effect of TTX on the regenerating system is entirely confined to its effect on nerve conduction.

The ability of two populations of regenerating axons to segregate on one tectum had been observed in the goldfish (Levine and Jacobson, 1975; Schmidt, 1978). In the doubly innervated tecta of adult goldfish, Meyer (1982) showed that binocular injections of TTX prevented the segregation of eye-specific columns. Other experimental models which have been used to determine the role of visual experience on map formation are three-eyed frogs, *Rana pipiens* (Constantine-Paton and Law, 1978), and the isthmotectal projection in *Xenopus* (Udin, 1985). The three-eyed frog model demonstrates that the ability to form eye-specific stripes is also present in the developing amphibian visual system, as the eye rudiment is transplanted before outgrowth of the retinal axons. Eye-specific bands of axon terminals run rostrocaudally through the dually innervated tectum, in a pattern

reminiscent of the goldfish eye-specific stripes mentioned above and of the ocular dominance columns seen in the mammalian visual cortex. The experiments carried out on this model and their possible significance are more fully discussed in Chapter 6, section 6:2.

The isthmotectal system in *Xenopus laevis* represents an example of map formation beyond the contralateral retinotectal projection. Fibres pass from the contralateral tectum to the *nucleus isthmi*, situated near the caudal tectum, and form a retinotopic map. Fibres then pass to the other tectum via the post-optic commissure of the diencephalon. They form a map which is in register with the retinotectal map. The tecta are therefore linked by further visual relays. This is of functional importance since metamorphosis brings about an alteration in the orientation of the eyes which yields a binocular field of 170^0. In contrast, the goldfish does not have a significant binocular field. Map alignment has been shown to be affected by the visual environment in this system. Dark-rearing prevents ipsilateral units from coming into register with contralateral units (Keating and Feldman, 1975). Although the topographic order of the ipsilateral map in a dark-reared animal is relatively normal with respect to the mediolateral tectal axis, it is disordered along the rostrocaudal axis (Keating and Feldman, 1975).

Rotation of one eye in midlarval tadpoles leads to rotation of the visuotectal map since each axon still projects to its normal tectal target . The ipsilateral map becomes rotated so that the two maps are in register. When these animals were dark-reared, they did not show a modified pattern of intertectal connections (Keating and Feldman, 1975). Again, visual experience was important for map alignment.

There is evidence in mammalian models that activity plays a role. For example, in kittens reared in stroboscopic light the characteristics of the responses from the visual cortex were altered (Olson and Pettigrew, 1974). When action potentials were silenced in one eye of neonatal kittens by repeated intraocular injections of TTX, receptive field properties of individual relay cells in the lateral geniculate nucleus (LGN) were abnormal. A large number of them were on-off cells: usually these cells have either on-centre

or off-centre receptive fields. Although retinotopic organisation was normal, LGN cells had significantly elongated receptive fields (Archer, Dubin and Stark, 1982). Binocular injections of TTX in kittens prevents the formation of ocular dominance columns in the visual cortex (Stryker, 1981), a system analogous to three-eyed frogs but with a greater number of synaptic relays.

Thus there was evidence at the time when the experiments to be described here were begun that activity plays a role in the sharpening of the map. Previous experiments had been limited by the methods used and by their degree of resolution. Further experiments were needed to get around this problem. It was for these reasons that the use of stroboscopic light was introduced. This has the effect of masking local correlations in neural activity by making non-neighbouring axons fire together. A modulation of lighting conditions would be less likely to have a non-specific toxic effect than TTX. Stroboscopic light is more specific in that it blocks the correlated firing pattern rather than obliterating neural activity altogether. In the experiments to be described in this thesis, stroboscopic light was used in optic nerve regeneration in the goldfish in order to test the hypothesis that locally correlated neural activity is important in map refinement.

Chapter 2: METHODS

2:1: Husbandry

2:1:1: Normal and experimental conditions

All observations were made on goldfish, *Carassius auratus*. For experiments involving anterograde and retrograde labelling with horseradish peroxidase (HRP) in normal animals, fish of length 35-74mm snout to tail-base were used. For experiments in which normal and regenerated projections were compared after exposure to different lighting conditions, fish of more standardised length were used (55-65mm). Stock fish were kept in 20-45 litre aerated plastic tanks at room temperature (14-24^{o}C). They were fed with TetraMin fish food every working day. Lighting conditions were those of the laboratory, approximately 12 hours of light per day.

Experimental fish for comparison of the effects of exposure to different lighting conditions were kept at a constant temperature of 20$\pm$ 0.3^{o} C. In these experiments, fish were kept in opaque plastic tanks of a neutral colour in light-tight cupboards and they were fed automatically every day with TetraMin. No attempt was made to eliminate visual contrast in the tanks.

2:1:2: Special lighting conditions

a) Diurnal. For assessment of the topographic refinement of the regenerating projection under standard laboratory conditions, fish were kept in diurnal lighting (12 hours dark/12 hours light) at approximately 600lux from a 'Northlight' fluorescent tube.

b) Stroboscopic. The tank was lit only by a 2 Joule xenon discharge tube flashing continually 300mm above the water surface. Flash intervals varied randomly in 20ms steps between 60 and 360ms, with an effective frequency of 2.8-16.7Hz (Cook, 1984). The mean illumination was approximately 140lux. A second tube was recruited automatically within 2 seconds if the first one failed. In case the mains supply failed, a battery was built into the system as a back-up.

c) Continuous light. The tank was lit continuously by a 20 Watt 'Northlight' fluorescent tube. The measured illumination was approximately 600lux.

2:2: Surgical procedures

2:2:1: Anaesthesia

All surgery was performed under MS222 (Sandoz) anaesthesia. Induction was by immersion in a 1% aqueous solution until the fish was immobile. Anaesthesia was then maintained by perfusion of the gills with a 0.012% solution via a mouth tube. At the end of surgery, fish were revived by passing fresh tap water over their gills via the mouth tube. When they started to move, fish were returned to their tank.

2:2:2: Dissection microscopy

All surgical procedures were performed under a dissecting microscope (magnification x 40).

2:2:3: Reflection of skull flaps

The hexagonal bony plate overlying the tecta and forebrain was freed along five of the six sides, the sixth (rostral) side being used as a hinge. For improved access to the tecta, fat and fluids were removed with either paper tissue swabs or by aspiration. In those animals in which rostral tectal lesions were made, the forebrain was removed by aspiration. In those animals in which ventral lesions were made, the tectum was displaced medially by insertion of a paper tissue swab between the lateral wall of the cranium and the tectum. After surgery, the skull flap was replaced, and, if necessary, the area was sealed with Nobecutane.

2:2:4: Optic nerve cuts

The fish on the holder was tilted away from the operator and the conjunctiva of the fish torn superiorly. The eye was displaced downward and the optic nerve exposed. Intraorbital fat and fluid were removed with paper tissue swabs to improve access to the optic nerve. The optic nerve was cut with iridectomy scissors and any remaining fibres teased apart. The

eye was reoriented in the orbit. Any fish which bled significantly were rejected.

2:2:5: Lens removal

The cornea was stabbed with a needle at a dorsal point a few millimetres in from its margin. This hole was extended circumferentially in both directions almost as far as the irideal darts. The lens was removed with forceps and the cornea replaced. The cornea healed rapidly with little scarring. The pupil contracted by an average of 28% by the end of the experiment. Regeneration of a functional lens was never seen, but occasionally small remnants of lens tissue could be seen attached to the ciliary body. These would have been insufficient to enable a focussed image to be formed.

2:2:6: Retinal dissection and preparation of retinal wholemounts

Fish were dark adapted for at least 45 minutes to ease the separation at dissection of neural and pigmented retina. Fish were then anaesthetised, still under dark adaptation. The eye was then punctured with a needle near the ventral fissure and the eye removed from the orbit by cutting the optic nerve. The eye was placed in 0.9% saline and the retina dissected free. The ventral cut was extended almost to the optic nerve head, and smaller cuts were made dorsally, nasally and temporally. The retinae were placed on hardened filter paper (Whatman no. 50) and fixed in 1% glutaraldehyde in 0.1M phosphate buffer for 30-45 minutes. They were then rinsed in 0.9% saline for 10 minutes before overnight immersion at 4^{0}C in a pre-soak solution (see appendix 1).

2:2:7: Dissection of tecta and preparation of tectal wholemounts

The bony plate overlying the tecta was freed and intracranial fat and fluid removed. The optic tectum was cut free at its anterior, medial and caudal borders with iridectomy scissors. The tectum was then squirted vigorously with distilled water to remove blood before being placed in 2% glutaraldehyde in 0.1M phosphate buffer at pH 7.4 for 30 minutes. Underlying tissue and blood vessels were dissected away before making a single rostral radial incision in order to ease flattening of the tectum: the

incision was caudal when the original tectal lesion had been rostral. The tectum was then flattened between hardened filter paper (Whatman no. 50) and a coverslip and left to fix for the remainder of the 30 minutes. The tectum was washed in 0.9% saline and pre-soaked for 8-18 hours at 4^{0}C in pre-soak solution (appendix 1).

2:3: HRP labelling techniques

2:3:1: Retrograde labelling from the optic tectum with HRP

The skull flap was reflected, exposing the optic tecta, and excess intracranial fat and fluid were removed. Lesions were made on the right tectum at various co-ordinates. The lesions were 100-120μm wide and were made by lowering a tungsten microknife perpendicular to the tectal surface on a micrometer screw gauge. The lesion was filled with horseradish peroxidase (HRP; Boehringer Grade I; 1μl of 30% solution in 0.1M phosphate buffer at pH 7.4) on gelatine foam placed over the lesion. Fish were maintained for 8-10 days at room temperature for retrograde transport to take place. The left retina was dissected free and processed to reveal HRP.

In some animals, HRP was applied via a micropipette. Micropipettes of external diameter 25-50μm were made by drawing out 1.5mm glass tubing in a Bunsen flame. HRP was introduced into a small group of fibres in the tectum by insertion of a micropipette filled with HRP into the tectum for 30 seconds, using a micromanipulator. Otherwise, processing of retinae was as described above.

2:3:2: Anterograde labelling from optic nerve lesions with HRP

The conjunctiva of the right eye was torn superiorly and the eye was displaced laterally and inferiorly to expose the optic nerve. Excess intraorbital fat and fluid were removed. A lesion was made in the right optic nerve with a needle and the area was packed with HRP on gel foam. The eye was replaced in the orbit and the fish maintained at room temperature for 24 hours before the tectum was processed for HRP.

2:3:3: Anterograde labelling from tectal lesions with HRP

The skull flap was reflected and excess fat and fluid removed as described above. For rostral tectal lesions, the forebrain was removed by aspiration. For ventral tectal lesions the tectum was displaced medially by insertion of a tissue swab between the cranium and the tectum. Micropipettes, diameter 25-50μm, filled with HRP were inserted into the tectum for 30 seconds using a micromanipulator. The cranial flap was replaced and the animal was maintained at room temperature for 4-6 hours and then the tectum was removed and processed for HRP.

2:3:4: Retrograde labelling from the optic tectum with WGA-HRP

A fuller analysis of this method is given in Chapter 3. In brief, iontophoretic injections of WGA-HRP were made into the tecta of fish anaesthetised and prepared as described above. Micropipettes of external tip diameter 15-25μm were filled with a 0.1% solution of WGA-HRP in 0.2M potassium chloride, buffered to pH 7.9 with 0.05M Tris. With the pipette tip in the *stratum fibrosum et griseum superficiale* of the tectum, 100-200μm beneath the *pia mater*, a current of 600μA was passed continuously for 10 minutes. The fish were then maintained at room temperature for two days, after which time the contralateral retina was removed and processed as for HRP.

2:3:5: Retrograde HRP labelling of entire retinae in order to perform cell counts

Fish with regenerating projections and normal fish matched for size were kept in either stroboscopic or constant light for 69-97 days. The optic nerve was cut or re-cut for fish with normal or regenerated projections, respectively. The cut nerve was packed with HRP on gel foam and 2 days later the retina was processed to reveal the labelled retinal ganglion cells (RGC).

2:3:6: HRP histochemistry for retinae and tecta.

HRP was revealed using the method of Hanker, Yates, Metz and Rustioni (1977), slightly modified for wholemounts (Pilgrim, 1981). Dissected retinae were immersed overnight at 4°C in a pre-soak solution of

1mg o-catechol and 0.5mg p-phenylenediamine in 100ml of Tris buffer (Sigma; 0.05M pH 7.4). The next day, retinae were developed for 21/2 hours at 4^{0}C in a solution of 100mg o-catechol and 50mg p-phenylenediamine in 100ml Tris buffer, with 5 drops of 25% hydrogen peroxide. Developed retinae were dehydrated in alcohol, first in 70% for 30 minutes, then 100% for 30 minutes, while the retinae were flattened under a coverslip. After flattening, the retinae underwent two further immersions in 100% alcohol of 30 minutes and 1 hour. Dehydrated retinae were cleared in two changes of methyl salicylate for 1 and 2 hours before being mounted in DePeX (Gurr).

Dissected tecta were immersed in a pre-soak solution as above. Tecta were immersed in developer solution (appendix 2) for 2 hours at 4^{0}C. The tecta were then dehydrated through graded alcohols for 21/2 hours and cleared for 3 hours in methyl salicylate as for the retinae. The tecta were then flat mounted in DePeX (Gurr).

2:4: Analysis of results

2:4:1: Drawing anterogradely labelled axons on the tecta

Optic axons filled with HRP reaction product were traced within the *stratum opticum* and the *stratum fibrosum et griseum superficiale* by means of a drawing tube. Measurements were made from tracings, the drawing tube having been calibrated with the aid of a stage graticule.

2:4:2: Plotting the distribution of labelled HRP and WGA-HRP retinal cells

A photomontage of the entire retina was made. Photographs were taken with an Olympus BHS photomicroscope system on Panatomic-X (Kodak). Magnification was 110-120 diameters. Retinae were scanned systematically at a magnification of 400 diameters. Ganglion cells were identified over a wide range of filling densities, with good agreement between observers. A cell was classed as labelled if 5 or more granules of reaction product were identified. Filled RGC were individually plotted with the aid of a drawing tube with adjustable magnification (Zeiss) onto the photomontage. Tracings were made from the photomontage for clarity.

2:4:3: Cell counts in retinae labelled with HRP

Counts of RGC were made in sample areas of the HRP-labelled retina, the ventronasal or ventrotemporal quadrants. Counts were made in 4 high power fields comprising an area of 1.075mm^2. The total retinal area was measured for each retina and a calculation of the total RGC number was made. Cell counts in fish with normal and regenerated projections which had been kept in stroboscopic or in constant light were compared.

2:4:4: Derivation of the area index

An objective and quantitative assessment of the degree of clustering of the retinal ganglion cells (RGC) filled after iontophoretic tectal injection of WGA-HRP was required. It was impractical simply to draw an envelope around a cluster of labelled RGC. The clusters were rarely well-defined enough for this to be practicable except in normal animals or in animals late in regeneration. Although efforts were made to centre the clusters within a quadrant by appropriate positioning of the tectal injection, some labelled RGC were separated from the majority by the cuts made in the retina in order to give a flatmounted preparation. A statistical approach based on nearest neighbour distance measurements was used (Clark and Evans, 1954). Clark and Evans derived the method to examine spatial relationships in animal and plant populations, and to compare these to a random distribution. Nearest neighbour distance measurements provide the basis for the method. Such measurements were made for all filled RGC and the value of the mean nearest neighbour distance was obtained for each set of observations. The mean distance to nearest neighbour that would be expected if the filled RGC were randomly distributed was also calculated. The ratio of the observed to the expected mean nearest neighbour distance gives a measure of the departure from randomness: this ratio is called the dispersion index. In a random distribution this ratio will be 1. Smaller values indicate a greater degree of clustering. Under conditions of maximum aggregation the ratio will be zero, since all of the individuals in the population occupy the same locus and the nearest neighbour distance is zero. A value for this ratio of greater than 1 indicates regular spacing of the individuals in the population, but does not apply to this experimental system.

This method has been extended to give a value for the area index which is the square of the dispersion index. The area index represents the fraction of the total retinal area that would be needed to enclose all the labelled cells, given that they were to be redispersed randomly inside a closed boundary while keeping their observed mean distance to nearest neighbour. It provides a direct estimate of the ratio between the area of the cluster of labelled cells and the total retinal area. Like the dispersion index, the area index is zero if all the individuals in the population are superimposed or 1 if they are randomly distributed.

2:4:5: Frequency distribution histograms

Frequency distribution histograms of normalised nearest neighbour distances were obtained by storing all the nearest neighbour distance values for the 26 retinae analysed in this way on computer. Each distance was then automatically divided by the expected mean for a random distribution with the same average density as that particular retina (Clark and Evans, 1954). This corrects for variations in labelled cell number and retinal area. These normalised distances were pooled by experimental group, transformed logarithmically to increase resolution in the body of the distribution and allocated to histogram bins.

Chapter 3: DEVELOPMENT OF AN ANATOMICAL METHOD OF DETERMINING MAP PRECISION

3:1: Introduction

Methods of assessing neuronal maps, in respect of both their anatomy and their degree of precision, have their advantages and disadvantages. In the earliest experiments on the regenerated projections of fish and amphibian optic nerves, the methods were behavioural (Sperry, 1943, 1944; Stone, 1944). First, the animals' responses to a food lure were measured. This method was very limited in its resolution, as it was difficult to measure the angle of the lure relative to the animal's eye; and the other eye had to be blinded in order to get as clear a response as possible from the experimental eye. Assumptions had to be made about the anatomy of the regenerated projection, since the behavioural response was at least a step removed from the visuotectal map itself. The degree of resolution of this method of mapping was limited to the quadrantal level (Stone, 1944). Second, the optokinetic response was measured. This involves measuring compensatory head movements in response to rotation of the visual field. The optokinetic response depends on the projection to the accessory optic nuclei and not to the tecta.

Historically the next important mapping method was electrophysiological (Gaze, 1958; Maturana, Lettvin, McCulloch and Pitts, 1960). This was a significant methodological step enabling the normal and regenerated projections to be characterised in greater detail, and allowing a greater range of experimental manipulations. However, while the upper visual field mapped systematically onto the upper surface of the contralateral optic tectum, the most lateral parts of the tectum were difficult to reach. In most cases the map obtained electrophysiologically has been described in terms of the relationship between a rectangular grid of tectal electrode positions and a corresponding array of visual receptive field centres. This technique records from multi-units, and some tectal points had a receptive field of up to 60^0, though precision was usually much greater. A potential disadvantage of this method has been the lack of certainty of the anatomical

basis of the responses which are measured. Whether the responses are pre- or post-synaptic has long been debated. For many years the recordings were thought to be pre-synaptic (Maturana, Lettvin, McCulloch and Pitts, 1960; Gaze, 1970). Gaze (1970) argued that the responses were of pre-synaptic origin. For example, he found that many characteristics of tectal units in the SFGS were the same as those recorded from the optic nerve, while deeper tectal units were very different from those in the optic nerve. More recently, however, the responses have been shown to be largely post-synaptic (Grant and Lettvin, 1991). Grant and Lettvin made recordings from the outer tectal neuropil in the frog, *Rana pipiens*, and they noted that some of the responses were binocularly driven, implying a post-synaptic origin. The multi-unit receptive fields were made up of the receptive fields of more than one distinct element. The findings were correlated with the presence of beaded dendritic appendages of tectal neurons demonstrated by the rapid Golgi method. Thus the electrophysiological maps which are made up of multi-unit receptive fields are a summation of local post-synaptic elements. This is an interesting finding, though it does not completely invalidate previous work.

The resolution is limited by the spacing of the grid, usually to a distance of 50-100μm between recording points. Since the responses have been shown to be post-synaptic, this may also reduce the resolution of the map. This factor may not be great, however, as the response is probably a local dendritic spike. The responses are again a step removed from the map which is being studied. This method requires the visual projection to be functionally normal, as this is the basis for the recordings. Therefore this method is not suitable for any experiments where retinal activity has been disrupted or conduction blockers used. The effects of conduction blockers must be allowed to wear off and the blockers may be toxic. Clearly this method would be unsuitable in some experiments designed to test the hypothesis that activity-dependent mechanisms are important in map formation. However, Schmidt has persisted with electrophysiological methods for map assessment after modulation of electrical activity (Schmidt and Edwards, 1983).

Anatomical methods would be preferable. Using the neuronal tracer HRP, there is no distinction between tracer taken up by axons of passage and by terminals. Deductions can be made about the source of the labelling using information obtained from anterograde tracing methods, for example. However, it would be difficult to draw any conclusions about the resolution of the map, especially in regeneration, because of the background "noise" of axons labelled as axons of passage.

Autoradiography, another anatomical method, is labour intensive and technically difficult, mainly because of the tissue processing and reconstruction of the projection which are necessary. Lesions are made within the retinae which are then labelled with tritiated proline, the lesions showing up as gaps in labelling. The resolution is limited, this time because of the size of the lesion needed to make a discernible breach in the map, and it is complicated by the presence of labelled axons of passage. In addition, uptake of tritiated amino acid into the circulation leads to background labelling, though background labelling is less with ^{3}H-proline than with other amino acids.

There was therefore a need for an anatomical method of high resolution which assesses the terminal arborisations themselves. The method should be quantifiable and easily reproducible. Ideally it should be possible to "map" several animals in a day, using standard surgical and histological techniques with reasonably priced reagents. The method should be non-toxic, to the operator or to the fish neurons. There should be good inter-observer agreement on the results of the mapping process, and it should be possible to carry out the technique in other laboratories so that results can be confirmed and experiments extended.

The non-toxic lectin wheatgerm agglutinin (WGA) can be conjugated to HRP covalently. The WGA-HRP molecule is taken up by adsorptive endocytosis at axon terminals (Gonatas, Harper, Mizutani and Gonatas, 1979), in contrast to the fluid phase endocytosis of free HRP. WGA binds specifically to N-acetyl-D-glucosamine and N-acetyl neuraminic acid residues in the neural membranes (Gonatas and Avrameas, 1973). WGA-

HRP conjugates were 40 times more sensitive than free HRP in tracing retrograde connections from the rat submandibular gland to the superior cervical ganglion. The greater efficacy of WGA-HRP as a neuronal tracer lies partly in the fact that conjugation may result in more than one molecule of HRP per molecule of WGA and also in the greater affinity of the lectin for nerve endings allowing a smaller dose of tracer to be used. The injection site can therefore be smaller, reducing the damage to axons: tracer uptake is therefore not in the fluid phase. The smaller injection site in turn improves the resolution of the method.

A mapping system using the non-toxic lectin wheatgerm agglutinin (WGA) conjugated to horseradish peroxidase was found to fulfil some of these criteria. It is proposed that WGA-HRP uptake can be restricted to the terminal arborisations since WGA can be adsorbed by axon terminals. Use of the lectin-peroxidase conjugate should allow use of a smaller injection site compared to HRP alone, and yet uptake of HRP should be sufficient for labelling, partly because of the more efficient tracer delivery and because more than one molecule of HRP may be conjugated to one molecule of WGA. The conjugate is transported to the cell soma, and the coloured reaction product formed when HRP acts on the chromagen(s) labels the cell whose terminal was at the injection site. If uptake were limited to axon terminals, then a limited and reproducible injection site should result, in the normal animal, in the labelling of a retinotopic cluster of cells. The cluster would represent the cells whose terminal arborisations were at the injection site and which could take up sufficient tracer for labelling to occur. Cells whose terminal arborisations were at a distance from the injection site would not take up any of the tracer and would not be labelled, while those whose terminal arborisations were close to the injection site might take up the tracer but not in amounts sufficient to cause detectable labelling. It was hoped that such restricted labelling could be obtained by these means.

3:2: Methods

WGA-HRP was injected into the tectum of 57 normal fish anaesthetised with MS-222 (Sandoz) as described in section 2:2:1. After

closure of the reflected cranial flap, the fish were revived and maintained until they were sacrificed and the retinae developed to reveal labelled cells. The tracer, which could be frozen in 10μl aliquots and could keep its activity, was iontophoresed into the *stratum fibrosum et griseum superficiale* and some days later the contralateral retina was removed and developed to show the reaction product. A selection of variables was chosen for testing in order to achieve the most clear and consistent labelling of the RGC, with a reasonable cluster size. These were:

a) concentration of WGA-HRP: concentrations of 0.1, 1.0 and 2.0% were tested. The WGA-HRP was dissolved in 0.2M KCl, buffered to pH 7.9 with 0.05M Tris.

b) pipette tip size: external tip diameters were estimated under the x25 objective of the microscope.

c) depth of the injection: depths of injection from 70-270μm were tested in eight retinae of normal fish at different tectal sites, front, middle and back. Greater injection depths were used at the front of the tectum compared to the back, allowing for the decline in laminar thickness from rostral to caudal (Raymond and Easter, 1983). The depth measurements were made with the pipette mounted on a calibrated micromanipulator, and were made from the point of first contact with the *pia mater*.

d) magnitude and duration of the applied current: 30 and 300nA of current (pipette positive) were compared in 6 retinae of normal goldfish, with durations of injection of 10 minutes or three pulses of 10 minutes each. Ten minutes was adopted as a reasonable duration of injection, and subsequently a broader range of current values was tested from 100-3000nA in 6 more normal retinae. In eight further normal retinae a possible reciprocal relationship between current size and duration was examined. Injection times of 5-40 minutes were tested versus a corresponding fall in current intensity from 1000-125nA.

e) time allowed for transport of WGA-HRP: transport times of two to

eight days were tested in twelve retinae of normal fish using 0.1% WGA-HRP injected for 10 minutes at 500 or 1000nA.

Tests of pipette exhaustion were made, with several injections being made from one pipette with an external tip diameter of 20μm.

The micropipettes were kindly supplied by Dr KAC Martin.

At a variable period after the injection, the contralateral retina was dissected free, flattened and fixed in glutaraldehyde. The transported WGA-HRP in the RGC was revealed by means of a modified Hanker-Yates reaction (Pilgrim, 1981) . A photomontage of each retina was then made and filled cells were plotted on this with the aid of a drawing tube. Nearest neighbour distances were measured for each labelled retinal ganglion cell.

Iontophoresis of 4% HRP (Boehringer Grade I) in 0.05M Tris and 0.2M potassium chloride was carried out for comparison. Current durations were 3-30 minutes and the current sizes were 30-1000nA.

3:3: Results

A standard concentration of 0.1% was chosen for subsequent injections of WGA-HRP. This concentration was chosen for economy and for effective control of delivery of the tracer over a reasonable period of time. At lower concentrations, the magnitude and/or duration of the current had to be increased in order to get sufficient labelling. An increase in the duration of the current would have been inconvenient because of the risk of the fish moving on the holder and because fewer animals could be mapped during the experimental period. Higher concentrations were not tried. At higher concentrations there might have been an increase in the diffusion component of tracer delivery and cost may have become a factor.

Pipette tip sizes of 15-25μm external diameter were found to be satisfactory. Larger tip sizes were found to cause labelling of larger clusters, either because of more axon damage leading to uptake from severed axons

as well as from intact axon terminals, or because there was a component of tracer diffusion as well as the current-dependent component. If the standard tracer concentration of 0.1% was used, with a pipette diameter of 15-25μm, then no labelled RGC were seen unless current was passed.

The most consistent results were obtained with the pipette depth being 100-120μm in caudal tectum, 120-150μm in mid-tectum and 150-200μm in the rostral tectum.

Pipette resistance was about 12MOhms.

A duration of injection of 10 minutes was chosen. The times of the experiments were therefore reasonably short so that several fish could be injected in one experimental session, and there was little risk of the fish moving and affecting the injection process.

A current of 600nA was chosen. Decreases in the current passed in the 10 minute injection period caused faint labelling of the cells. Cluster area was reduced only slightly before the identification of labelled cells became difficult. Increases in the amount of current passed resulted in proportional increases in the area of the patch of filled cells.

When pulses of current were passed, the usual consistency in the labelling of the cells was lost.

There was no visible difference in the quality of labelling of the cells at the transport times tested between 2 and 8 days. Therefore 2 days was chosen as the briefest and most convenient tracer transport time for subsequent experiments.

Direct application of HRP to a tectal lesion or iontophoretic injection of HRP resulted in a pattern of labelling which differed radically from the compact cluster of labelled cells seen following iontophoresis of WGA-HRP (Figures 9 and 15). The two types of HRP administration resulted in similar patterns of labelling. The patterns of labelled RGC after iontophoresis of

HRP comprised arcs and bars, with the temporalmost end of the bar corresponding to the cells which had their terminal arborisations at the iontophoresis point. Thus they were similar to those retinae which were processed following direct application of HRP to a tectal lesion (section 4:2).

3:4:Discussion

The clearest and most consistent labelling was obtained with a concentration of WGA-HRP of 0.1%, iontophoresed through a pipette of tip size 15-25μm external diameter, at a current of 600nA applied for 10 minutes. The pipette depth varied with tectal position, but was most effective at 150-200μm in the rostral tectum and at 100-120μm in the caudal tectum. Tracer transport time was not critical but was most conveniently two days. Thus assessment of the precision of the map using this method is practicable.

The results indicate that, under the conditions described above, uptake of WGA-HRP is restricted to terminal arborisations. Evidence for this comes in particular from comparison of the patterns of labelled RGC seen when HRP and WGA-HRP are used. The patterns of labelling are completely different. In the former the pattern can be explained if it is argued that severed axons of passage take up the tracer as well as the terminal arborisations. The patterns of labelled RGC obtained after iontophoresis of HRP are very similar to those obtained following direct application of HRP to a tectal lesion (section 4:2). In general, the pattern is made up of an arc and a bar which join each other at the ventralmost end of the arc. It has been argued (sections 4:2 and 5:2) that the arc of labelled RGC is the result of the labelling of fascicular axons of passage at the tectal lesion site, while the bar is the result of the labelling of fibres running rostrocaudally in the SFGS, again as axons of passage. In contrast to the pattern seen after iontophoresis of HRP, iontophoresis of WGA-HRP results, in the normal fish, in the labelling of a compact cluster of RGC at a retinal site corresponding to the tectal iontophoresis site. Thus, the labelling is retinotopic and the

labelling of axons of passage has been excluded. Comparisons of these patterns alone provide strong evidence for the restricted uptake of WGA-HRP.

It has been shown that for retrograde tracing of neuronal connections, weight-for-weight of HRP, small amounts of WGA-HRP demonstrate connections which are not demonstrated by HRP alone (Mesulam, 1982, p43). In addition, there are precedents in other systems for the restricted uptake of lectin conjugated HRP (Mesulam, 1982). Therefore it has previously been shown that the greater efficacy of WGA-HRP as a tracer allows a much smaller injection site to be used which should allow restricted uptake.

While there are strong arguments for the labelling of a cluster of RGC in the normal goldfish being the result of the restricted uptake of WGA-HRP, the use of this method as an anatomical and quantitative method of mapping under different experimental conditions must take into account possible differences in the properties of terminal arborisations under these conditions. For example, the size and branch number of a terminal arborisation may alter in regeneration compared to the normal animal and this might alter the uptake of WGA-HRP. For this reason, while comparisons between experimental groups may well be possible, it may be more difficult to make comparisons within an experimental group. Also, another terminal structure, the growth cone, is able to take up HRP (Chu-Wang and Oppenheim, 1980) and more of these may be present in the regenerating projection. Thus, this mapping method cannot be assumed to reveal only mature terminal arborisations. The different properties of the terminal arborisations under different experimental conditions may provide interesting data about those properties, but this method may not be able to be used to make direct comparisons as if all other things were equal.

Nevertheless, as will be discussed in sections 5:4 and 5:5, this method of determining the precision of the retinotectal map does provide information about the map which results after regeneration of the optic nerve in different lighting conditions. Comparisons between the groups show that

the map is less precise after regeneration in stroboscopic light compared to diurnal light. The quantitative nature of the method, although time consuming at the time these experiments were carried out because the large number of nearest neighbour measurements had to be made by hand, stands up well to statistical analysis.

Chapter 4: RESULTS

4:1: Anterograde labelling with HRP in normal fish

Lesions were made in the right optic nerve (14 fish) or axons were severed as they crossed the tectum towards their tectal termination sites (68 fish). Filled axons were traced in tectal wholemount preparations. Optic nerve lesions resulted in larger numbers of filled fibres which were denser and more difficult to follow. Some optic nerve lesions resulted in complete rings of tectal axon terminals being labelled (Figures 2A and 2B), with fascicles coursing over the tectal surface in a symmetrical array. Tectal lesions however resulted in more discrete patterns of labelled axons, comprising at most a few fascicles, a partial annulus of terminals and axons coursing caudally for varying distances towards termination sites (Figure 3). The lateral half tectum was less accessible at operation therefore most of the lesions were made medially. Thus most of these patterns were confined to the medial half tectum. In these patterns fibres did not cross the rostrocaudal tectal axis. Interestingly, others have since shown misrouted axons which take the wrong brachium and therefore must cross the rostrocaudal axis (Becker and Cook, 1987).

The tectal injection site was visible on the surface as an area of dense labelling, elliptical in shape with the long axis along the trajectory of the fascicle (Figure 4). Fibres were filled both rostrally and caudally from the injection site. From the injection site, fibres were seen running rostrally in discrete bundles into the brachium, medial or lateral depending on the site of the lesion. These bundles spread into fascicles as they passed caudally onto the tectum. The fascicles continued through the injection site and passed caudally onto the tectum with a curved trajectory until they reached an imaginary midline joining the rostral and caudal tectal poles. The distances between the fascicles widened towards the caudal pole (Figure 2A). At the midline they became attenuated. Along the length of the fascicle, axons were seen to leave it. Most passed caudally to terminate at a distance from the fascicle. These latter fibres ran in a deeper layer compared to the fascicles which were easily seen near the surface of the optic tectum. The

Figure 2A: Photomicrograph of tectal wholemount. Fascicles (single arrow) and terminal arborisations (double arrow) have been labelled with HRP following a partial optic nerve lesion. Fascicles fan out over the tectal surface from the two brachia, and their trajectories are in contrast to the ring of terminal arborisations. The direction of the rostral tectal pole is marked. Bar represents 100μm.

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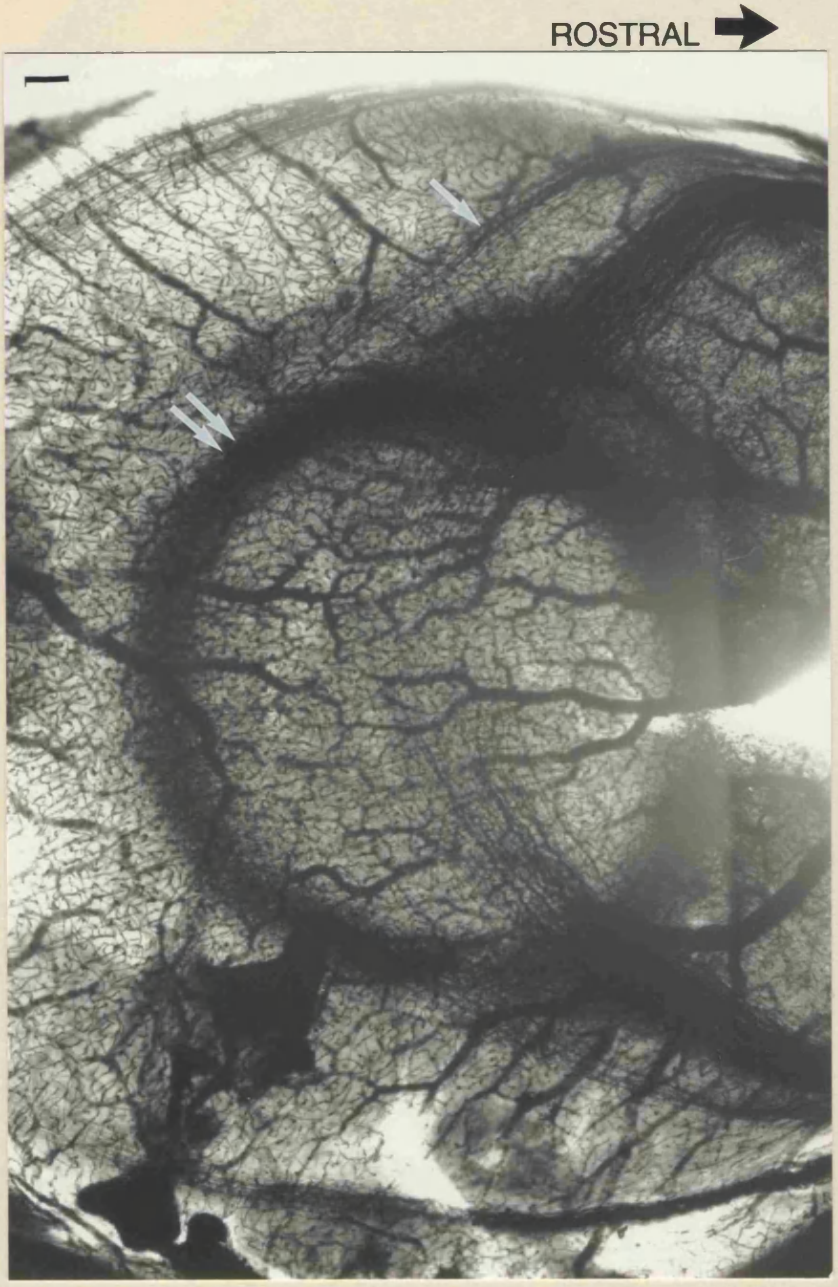
ROSTRAL

Figure 2B: Photomicrograph of tectal wholemount. The symmetrical array of fascicles and concentric rings of terminal arborisations are shown following HRP labelling of a partial optic nerve lesion (more extensive labelling than in figure 2A). The direction of the rostral tectal pole is marked. Bar represents 100μm.

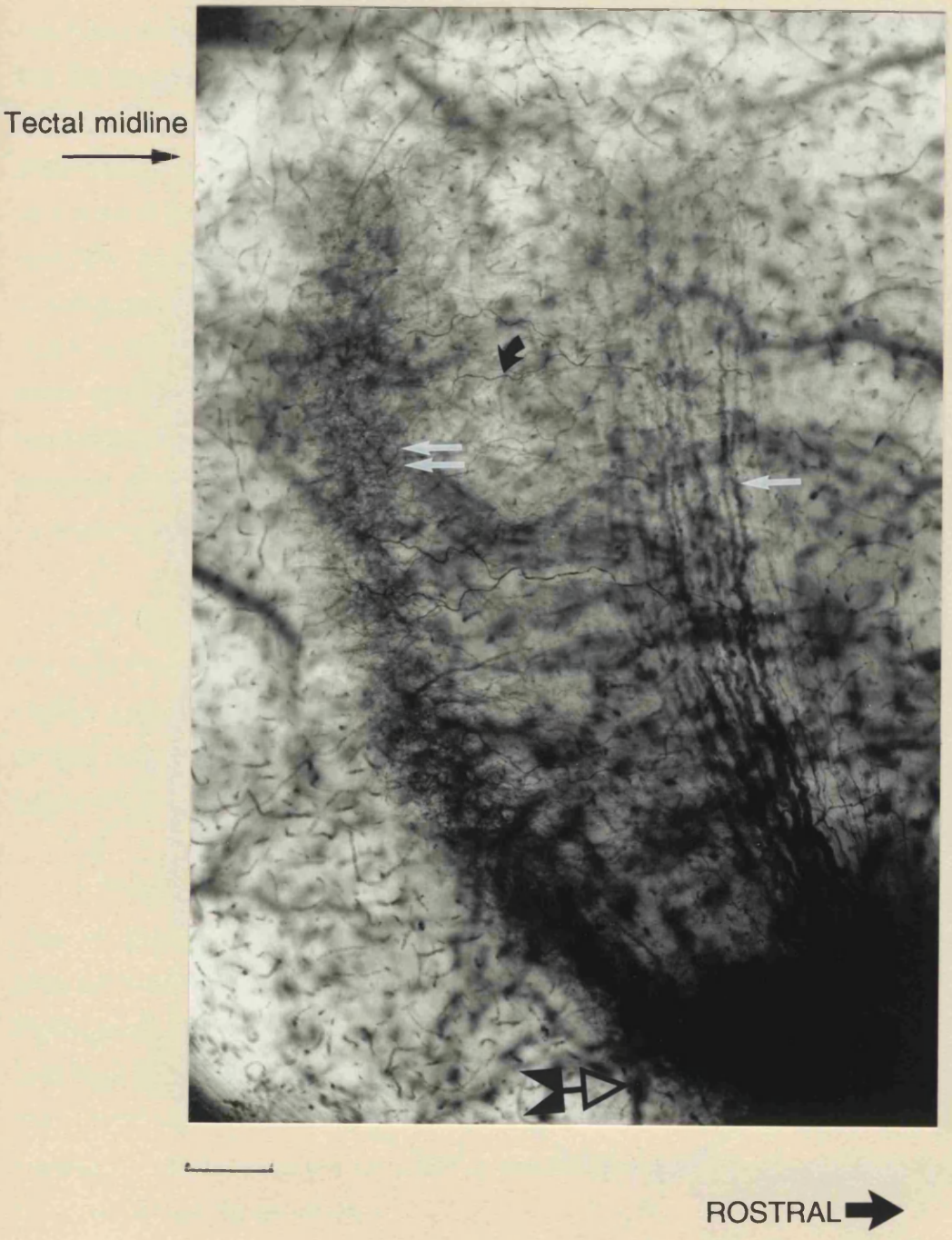

Figure 3: Photomicrograph of part of tectal wholemount showing result of HRP labelling via a tectal lesion (large arrow). Fascicles (single arrow), terminal arborisations (double arrow) and axons joining them (curved arrow) are seen. The distribution stops at the midline, which has been marked, between medial and lateral tectum. The direction of the rostral tectal pole is marked. Bar represents 100μm.

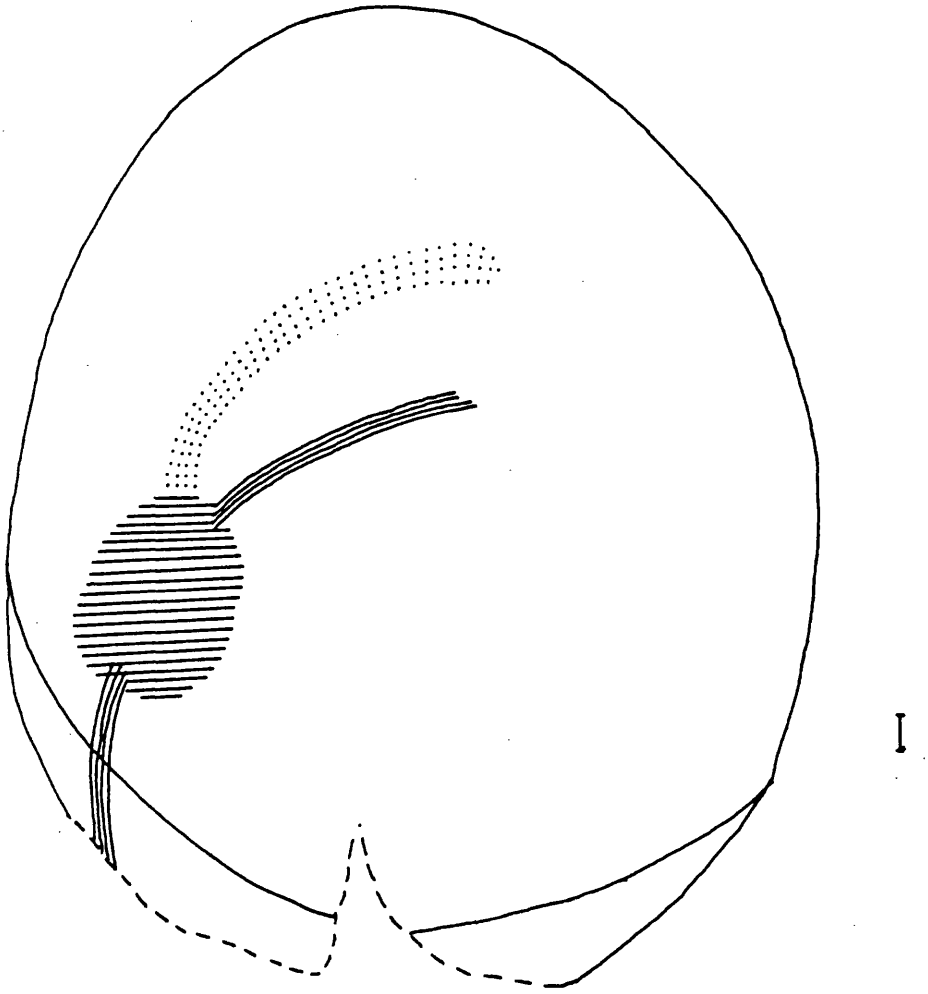

Figure 4: Low power *camera lucida* drawing showing the site of a tectal lesion (cross hatching), the fascicle and the sites of terminal arborisations (stippling). A cut was made at the rostral pole of the tectum to facilitate flat-mounting. Bar represents 100μm.

fascicles are known to run in the *stratum opticum* (SO; Sharma, 1972) and the fibres which run caudally are in the deeper *stratum fibrosum et griseum superficiale* (SFGS). Axons left the fascicle at fairly even intervals. The attenuation of the fascicle at the midline was a reflection of the lower number of axons it contained at this point because the majority had already left the fascicle and turned caudally. Termination sites, seen as a mass of overlapping terminal arbors, filled from a single tectal lesion also formed a curve over the tectal surface, and stopped at the midline (Figure 3). They were also at a distance below the fascicle in the SFGS. Optic nerve lesions frequently labelled concentric rings of terminal arborisations. These were centred approximately on the tectal midpoint. Tectal lesions labelled arcs of terminal arborisations, again centred on the tectal midpoint.

In addition to the axons leaving the fascicles, there was, arising from the more rostral lesion sites, a sheet of axons running directly caudally. These axons were in the SFGS and did not appear to arise from the fascicle at the lesion site (Figures 5A and 5B). They did not appear to extend beyond the rostrocaudal midline.

The precise trajectory of the fibres running from fascicle to termination site in the caudal tectum was difficult to ascertain because of the wholemount technique. Generally these fibres ran caudally (Figure 3). Retrograde studies described later in this section and later work in frog (Hitchcock and Easter, 1987) suggested that axons of passage labelled in the rostral tectum may have a centripetal direction at some point along their trajectory. There was no convincing evidence from the anterograde findings to complement those results.

The distance travelled by axons in the SFGS which had arisen from fascicles varied according to the position of the fascicle on the tectum. Axons from rostral fascicles ran almost half the rostrocaudal tectal length before terminating in the caudal half-tectum. Axons from a fascicle midway back on the tectum ran a shorter distance before terminating. Axons from the marginal fascicle were seen to terminate rostral to the fascicle (Figure 6). Retrograde HRP tracer studies, interpreted in the light of earlier studies of

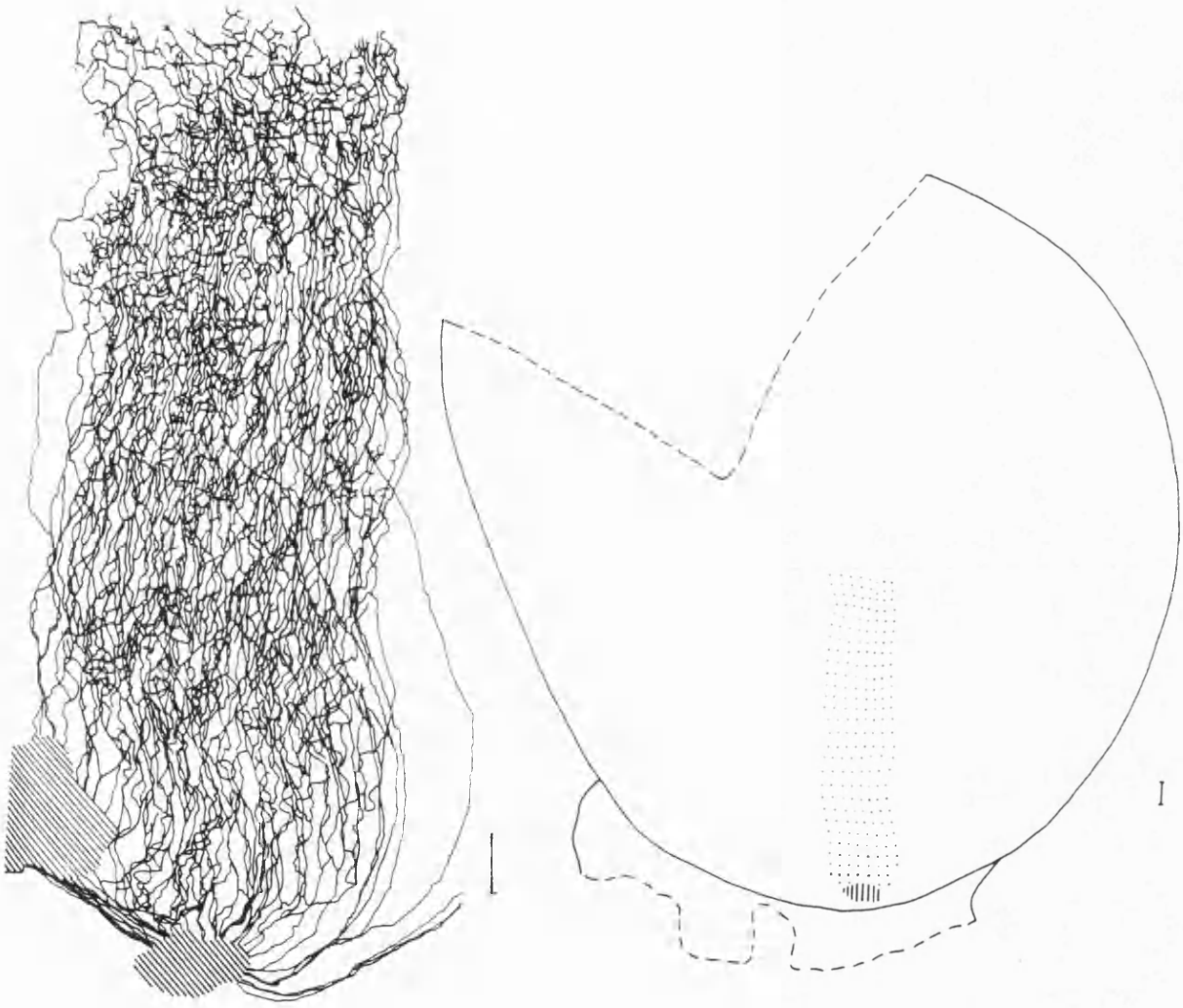

Figure 5A: *Camera lucida* drawings of axons labelled from a lesion (cross hatching) at the rostral tectal pole. High power drawing (left) shows RGC axons running caudally from the lesion in a grossly parallel array; terminal arborisations were seen amongst the axons. Low power drawing (right) shows the extent of labelled fibres and arborisations on the tectum. The pattern of labelled axons is in contrast to the array of fascicles seen after a lesion further back on the tectum. Bars represent 100μm.

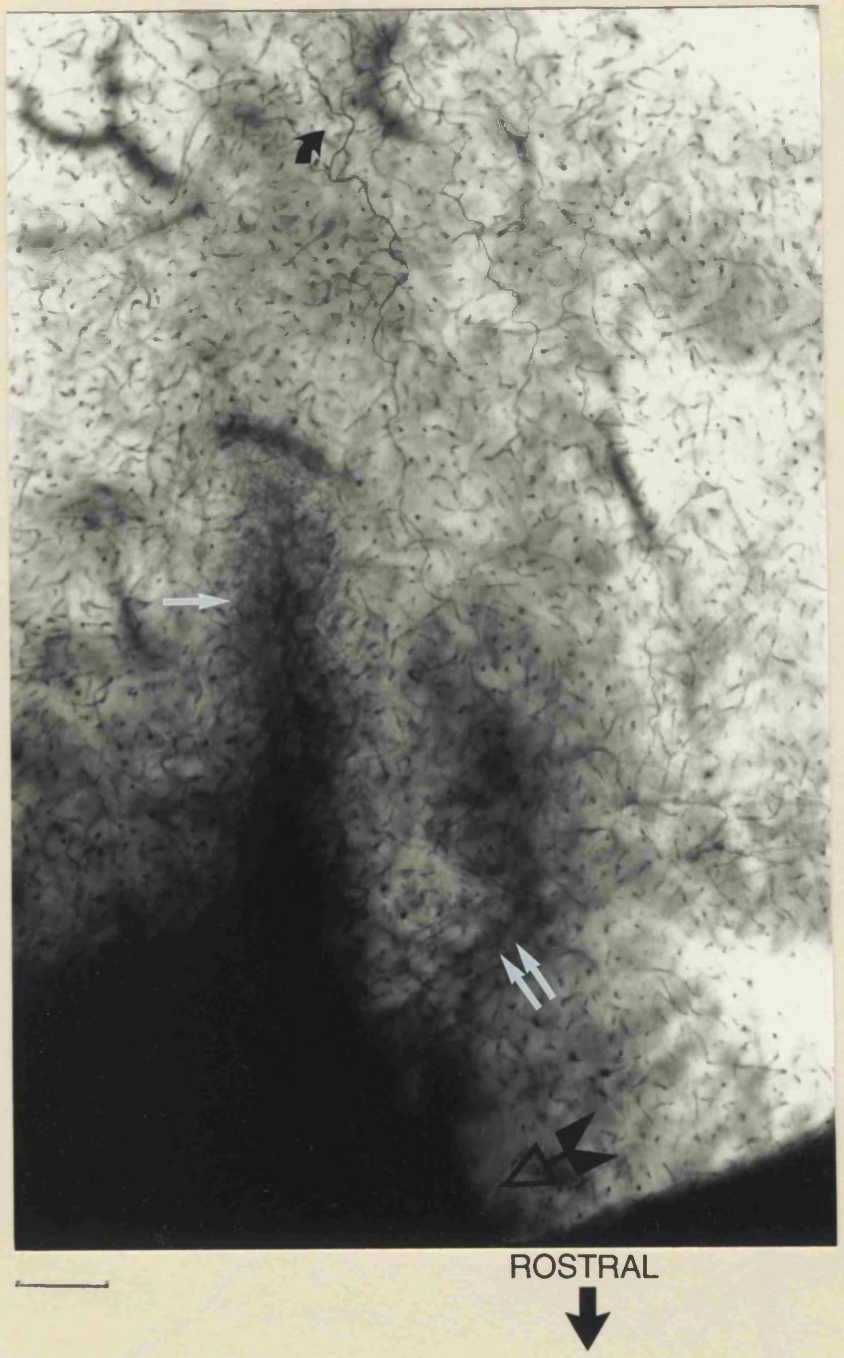

Figure 5B: Photomicrograph of part of tectal wholemount with HRP labelling from rostral tectal lesion (large arrow) showing axons and terminals in the SFGS running directly caudally (single arrow) and a separate group of fascicles (double arrow). Axons extending from the fascicles run for a considerable distance on the tectum before terminating (curved arrow). The contrast between tectal innervation arising from the rostral tectal margin and from the fascicles is well shown. The direction of the rostral tectal pole is marked. Bar represents 100μm.

Figure 6: *Camera lucida* drawing of retinotectal axons in a peripheral fascicle with arborisations terminating rostral to their fascicle of origin (stippling). Bar represents 100μm. Accompanying high power drawing (top) shows several terminal arborisations from the stippled area.

ganglion cell birthdate (Straznicky and Gaze, 1971), showed that rostral fascicles are older than caudal ones, and the youngest fascicles extend, one from each brachium, to the caudal periphery. Therefore older axons travel further caudally from their fascicle to their termination sites compared to the youngest, which turn rostrally.

This relationship was quantified. Close to the rostrocaudal axis two measurements were made on a series of fibres chosen at random from various tectal preparations showing different patterns of labelled axons. The first was the rostrocaudal distance along the fibre's trajectory from its turning point as it left the fascicle to the most distal branches of its terminal arborisation (A; Figure 7). This point was chosen because it was the most easily identifiable and reproducible point on the arborisation. The second measurement was the rostrocaudal distance from the line joining the most distal branches of the terminal arborisation to the caudal tectal edge (B; Figure 7). The first measurement was plotted against the sum of the two measurements (Table 1; Figure 8).

The graph shows a straight line relationship between the distance from the fascicle to the termination site and the length of tectum caudal to the fascicle. That is, the greater the distance from turning point to termination site (more rostral fascicles), the greater the distance from the turning point to the caudal tectal pole. The gradient of the graph reflects the constant relationship between these two variables. This relationship is constant within a fish and also constant between fish, even fish of differing sizes and presumed ages. If the distance from fascicle to termination site reflects migration of the axon from the fascicle, which is necessary according to the hypothesis of shifting connections, then this migration is constantly related to the distance from the fascicle to the caudal pole. This latter measurement is in turn a reflection of tectal growth, since the tectum grows by the addition of cells at the caudal pole. Therefore caudal migration is constantly related to tectal growth.

That migration has occurred is an assumption based on the pattern of axons observed and also on the hypothesis of shifting connections, and this

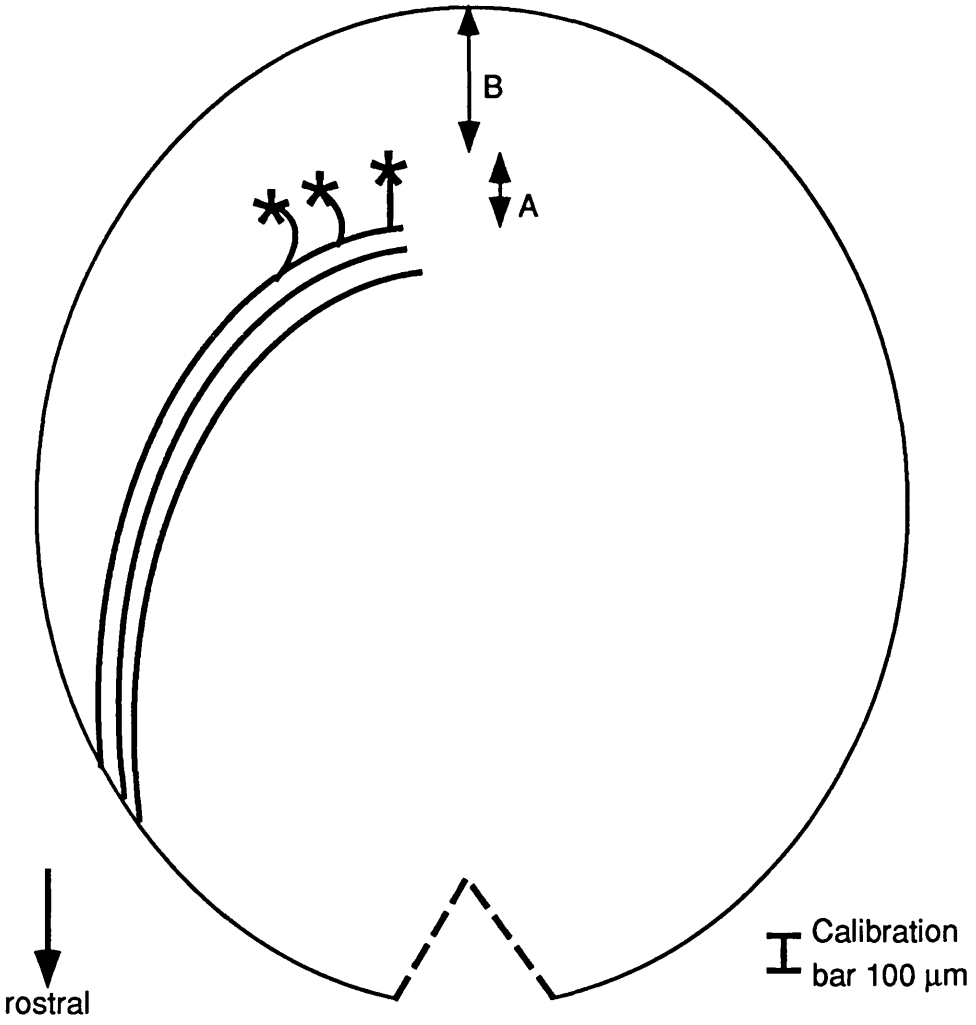

Figure 7: diagram of tectum showing axon leaving fascicle in tectal midline.
A: distance from turning point as it leaves the fascicle to the most distal branches of its arborisation.
B: rostrocaudal distance from distal branches of terminal arborisation to caudal tectal edge.

Specimen	length to edge (sum of A+B) (μm)	length to terminal (A) (μm)	tectal length (μm)
A3	183	-163	3360
A3	164	-203	3360
B1	873	189	3520
B1	952	237	3520
B2	726	100	3400
B2	723	142	3400
B3	639	100	2960
B3	621	132	2960
B4	884	157	3060
B4	957	226	3060
B5	952	252	3400
B5	731	110	3400
C1	942	355	3220
C1	1026	484	3220
C2	947	321	3260
C2	968	363	3260
C3	942	368	2780
C3	1063	400	2780
C3	547	0	2780
C4	1105	384	3100
C4	1128	473	3100
C4	1280	479	3100
C5	847	299	2920
C5	820	263	2920
C6	694	210	2980
C7	1184	431	3260
C7	489	137	3260
C9	605	205	3100
C9	694	274	3100
C9	752	331	3100
C10	842	394	3200
C10	747	337	3200
C12	510	42	3300
D1	1462	552	2960
D1	1540	721	2960
D1	1400	584	2960
D2	1068	563	2960
D3	610	147	3280
D3	500	79	3280
D4	710	237	2980
D4	863	389	2980
D4	179	-105	2980
D5	968	268	3080
D5	1015	300	3080
D5	316	-17	3080
D8	486	132	-
E6A	222	-81	3360
E6A	167	-131	3360
E11	341	83	3060
E11	109	-175	3060
J1	2393	1015	3880
J1	2206	970	3880
J4	2104	989	4020
J4	2223	1005	4020
J6	2372	1131	3900
J6	2388	1147	3900
J12	1815	745	4280
J12	1817	765	4280
J12	1801	765	4280
J19	2588	1270	3800
J30	1680	810	3560

Table 1: Measurements of distances from fascicle to caudal tectal edge (A+B) and from fascicle to terminal arborisation (A). Total tectal lengths are also shown.

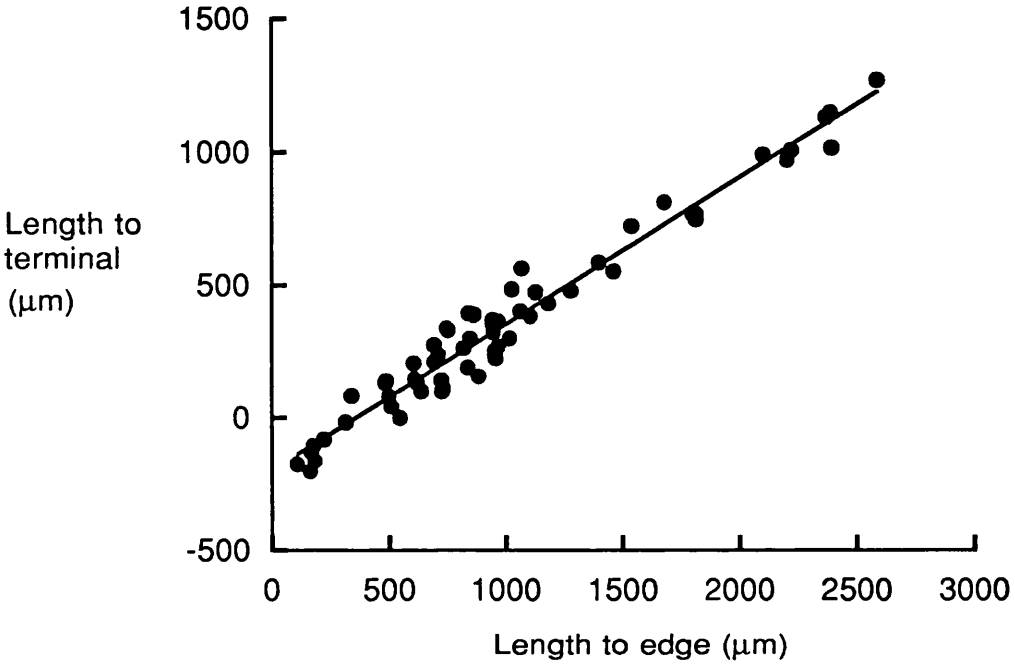

Figure 8: graph of length of axon from fascicle of origin to terminal arborisation (A) against distance from fascicle of origin to caudal tectal edge (A + B). Regression line described by the equation: $y = 0.55 x - 198$

assumption is implicit in the discussion of these results. Caudal migratory distance was measured as a positive value, and conversely rostral migratory distance from the most peripheral fascicles was taken to be negative. The intercept on the x axis was 360μm. Therefore the site at which axons terminate directly beneath their fascicle (zero caudal migration) is estimated to be 360μm from the caudal tectal edge. The intercept on the y axis was -198μm. Therefore at the caudal tectal pole, terminals are on average 198μm rostral to the most peripheral fascicle.

The gradient of the line was 0.55. Therefore, the caudal migration of terminals lying close to the rostrocaudal axis was approximately half the amount of tectum added since their fascicle of origin was laid down at the tectal edge.

The gradient of the graph was independent of the tectal length which varied from 2780 to 4280 μm. Therefore, whatever the size of the tectum, the relationship of caudal migration to amount of tectum added by growth was constant.

The graph also implies that fascicles contained fibres destined to synapse on the caudal half-tectum only. When the distance to the caudal rim was greatest (rostral fascicles), the caudal elongation of these fibres took them beyond the rostrocaudal midline. This is a surprising finding. It corresponds to the results obtained from retrograde labelling (section 4:2): retinal patterns contained arcs only in the ventronasal quadrant and arcs correspond to fascicular axons. Previously, the fascicles were thought to contain all the fibres destined to synapse on the tectum. For example, this assumption had been made by Attardi and Sperry (1963). The results from these anterograde tracer studies refute this assumption.

The fascicles are neither concentric with the map nor with each other: they form a fan-like array which is clearly seen following optic nerve lesions (Figures 2A and 2B). The results described here would be consistent with the hypothesis that each fascicle represents the relative position of the caudal half tectal margin at the developmental stage when those fibres were

caudal half tectal margin at the developmental stage when those fibres were newly generated and at the tectal periphery. As the tectum grew, each fascicle would become displaced rostrally, relatively. Tectal hypertrophy, which is thought to contribute to tectal growth (Raymond and Easter, 1983), would contribute to the distortion of the tectum. Fascicles which were almost semi-circular in their trajectory, when they marked the caudal tectal boundary, would adopt a more mediolateral trajectory as they 'moved' relatively rostrally. This is an assumption based on observation of the fascicular pattern on the tectum and on the known pattern of tectal growth.

What is the origin of the terminals that fill the rostral half-tectum? Lesions at the most rostral point of the tectal pole, near the rostrocaudal axis, succeeded in labelling axons which coursed directly caudally within the SFGS (Figure 5A). Arborisations were seen throughout the column of labelled axons which ended at the rostrocaudal midpoint. Axons could be seen passing from the brachia along the rostral tectal margin and then turning caudally to run in the SFGS.

Given that approximately half the newly generated fibres arrive at the rostral tectal pole, the distance which the caudal fibres must migrate to allow these fibres to synapse will be about half the distance which has been added by growth at the caudal half-tectum. This is consistent with the gradient of the graph (0.55).

It is not known why the newest fascicular fibres turn rostrally at the caudal tectal pole though it is possible that this portion of tectum that has been generated most recently does not contain mature cells capable of forming synapses. Therefore the axons may have to pass further rostrally to form effective synapses. This hypothesis has not yet been tested.

4:2: Retrograde labelling with HRP in normal fish

Retrograde and anterograde HRP tracing methods were used as complementary means of characterising further the anatomy of the normal goldfish retinotectal projection.

For retrograde labelling, all lesions were made on the right tectum yielding filled cells in the left retina. Eighty-three retinae were examined and 19 representative distributions plotted. Six patterns of particular interest are reproduced in full (Figures 9 and 10). Lesions passed through the *stratum opticum* and the *stratum fibrosum et griseum superficiale* of the tectum. The lesions were made at a series of tectal positions noted on a diagram of the tectal surface, and a corresponding series of patterns of filled retinal ganglion cells (RGC) was developed.

In normal animals, the patterns of filled RGC were restricted to a small percentage of the retinal area. This implies that the axons and terminals labelled at the tectal injection site comprised a small proportion of the total retinotectal projection. It is partly a reflection of the size of the injection site. However, it also implies a certain degree of orderliness of the fibre pathway. At the other extreme, an alternative would be that fibres criss-cross the tectum in a disorganised way with many turns close together. This would lead to a greater number of RGC being labelled over a wider retinal area, and the pattern of filled cells would appear to be more disorganised.

The majority of filled RGC were labelled as axons of passage since the section of the pattern which corresponded to terminal arborisations accounted for only the temporal end of the 'bar' (see below). The pattern was on the whole confined to the ventral retina, corresponding to cells which terminate in medial tectum, implying that ventral RGC confine their paths to medial tectum in the normal animal.

Very few filled RGC were seen in the dorsal half-retina, though the dorsal tips of arcs at the nasal retinal pole sometimes appeared to extend into the dorsal half-retina (Figure 10C). The definition of a half-retina has

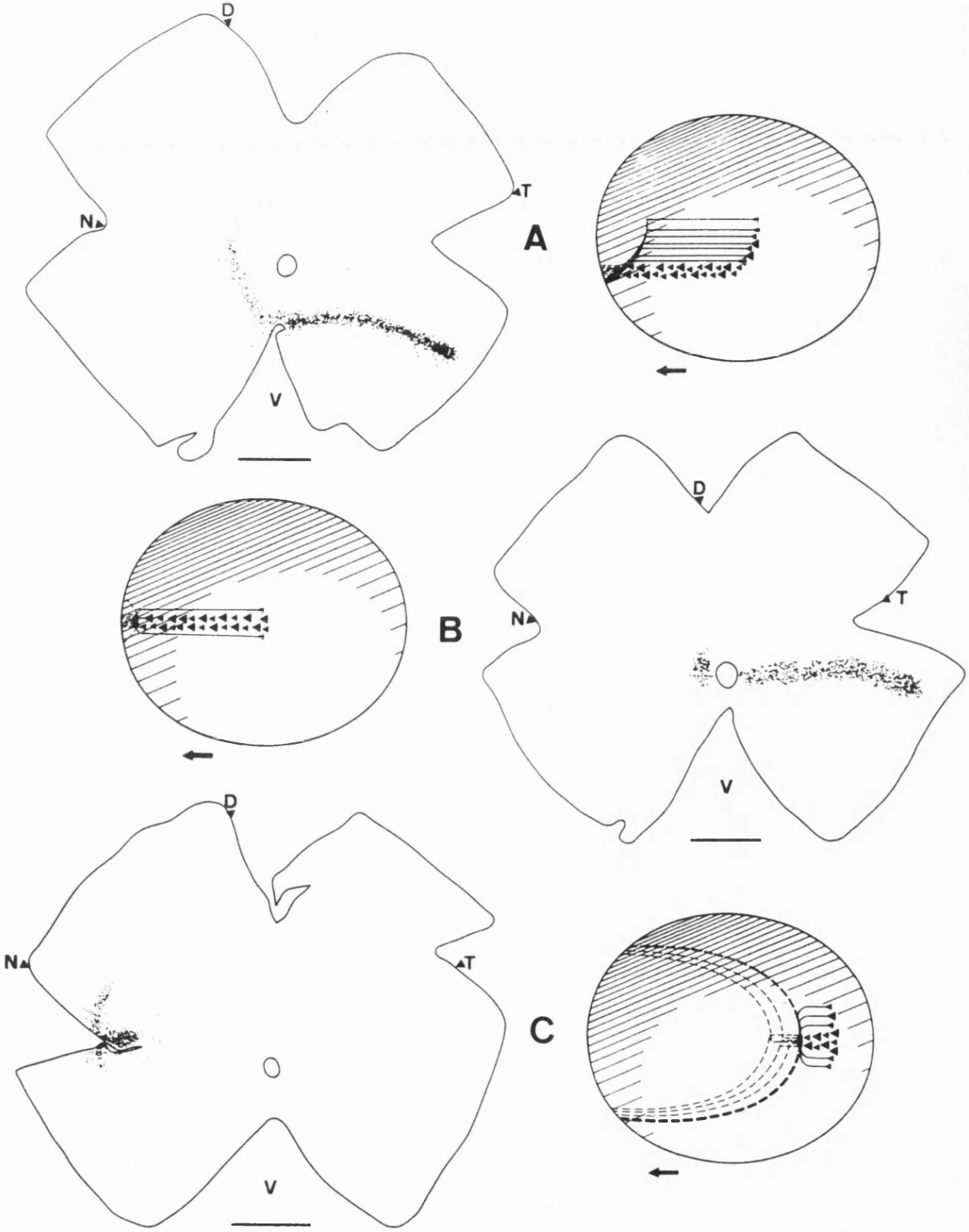

Figure 9: Tracings of flat-mounted goldfish (left) retinae showing patterns of labelled RGC following application of HRP to contralateral tectal lesions. Diagrams of corresponding flat-mounted tecta with the axons' tectal paths are shown. Retinae: V=ventral, N=nasal, T=temporal, D=dorsal. Bar=1mm. Tecta: arrow in midline pointing rostrally. Cross-hatching represents tectal surface hidden at operation. Lesion shown by transverse bar. Terminal arborisations shown by triangles and extrafascicular axon segments by continuous lines.

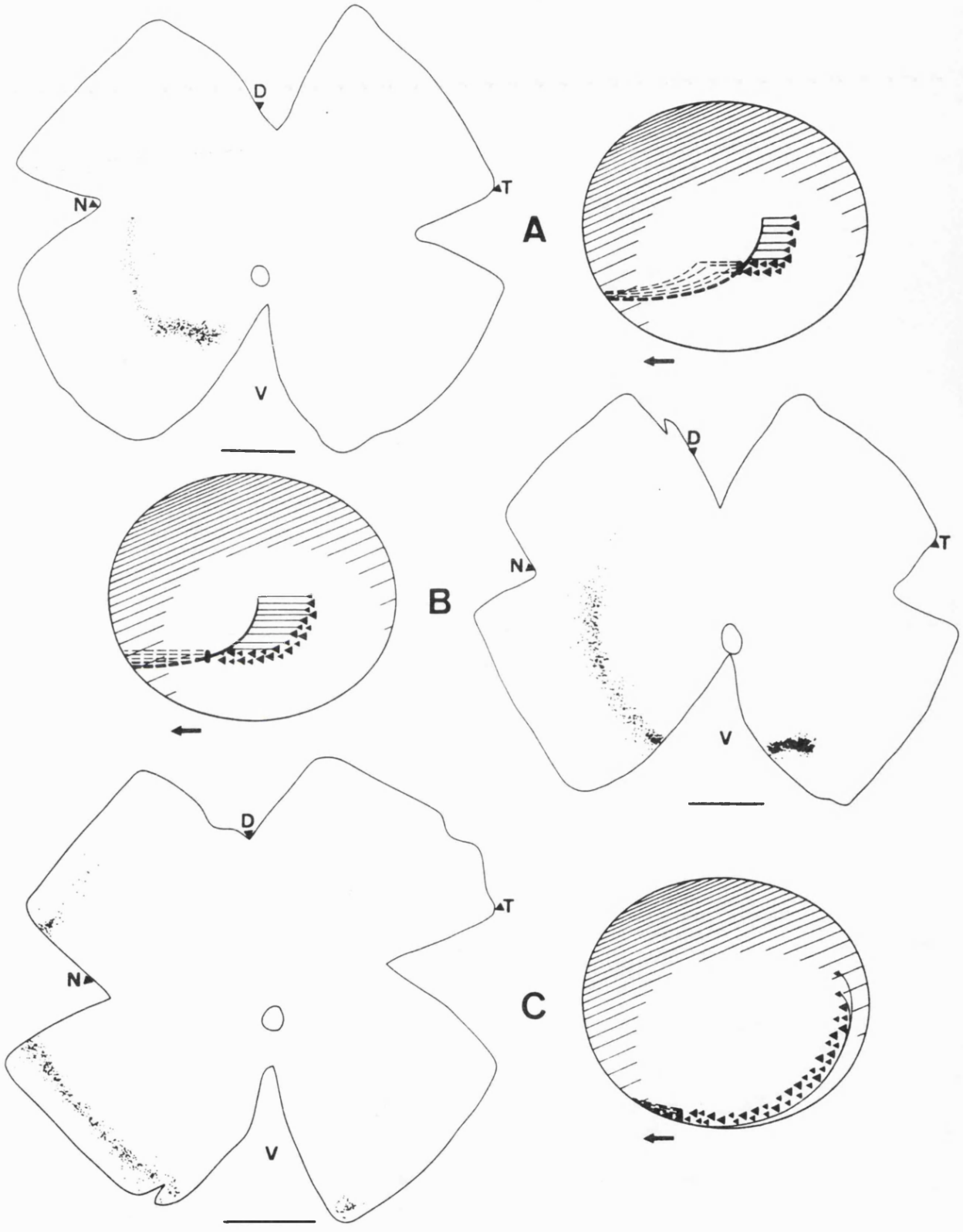

Figure 10: Tracings of flat-mounted goldfish retinae showing patterns of RGC following HRP labelling of contralateral tectal lesions with diagrams of their axons' tectal paths. For conventions, see Figure 9.

been debated (Springer and Mednick, 1986). In the experiments described here, the retinae were divided into quadrants on the basis of a ventral cut made at the choroid fissure at the time of dissection and by area. The other cuts marked approximate quadrants only, and were for the purpose of giving a flat mounted preparation. Therefore the significance of these filled cells which apparently extend into the dorsal retina is unclear. However, it is hard to argue that the cells labelled towards the dorsal pole of the retinae in Figures 10B and C are a result of the cuts made during dissection for the purposes of flat-mounting. It is possible that such cells are labelled because they originally reached their termination sites via the medial instead of the lateral brachium. Cells which are destined to terminate around the caudal pole of the tectum would not reduce their chances of finding the correct termination site unduly if they took the "incorrect" brachium. The proportion of cells taking the incorrect brachium has since been quantified (Becker and Cook, 1987), and this work will be referred to in the Discussion.

Generally, the pattern of filled cells comprised an "arc" and a "bar". The arc had its centre at or near the optic nerve head and was situated in the ventronasal retinal quadrant. Its radial distance from the optic nerve head varied from almost central retina (Figure 9B) to the retinal periphery (Figure 10C). Its length also varied, as shown in these two figures. The arc was usually attached to a bar at its ventralmost end. Exceptions included the example shown in Figure 9C in which the bar arose partway along the arc, though nearer the ventral tip, and also the retina shown in Figure 10C in which a bar is not easily apparent.

In some cases, (Figures 9A,B and 10A,B), an increase in the density of filled cells could be seen at the temporal end of the bar which was, in most cases, in the ventrotemporal retinal quadrant.

The bar was not always straight. For example, in Figure 9A there was a slight curve in the bar with a ventral concavity. In examples where the bar was especially short (eg Figure 9C) no curve was visible. One possible cause of this curvature was the flat-mounting process. In Figure 9B where the curve in the bar was under the cut made at the temporal pole, it might be

assumed that the technique had caused the curvature. However, this might not be the case in Figure 9A where the bar was well within the ventronasal quadrant, though in this example it is possible that the distortion arose as a result of the ventral cut.

These findings were interpreted as follows. When the lesion is made on the tectum, there are at least three potential sources of tracer uptake. Axons of passage will be damaged in the *stratum opticum* (SO) which contains axons within fascicles. In addition, axons of passage that have left their fascicles will be damaged within the *stratum fibrosum et griseum superficiale* (SFGS). Lastly, terminal arborisations at the lesion site will be able to take up the tracer, whether or not they are damaged. Therefore the patterns of labelled RGC represent cells filled with HRP taken up by each of these potential sources. From previously used mapping methods it is known that a lesion made at the rostral tectal pole, for instance, will label terminals whose cell bodies of origin are at the temporal retinal pole (Figure 9B). Extrapolating to other patterns, it is inferred that cells at the temporalmost end of the bar represent axon terminals at the lesion site. The increased density of the filled RGC in this part of the pattern in some examples could arise because there is much overlap of terminal arborisations. Therefore there would be greater opportunities for the cell bodies to be labelled. Another likely possibility is that there is diffusion beyond the area of direct axon injury leading to further endocytotic uptake. The lower density of cells labelled as axons of passage could arise because of a technical artefact. If the needle which made the lesion ran parallel to or pushed aside the axons in the fascicles or in the SFGS there would be a lower number of severed axons.

The bar, which ran in a temporonasal direction, comprised filled cells of many ages, since the retina grows circumferentially. These cells' axons passed through the lesion site in the SFGS *en route* to termination sites which were more caudal on the tectum, corresponding to cells of origin towards the nasal retinal pole. When the lesion was made rostrally, the bar ran in most cases nearer to the optic nerve head, representing older cells, as well as nasally. For example in Figure 9B, where the tectal lesion was made

almost at the rostral tectal pole, the bar ran right up to the optic nerve head. In other cases, when the lesion was made caudally, the bar extended peripherally as well as nasally, representing younger cells (eg Figure 9C) . Thus the cells comprising the bar, except for its temporal tip, were filled from axons of passage.

In those instances where the curvature of the bar was not easily explained by the flat-mounting technique, (Figures 9A, 10A), what might the curvature represent? It is possible that the axons of passage in the SFGS were not taking a directly rostrocaudal route, since such a route would lead to a straighter temporonasal retinal bar. It appears that the axons of passage might have a centripetal trajectory within this layer in cases where the lesion is made rostrally (Figure 9A), for a short distance immediately caudal to the lesion site. In the example shown in Figure 10A where the lesion was made in the caudal tectum, the trajectory of the axons of passage in the SFGS might bend slightly medially before turning towards the caudal edge of the tectum. These axons of passage arise from older fascicles, not from the new fascicles at the tectal edge. Therefore older axons, when they shift in a generally caudal direction on the tectum, in some cases may take a slightly curved route.

As a result of the different modes of growth of retina and tectum, there will be a regional variation in the amount of new space on the tectum, and therefore a regional variation in the competition among the RGC for space to terminate. There is more new space caudally than laterally, so there may be greater "pressure" for space laterally. This might be reflected in a centripetal trajectory among the older, more central fibres shifting caudally in the SFGS.

The arc represents a portion of a generation of RGC. It is known that the goldfish retina grows throughout life by generating cells in a circumferential pattern, the dividing cells being at the periphery (Straznicky and Gaze, 1971). Therefore the arc near the centre of the retina (Figure 9B) contains some of the oldest RGC while the arc at the periphery (Figure 10C) contains some of the newest. The arcs fall within the ventronasal quadrant, and therefore their axons terminate in the caudomedial region of the tectum.

The termination sites are therefore at some distance from the lesion sites, as in Figures 9A and 9B, where the lesions are near the rostral tectal pole. The arc, like most of the bar, is formed as a result of uptake of label by axons of passage. It is proposed that, unlike the bar, it is labelled when the lesion breaches the fascicle(s) in the SO. The curve of the arc is partly a reflection of the curve of the fascicle, but only in so far as it contains axons of the same age whose terminals form an arc on the tectum. I propose that the fascicles fail to form precise arcs because of the mode of tectal growth.

If it is argued that the fascicles do contain the axons which correspond to the arcs of RGC, a lesion near the tectal midline should yield a pattern with a short arc since fewer axons will be severed (Figure 9A). A lesion far from the tectal midline should yield a long arc since a larger number of fascicular axons of passage will be severed (Figure 10C).

The fact that arcs are confined to the ventronasal quadrant implies that fascicles only contain fibres destined for the caudal tectum. Fibres that innervate the anterior tectum pass from the rostral edge directly caudally (with the possible path curvatures described above) and are not situated in the fascicles at all. This is despite the fact that fascicles are present all over the visible tectal surface, a finding confirmed by the observation that lesions in all regions generate arcs.

I propose that this reflects tectal growth patterns, since tectal cells are added only at the caudal tectal border (Straznicky and Gaze, 1972), and that fascicles mark the position of this border at various developmental stages. Since fascicles cover the entire tectum, the fibres from the rostral pole must run in a lower layer, the SFGS.

Figure 9C, where the bar arises along the arc, may represent some interdigitation of fascicles around the tectal midline and therefore overlap of terminations of the axons they contain.

It was difficult to label the fascicles which were on the other, symmetrical side of the array, because of the curvature of the lateral tectal

surface. However, labelling a fascicle on this side of the tectum should result in labelling of RGC in the dorsonasal quadrant.

The distance an axon runs caudally after leaving its fascicle is a function of its age. This can be inferred from both the anterograde and retrograde HRP studies described here. In the former, the axons which left the more rostral, older fascicles were shown to extend further before terminating than axons from more caudal, younger fascicles. In the retrograde studies, Figure 9A represents an old fascicle since the radius of the arc is small. The fascicular axons terminate on caudal tectum at some distance caudal to the lesion site. In Figure 10A, the fascicular axons are younger since the arc is further from the optic nerve head compared to figure 9A. The distance from the fascicle to the termination site is shorter than in Figure 9A, as predicted in the accompanying line drawings of the tecta. Finally, in figure 10C, there is no discernible bar and the arc is composed of young RGC at the retinal periphery. The fascicular axons must therefore terminate near the fascicle. Thus, the older the axons, the greater the fascicle-to-termination-site distance. Since retina and tectum have different modes of growth, it is proposed that older axons will have to shift further than younger ones.

Some of the patterns of filled RGC contain some scattered cells at a distance from the body of filled cells which make up the arc and the bar, as in Figure 9A. This implies that although the paths of axons are in the main highly ordered, occasional axons take circuitous tectal routes to their termination sites. The filled cell to the right of the optic nerve head in Figure 9A can be presumed to terminate in the tectal midline in the rostral half-tectum. As it has been labelled by a lesion towards the rostromedial tectal margin, its path (or its termination) must pass through this point. According to our understanding of tectal paths, this cell's axon would normally run directly caudally in the SFGS and would not pass through the lesion site. Therefore this axon's path must be circuitous. If this labelled RGC was terminating at the lesion site, by definition both its path and termination would be erroneous.

Thus the patterns of labelled RGC following tectal lesions are consistent with the hypothesis of shifting connections and provide strong circumstantial evidence for it. The fact that these results are consistent with the patterns shown by anterograde HRP labelling supports the validity of the method.

4:3: A quantitative WGA-HRP study of the regenerating projection in standard laboratory conditions

4:3:1: Retrograde labelling

Iontophoretic injections of wheatgerm agglutinin conjugated to horseradish peroxidase (WGA-HRP) were made into the tecta of normal goldfish or goldfish at a series of stages of optic nerve regeneration. The injections were made at one of two standard tectal sites corresponding to the ventral quadrants of the retina: they were injected on the dorsomedial tectum, either rostrally or caudally (Figure 11A). This facilitated the measurement of nearest neighbour distances because it maximised the number of filled cells seen in the centre of the quadrant and thereby minimised the pairing of nearest neighbour cells across retinal cuts which could potentially give rise to errors. It also permitted the injections to be made at a standard depth for optimal filling of retinal ganglion cells (RGC): the standard site meant that the standard depth corresponded to a standard laminar position, thus allowing for the curvature of the tectum and an actual decline in laminar thickness from rostral to caudal.

Labelled RGC in normal and late regenerate retinae were of similar appearance, containing many small brown granules of reaction product in the cytoplasm. Labelled cells in early regenerates had a more swollen appearance, this being a well recognised response to axotomy (Murray and Grafstein, 1969). The granules were rather less dense in these animals. Cells were considered to be labelled if they had five or more granules present, though for the majority of filled RGC such a distinction was not necessary since they contained many more than five granules.

4:3:2: Retrograde labelling in normal fish

Eleven retinae of normal fish were examined. In all, the injection yielded a compact cluster of labelled RGC. The clusters were situated in either the ventrotemporal quadrant (rostral injection) or the ventronasal quadrant (caudal injection; figure 11B). The number of labelled cells ranged from 223 to 719. The average number of labelled cells was 445. No filled cells were seen distant from the cluster: the largest value for mean nearest

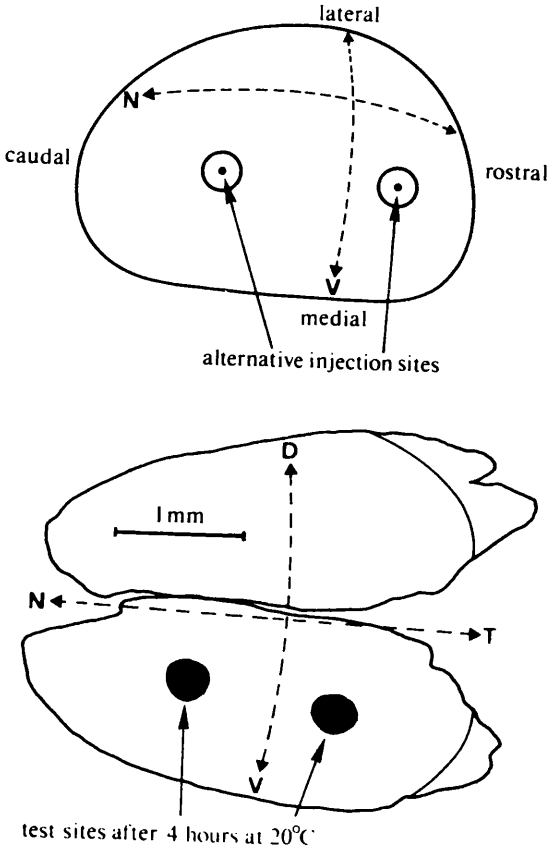

Figure 11A: Diagram (top) of optic tectum from above, showing rostral and caudal injection sites. V=ventral, N=nasal retinal pole projection sites. Tracing (bottom) of flat-mounted tectum showing the limits of two test WGA-HRP injection sites. Even after 4 hours there is little diffusion of tracer into the surrounding tectum. N=nasal, T=temporal retinal pole projection sites.

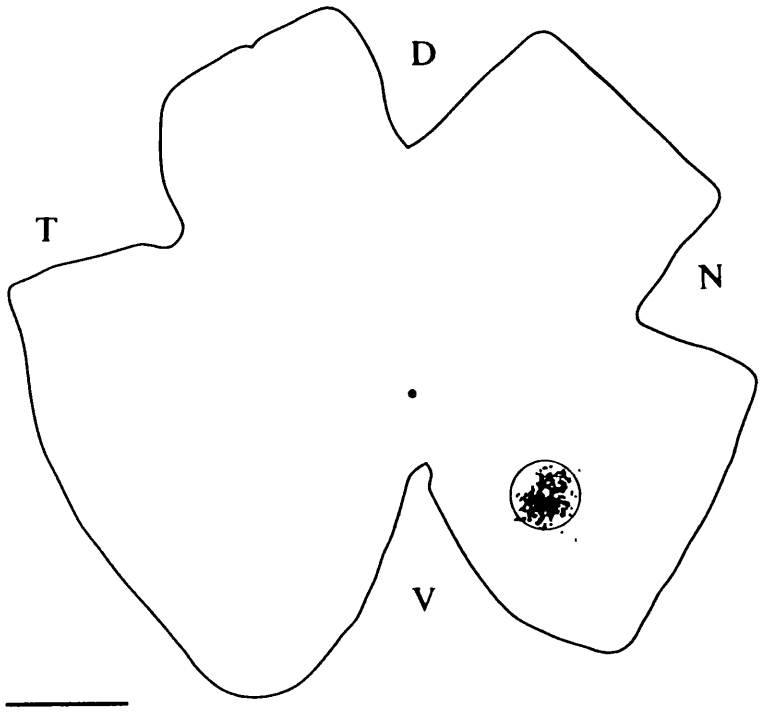

Figure 11B: Tracing of retinotopic (in ventronasal quadrant) cluster of labelled RGC following injection of WGA-HRP at caudal tectal site in a normal goldfish. The cluster is compact. Area of circle around cluster as a proportion of the retinal area is equivalent to the area index calculated for this cluster. Bar=1mm.

neighbour distance was 16.08μm. There was slight variation in the site of the cluster within the quadrant, and the radius of the clusters varied from 238.1 to 352.8μm, averaging 305.3μm. This could reflect slight variations in the depth of the injection site between animals: when the micropipette was applied to the tectal surface, the surface was occasionally depressed intact before it was penetrated and the recoil of the tissue could have been a source of variability. Slight variations could alter the distance from the surface to the terminal arborisations. Variations in cluster size could also have arisen from differences in the amount of tracer injected.

4:3:3: Retrograde labelling in fish after optic nerve cut

Retinae were examined at the following stages after optic nerve cut: 18, 24, 28, 35, 41, 42, 49, 56, 70, 98, 125, 239 and 524 days. Twenty-three retinae were examined after rostral injection. Three of these at 239 days were not plotted for economy of time, and a further two were not selected for quantification because they had an unusual distribution of cells (see below). Therefore 18 retinae were used for quantification following rostral tectal injections. Thirty-two retinae were examined after caudal injection, and measurements were made in all of these. Rostral injections were performed from 18 to 239 days, and caudal from 28 to 524 days. The rostral injections were performed at earlier stages than the caudal because the regenerating axons grow in at the rostral tectal margin and therefore there was a greater likelihood that a rostral injection would yield labelled cells at this stage.

Labelled cells were seen as early as 18 days post optic nerve cut, following rostral tectal injections. The number of labelled cells varied from 10 to 802. The labelled RGC were widely distributed throughout the retina in all retinae examined at 18 days, with no visible clustering (Figures 12A and B). However, there was a bias toward the ventral half retina in two cases and toward the appropriate quadrant in one case. No such bias was detected in the remaining 2 cases. The area of the "clusters", so-called for statistical purposes, ranged from 7.46mm^2 to 16.32mm^2, and the mean nearest neighbour distance from 92.5μm to 236.4μm.

A similar pattern was seen following caudal injections at 24 days (five

animals). The number of labelled cells ranged from 5 to 998. Three retinae contained 5, 5 and 8 filled cells (Figure 13A). The remaining two retinae contained 241 and 998 filled cells and in these there was a bias toward the "correct" (ventronasal) quadrant (Figure 13B).

Injections were made at both rostral and caudal sites at 28 days, in three fish at each site. The range of filled cells was 50 to 1366 per retina, and there was a bias toward the correct quadrant. There was no evidence of true cluster formation.

Examples were obtained at 35-56 days of regeneration from caudal injections only (12 fish). Numbers of filled cells ranged from 37-777. Clusters were detectable in the ventronasal quadrant in 9 of these 12 animals (Figure 14).

At 70 days of regeneration, 6 rostral and 4 caudal injections all produced retinotopic clusters (179-456 filled cells). Scattered cells were still seen in all cases but one (Figure 15). Little change was then seen in the distribution of cells in 22 more animals examined up to 524 days. In 5 retinae the clusters were compact and resembled those of normal animals.

In eight cases examined between 56 and 524 days, single injections resulted in the labelling of two adjacent clusters. Five of these retinae were the result of caudal injections. Both clusters were seen in the retinotopic (ventronasal) quadrant, and a line joining the clusters had a radial direction (Figure 16). This was not the case in the double clusters which arose after rostral injections, where, although both clusters were in the appropriate quadrant, a line joining the clusters had no consistent orientation (Figures 17A and B). In four of the eight retinae showing double clusters, the clusters were of approximately equal size and density; whereas in the remaining four retinae one cluster was clearly larger or denser than the other.

As already noted there was a wide variation in the number of labelled cells. There was no systematic relationship between the number of labelled cells and regeneration time (Figure 18).

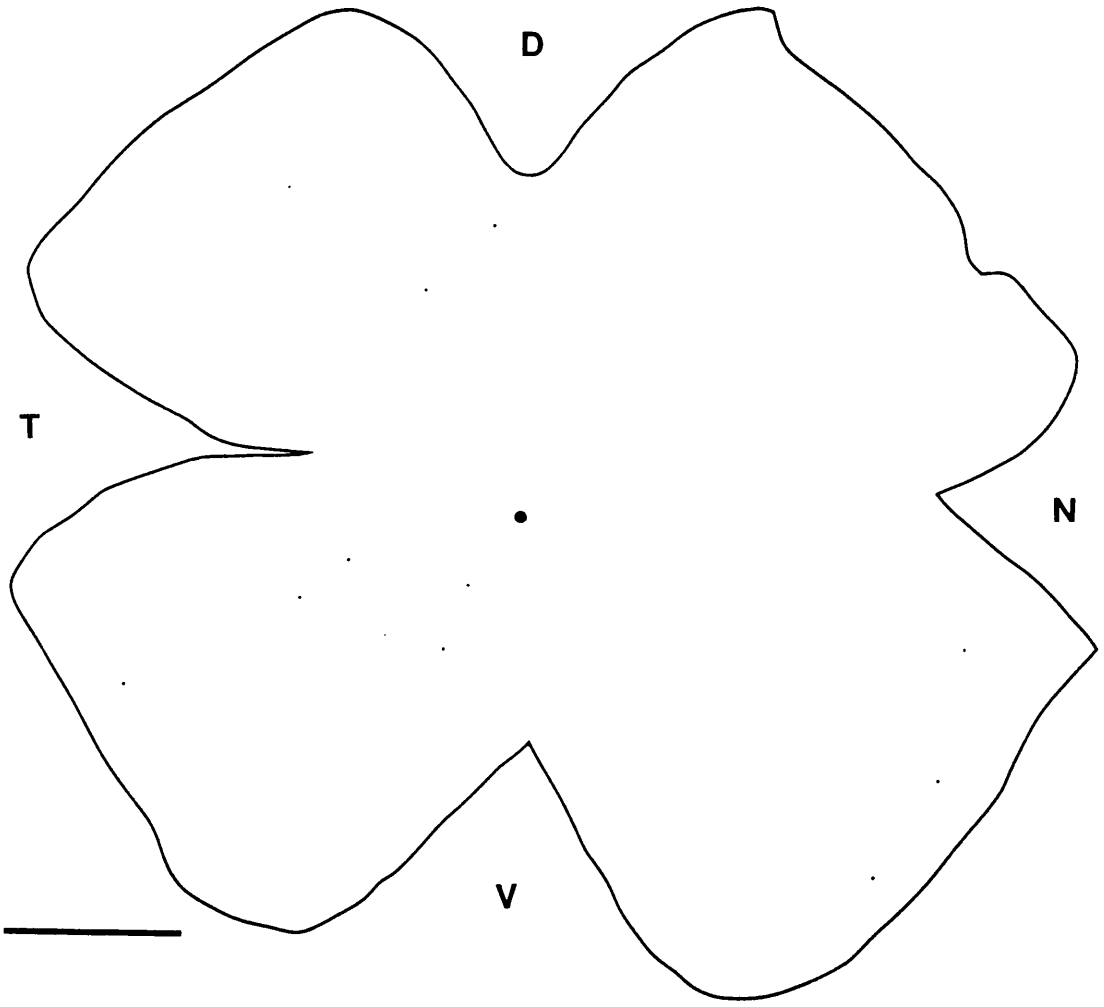

Figure 12A: Tracing of flat-mounted goldfish retina showing small number of labelled RGC following WGA-HRP labelling at rostral tectal injection site at 18 days of regeneration. Bar represents 1mm.

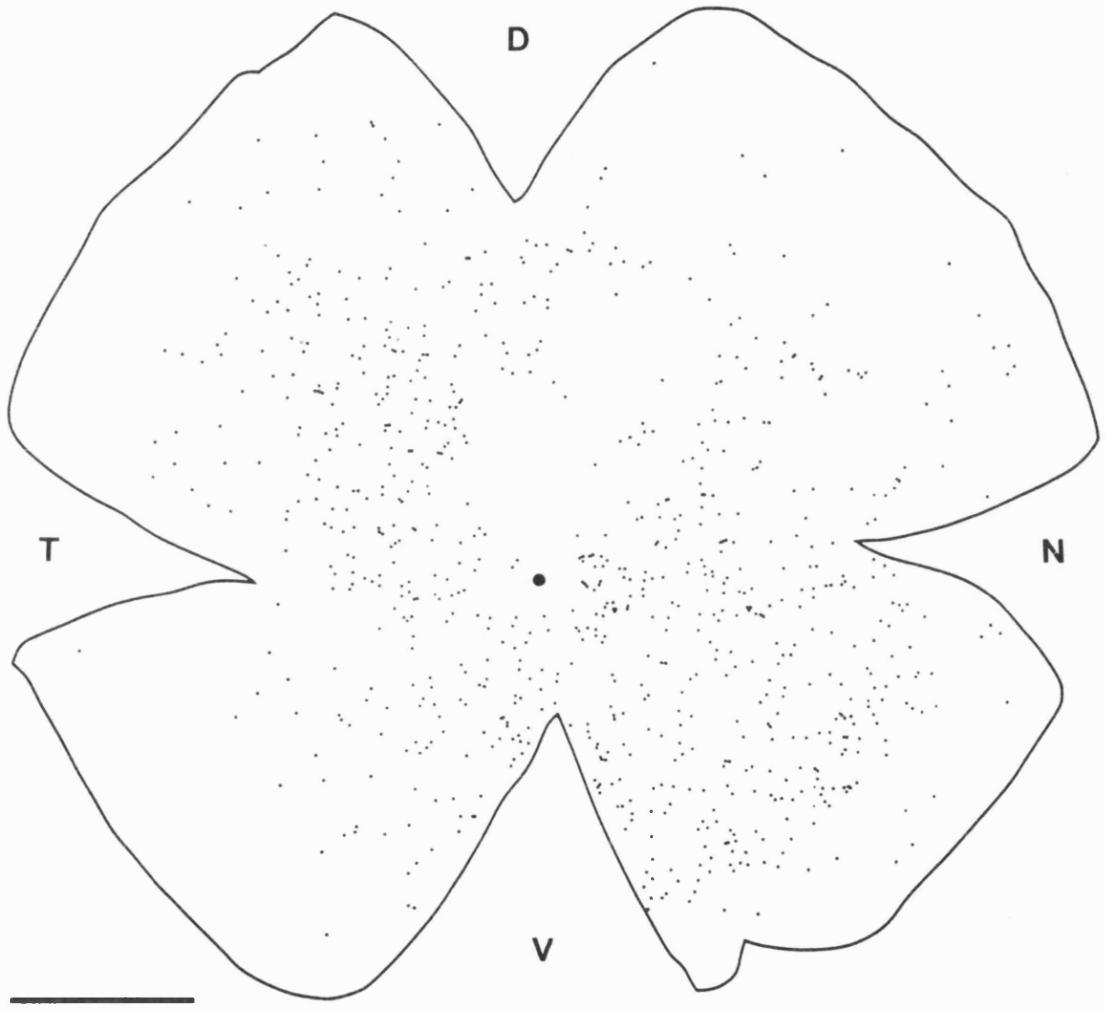

Figure 12B: Tracing of flat-mounted goldfish retina showing large number of labelled RGC following WGA-HRP labelling at rostral injection site at 18 days of regeneration. Bar represents 1mm.

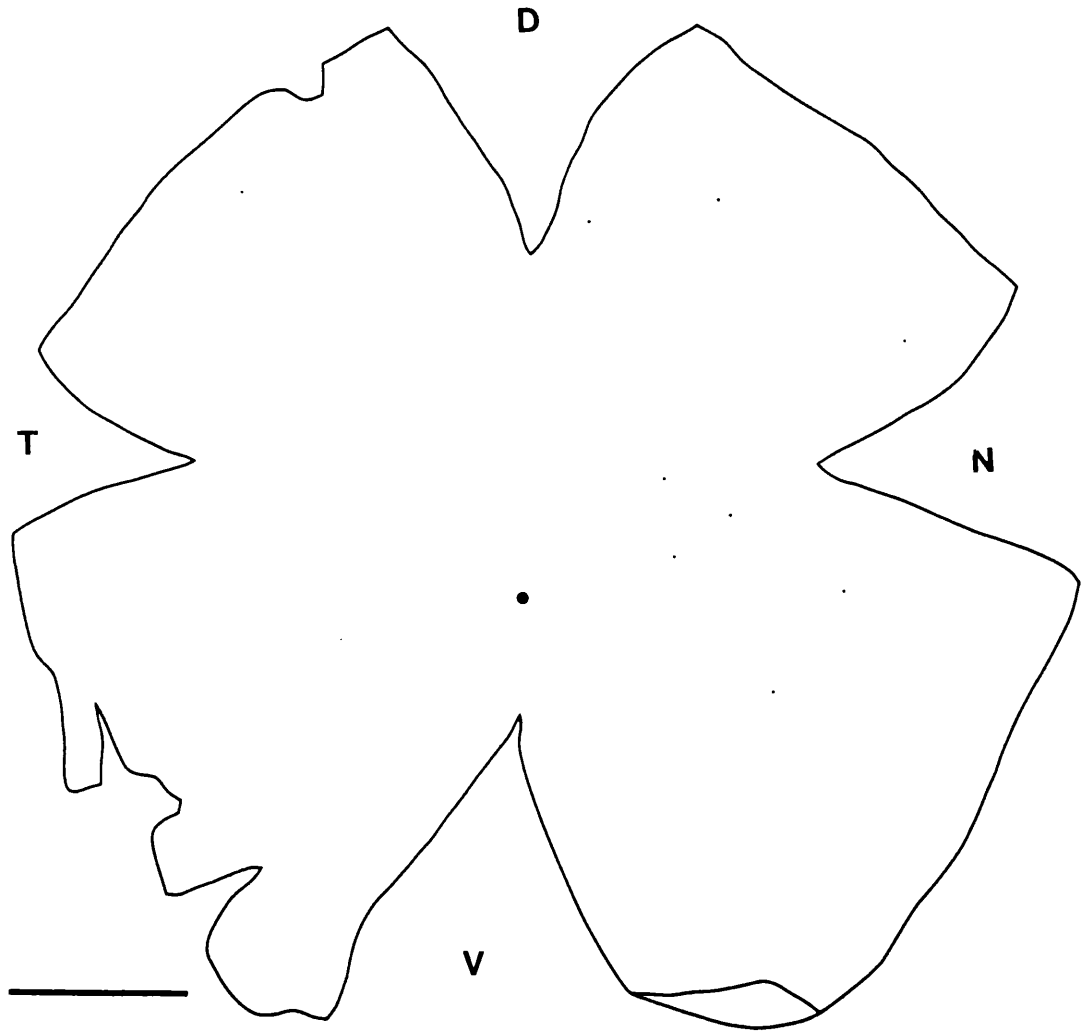

Figure 13A: Tracing of flat-mounted goldfish retina showing small number of labelled RGC following WGA-HRP labelling at caudal tectal injection site at 24 days of regeneration. Bar represents 1mm.

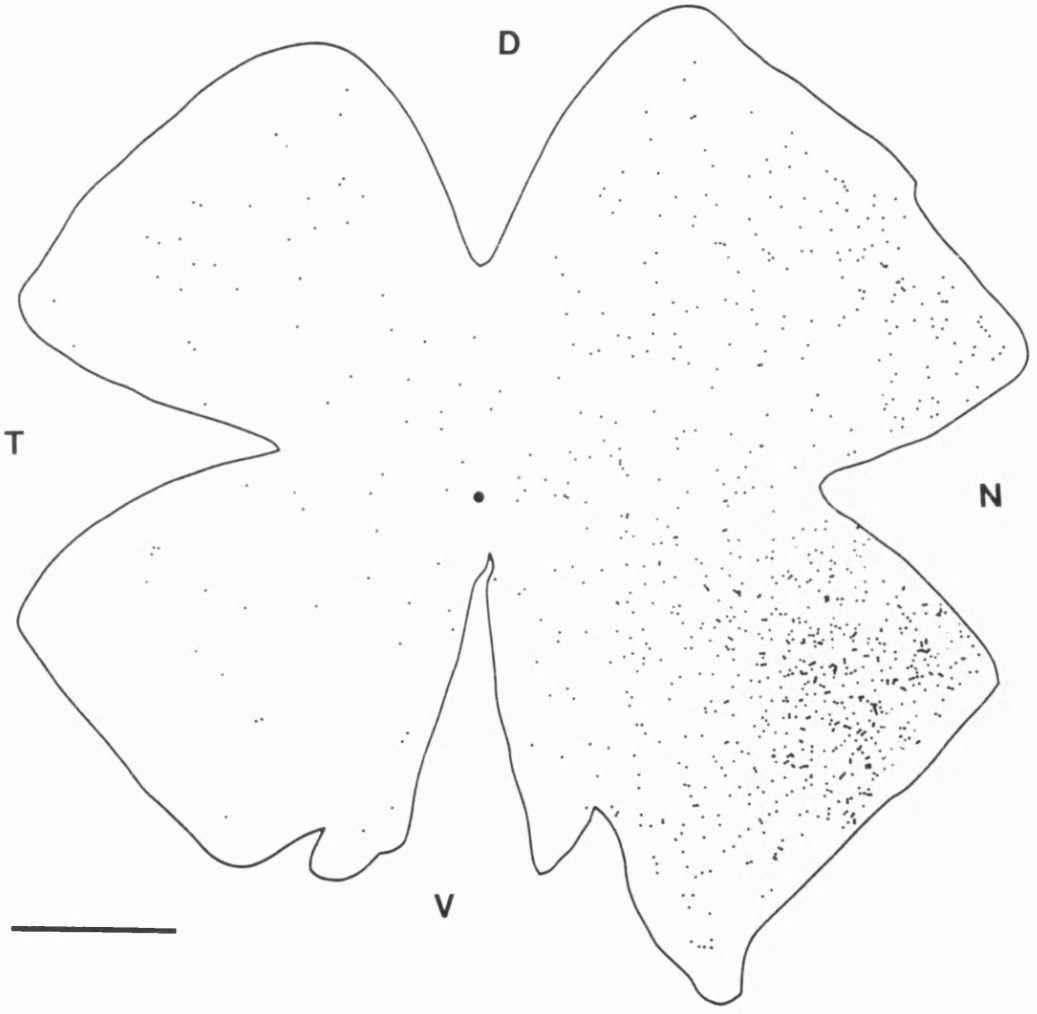

Figure 13B: Tracing of flat-mounted goldfish retina showing large number of labelled RGC with ventronasal quadrantal bias following WGA-HRP labelling at caudal tectal injection site at 24 days of regeneration. Bar represents 1mm.

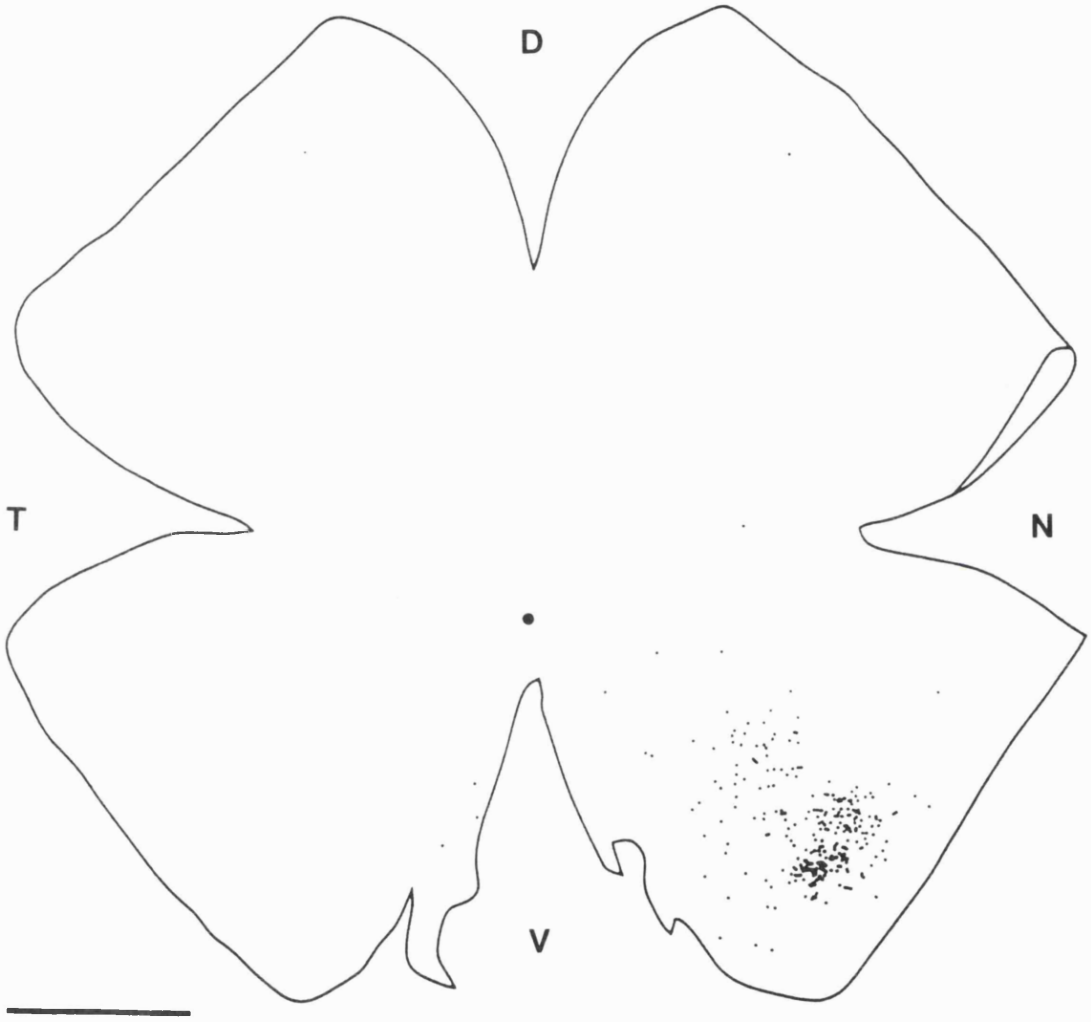

Figure 14: Tracing of flat-mounted goldfish retina showing labelled RGC with ventronasal quadrantal bias and early cluster formation following WGA-HRP labelling at caudal injection site at 41 days of regeneration. Bar represents 1mm.

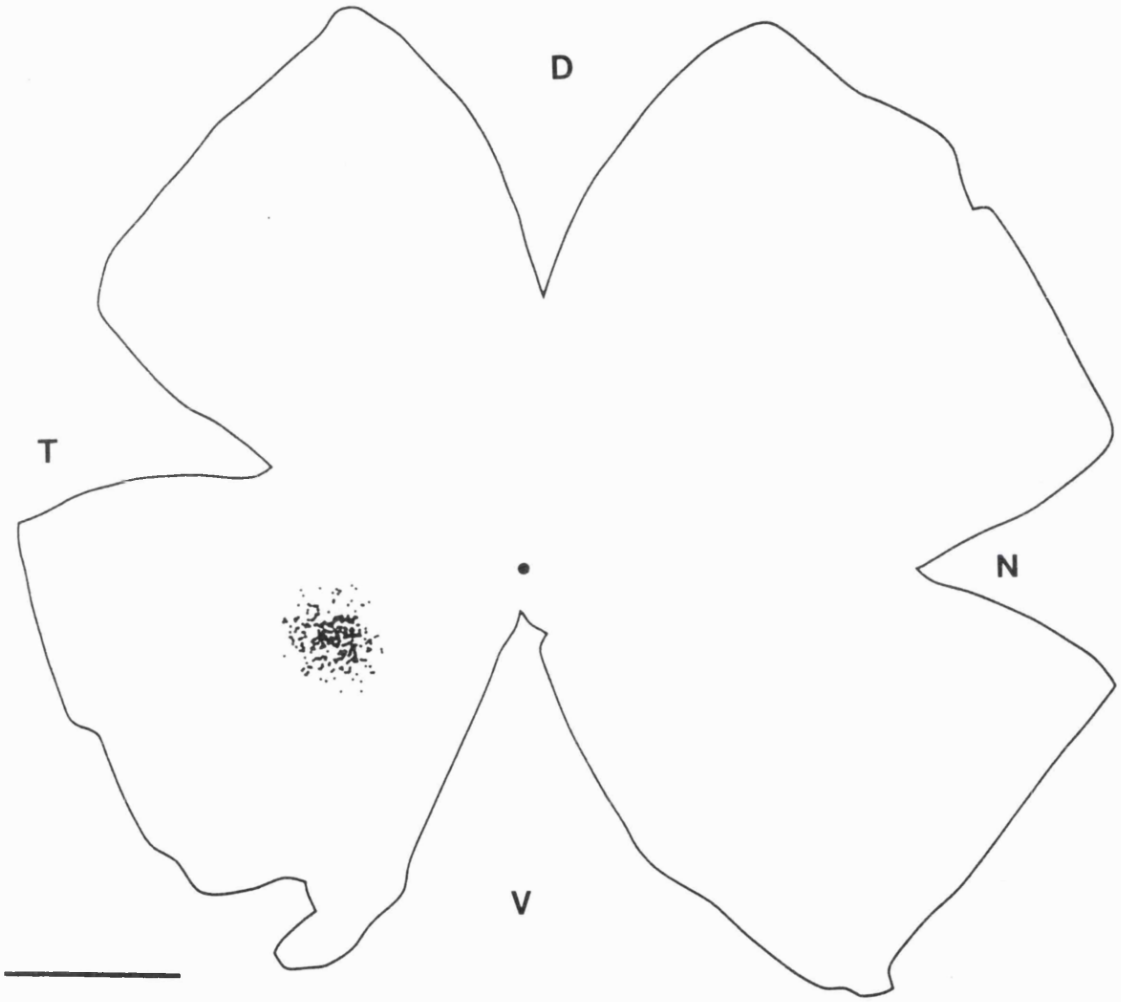

Figure 15: Tracing of flat-mounted goldfish retina showing cluster of labelled RGC in the ventrotemporal quadrant following rostral tectal injection of WGA-HRP at 70 days of regeneration. Bar represents 1mm.

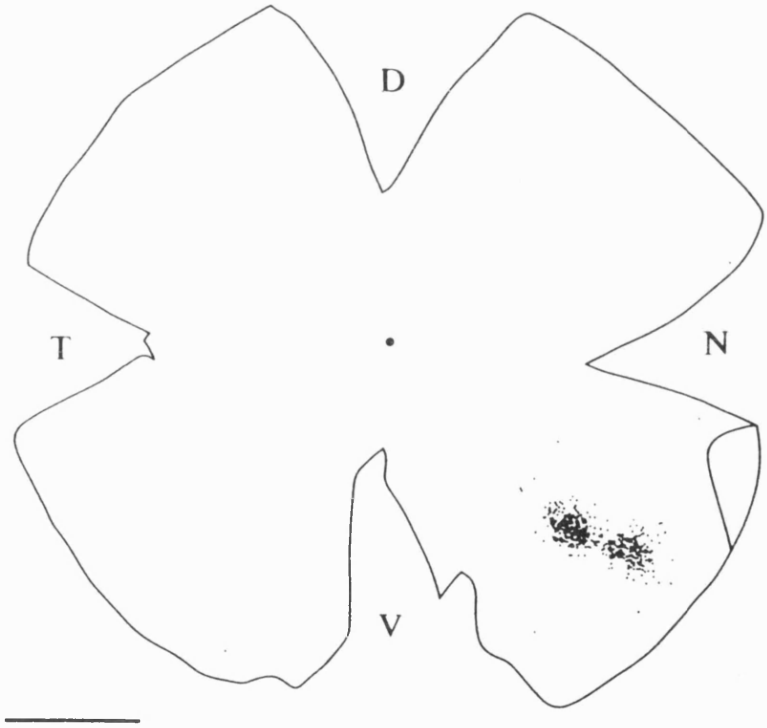

Figure 16: Tracing of flat-mounted goldfish retina showing a double cluster (radial orientation) of labelled RGC in the ventronasal quadrant following a single caudal injection of WGA-HRP after 70 days of regeneration. Bar represents 1mm.

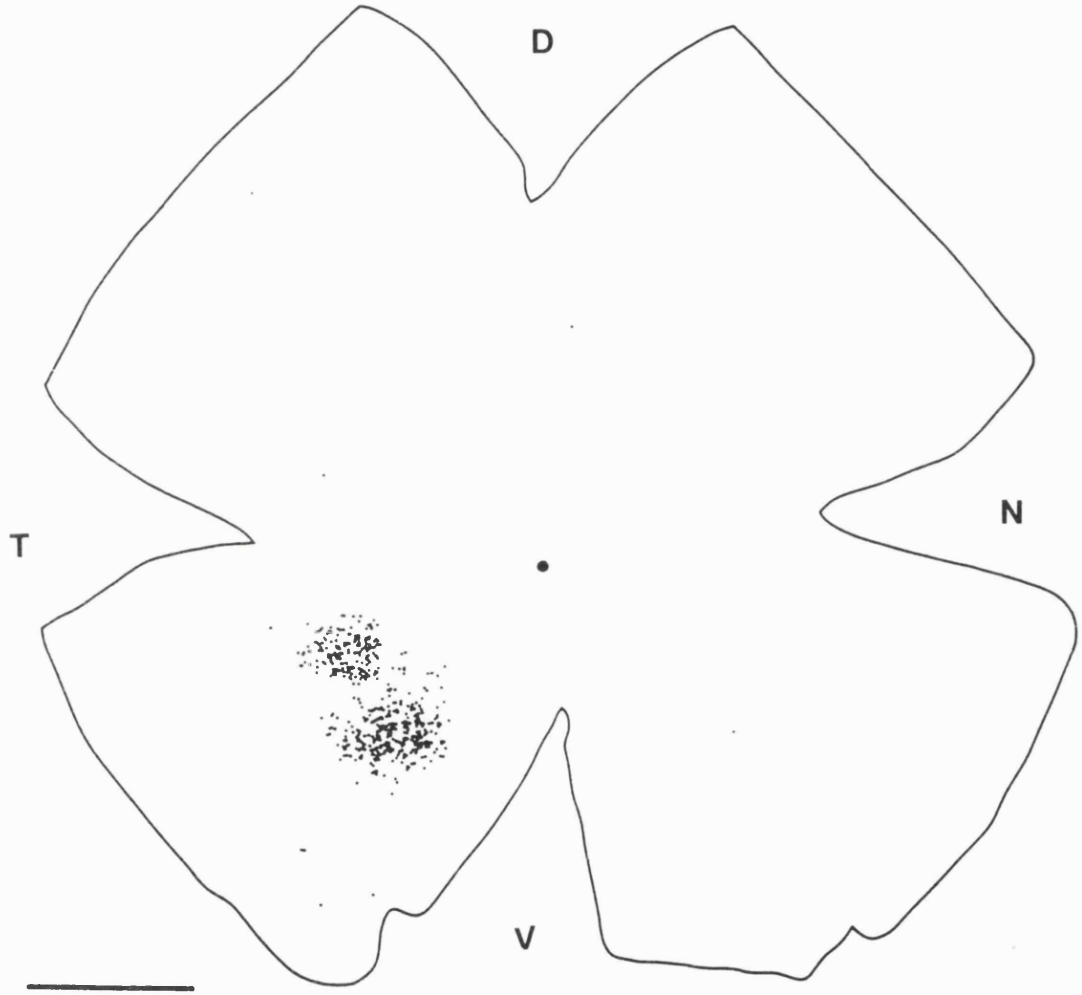

Figure 17A: Tracing of flat-mounted goldfish retina showing a double cluster of labelled RGC in the ventrotemporal quadrant following a single rostral injection of WGA-HRP at 125 days of regeneration. Double clusters following rostral tectal injections showed no consistent orientation (see also Figures 16 and 17B). Bar represents 1mm.

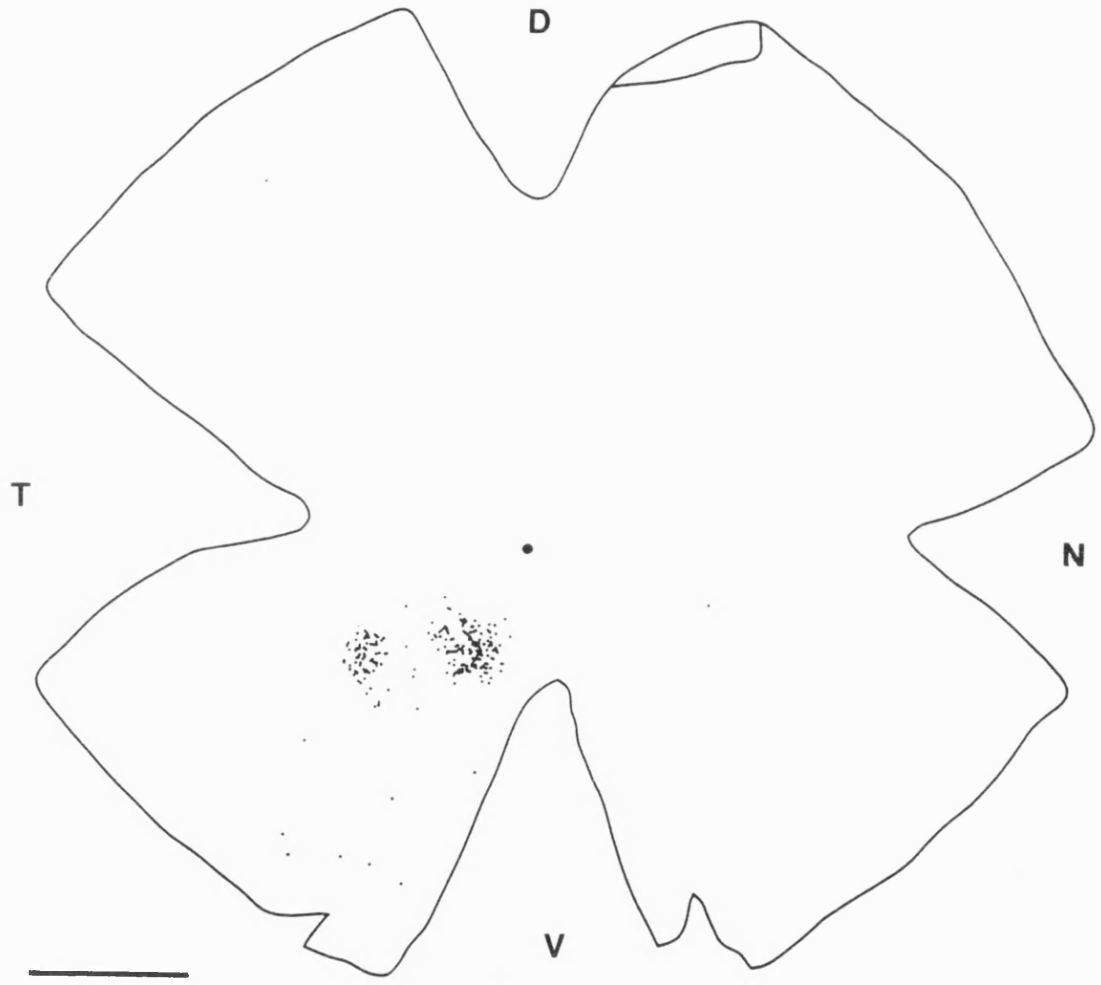

Figure 17B: Tracing of flat-mounted goldfish retina showing a double cluster of labelled RGC in the ventrotemporal quadrant following a single rostral injection of WGA-HRP at 239 days of regeneration. Bar represents 1mm.

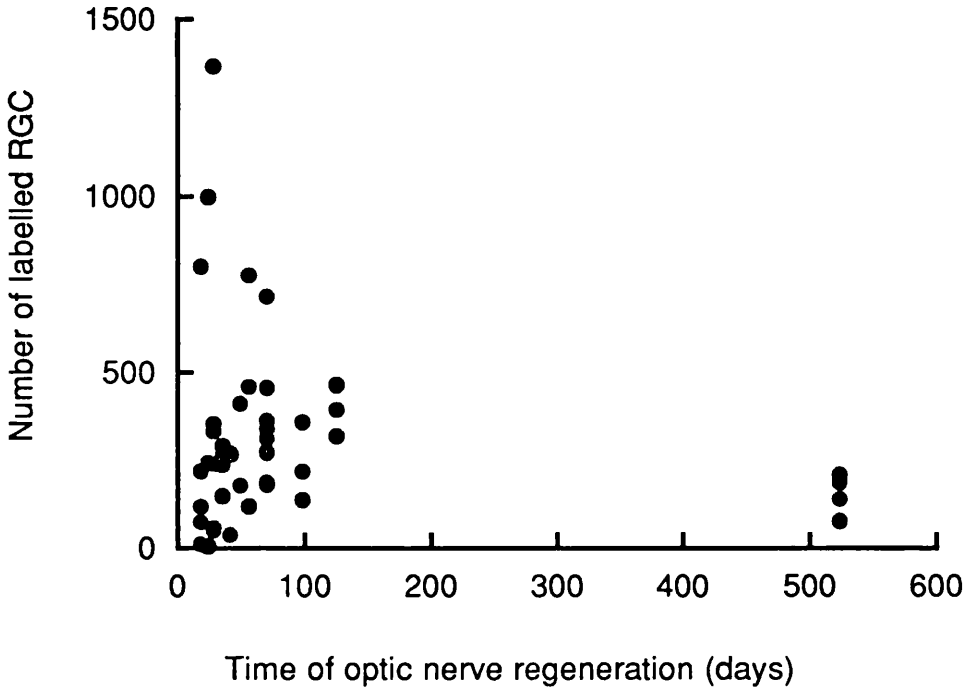

Figure 18: Graph showing number of labelled RGC at various time points of optic nerve regeneration under standard laboratory conditions.

4:3:4: Quantitation of retinal distribution

A statistical method for the quantitation of the clustering of labelled cells was needed. The area of retina over which filled cells were distributed was variable especially in mid- and late regeneration. The number of filled cells was also very variable, and the injection site was either rostral or caudal. A method based on the nearest neighbour distance was chosen (Clark and Evans, 1954) so that these variables could be taken into account and an area index calculated for each pattern of labelled cells. By planning the injection sites so that the clusters fell as centrally as possible in the appropriate quadrant, difficulties in the measurement of nearest neighbour distances were minimised. In brief, the area index is a measure of the area of the cluster compared to the area of the retina (for details see Methods). Therefore the more compact the cluster, the smaller the area index. The size of the area index was taken to be a measure of map refinement. WGA-HRP is taken up by the terminals only, a point discussed in section 3:4. Therefore a standard injection at different stages of regeneration will compare the arrangement of terminals at these stages. It has been observed that clustering increased as regeneration progressed (see above), though there was little increase after 70 days. The area index was used to quantify this observation.

The values of mean nearest neighbour distance, cluster radius, cluster area and linear index were individually plotted against time of regeneration, as was the area index and the logarithm of the area index (Table 2; Figures 19 and 20). Figure 19 shows various transformations of the data and indicates the progression of map refinement.

Values of the area index were obtained from 50 retinae after optic nerve cut and from 11 normal retinae. The area index was plotted on a logarithmic scale against the time from optic nerve cut (Figure 20B). Normal values were plotted as if at day 0. The graph shows that the area index fell with time until 70 days at which point it levelled out, the breakpoint being adopted here for regression analysis. There was no significant difference between the regression constants and coefficients for the lines obtained from rostral and caudal injections, therefore the values from all the injections

Caudal injections

No. of days of regeneration	No. of labelled RGC	mean nearest neighbour distance (µm)	cluster area (μm^2 x 10^{-6})	cluster radius (µm)	linear index	area index
24	998	58.4	2081	13.6	0.77	0.6
24	241	107	1874	11	0.73	0.54
24	5	682.3	1722	9.3	0.72	0.51
24	5	1334.5	3367	35.6	1.31	1.71
24	8	688.1	2196	15.1	0.81	0.66
28	57	165	1406	6.2	0.56	0.31
28	239	88.3	1540	7.5	0.58	0.34
28	50	295.8	2360	17.5	0.81	0.66
35	290	62.4	1198	4.5	0.51	0.26
35	262	94	1717	9.3	0.6	0.36
35	235	52.6	910	2.6	0.37	0.14
35	147	66.8	913	2.6	0.32	0.1
41	37	335.8	2305	16.7	0.81	0.65
41	271	49	911	911	0.34	0.12
42	265	49.8	914	914	0.36	0.13
49	177	64.7	971	971	0.36	0.13
49	411	44.5	1018	1018	0.41	0.17
56	777	28.3	891	891	0.37	0.13
56	118	73.4	899	899	0.38	0.14
56	459	36.3	878	878	0.35	0.12
70	310	25.8	513	513	0.18	0.03
70	361	24.9	534	534	0.21	0.04
70	181	53.9	818	818	0.31	0.1
70	275	36.4	682	682	0.25	0.06
98	358	42.7	913	913	0.31	0.1
98	217	12.2	202	202	0.07	0.01
98	136	20.5	269	269	0.1	0.01
524	139	42.3	563	563	0.24	0.06
524	76	47.3	465	465	0.13	0.02
524	185	37.2	571	571	0.17	0.03
524	205	19.8	320	320	0.1	0.01
524	208	17.8	290	290	0.1	0.01

Table 2: Results from WGA - HRP labelling during optic nerve regeneration under standard laboratory conditions. This page, caudal injections; next page, rostral injections.

Table 2 : continued

Rostral injections

No. of days of regeneration	No. of labelled RGC	mean nearest neighbour distance (μm)	cluster radius (μm)	cluster area (μm^2 x 10^{-6})	linear index	area index
18	802	67.1	2146	14.5	0.81	0.65
18	117	148	1807	10.3	0.74	0.54
18	10	577.1	2059	13.3	0.76	0.57
18	218	92.5	1541	7.5	0.58	0.34
18	73	236.4	2279	16.3	0.87	0.75
28	1366	44	1833	10.6	0.57	0.33
28	332	89.9	1848	10.7	0.65	0.42
28	353	72.1	1528	7.3	0.58	0.34
70	186	30.2	464	0.7	0.17	0.03
70	179	41.4	625	1.2	0.25	0.06
70	456	21.2	511	0.8	0.2	0.04
70	270	16.4	304	0.3	0.11	0.04
70	339	26.1	543	0.9	0.21	0.04
70	717	15	453	0.6	0.18	0.03
125	465	19.4	472	0.7	0.18	0.03
125	463	16.2	393	0.5	0.15	0.02
125	393	24.8	555	1	0.21	0.05
125	318	24.5	492	0.8	0.18	0.03

Figure 19A:

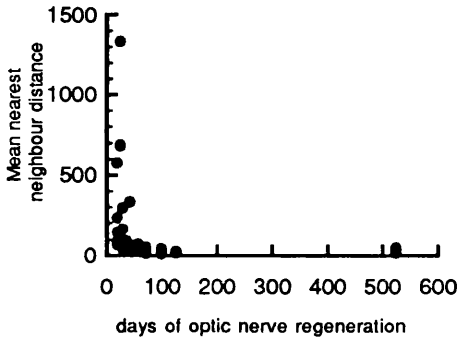

Figure 19B:

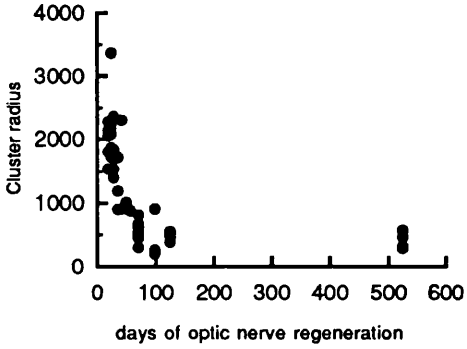

Figure 19C

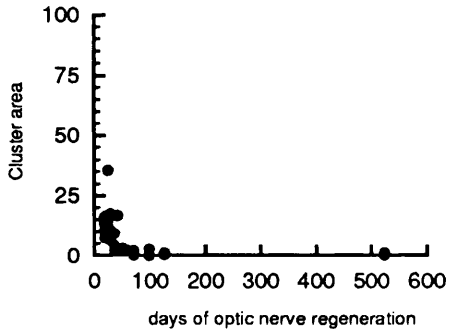

Figure 19D:

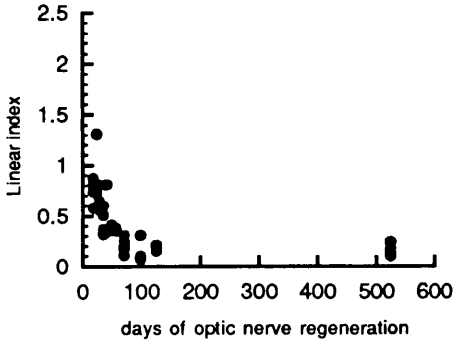

Figure 19A, B,C,D: Graphs showing resolution of retinotectal map during optic nerve regeneration as demonstrated by the following variables:
A : mean nearest neighbour distance.
B : cluster radius.
C : cluster area.
D : linear index.

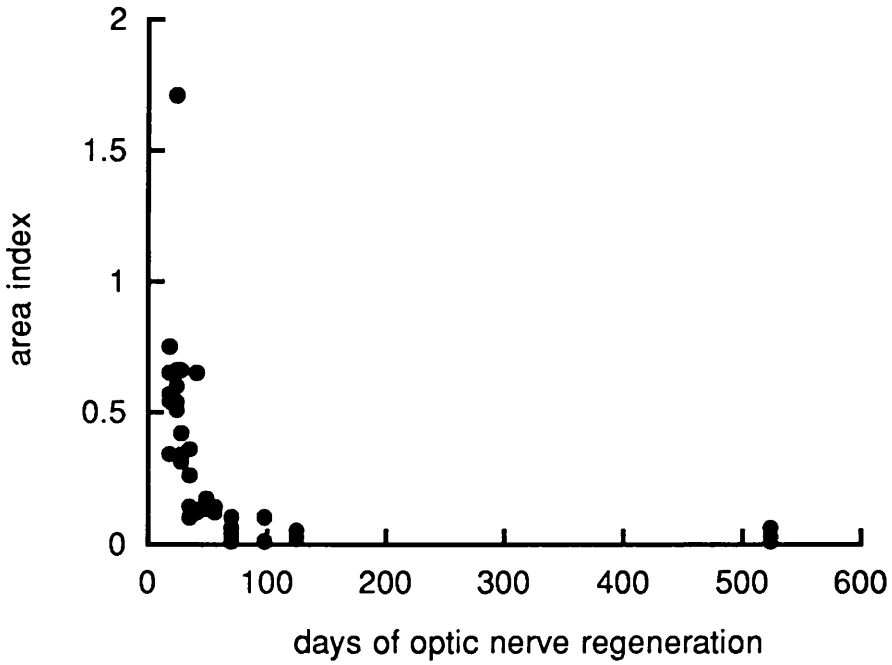

Figure 20A: Graph showing variation of area index with time of optic nerve regeneration under standard laboratory conditions. An exponential fall in area index is demonstrated.

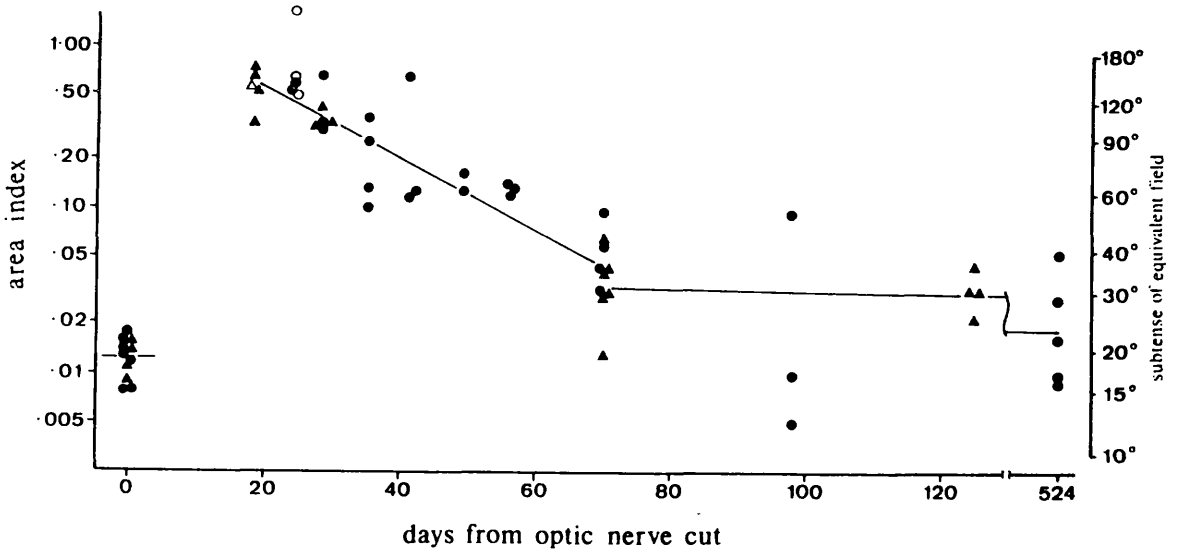

Figure 20B: Graph showing area indices plotted on a logarithmic scale against time from optic nerve cut. Triangles=rostral injections, circles=caudal injections, open circles=fewer than 30 labelled cells (omitted from regression line analysis). A short line through the normal area indices (at 0 days) shows mean value of log area index. Axis discontinuity between 125 and 524 days caused a shift in the regression line. Ordinate on the right shows calculated angular subtenses for circular receptive fields with equivalent retinal image areas.

are plotted together. After 70 days of regeneration there is a further decline in the area index value as shown by the regression line which is continuous and which approaches the x axis gradually. Although the area index values fell slightly for both rostral and caudal injections, the regression coefficients were not significant. The scatter of the points above and below the regression lines is not as equal as it may appear because of the logarithmic nature of the y axis. The straight line of the logarithmic plot represents an exponential fall in the area index. The area index halved every 13.75 days, on average, for the period 18-70 days. There was, however, a significant difference ($p=0.0008$, one-tailed Mann-Whitney U test) between the area indices of the late regenerates (days 70-524 of regeneration) and the normal animals, those of the latter being smaller (mean=0.0126 versus 0.0373).

4:4: A quantitative WGA-HRP study of the regenerating projection in continuous and stroboscopic light

WGA-HRP was injected iontophoretically into the caudomedial quadrant of the left tectum of 64 fish, and labelled cells were detected in the right retina. All fish had undergone optic nerve cut. In 48 animals the right lens was removed at the time of optic nerve cut in order to blur the retinal image. In the remaining 16 fish, the lens was left intact and these animals were kept in diurnal light. These animals were described in section 4:3:2. The precision of the regenerated map was assessed by the method described in Chapter 3. Comparisons were made between groups of animals, those which had been maintained in diurnal, continuous and stroboscopic light after optic nerve cut. Iontophoretic injections were made at 42-98 days after optic nerve cut.

After iontophoretic injection of WGA-HRP, labelled RGC were seen in the retinae of all 64 fish. In general the labelled cells were found in the ventronasal retinal quadrant as the injection was in the caudomedial aspect of the tectum. The intensity of the labelling and the morphology of the labelled cells did not differ between the experimental groups.

Inspection of retinae labelled at 42-49 days after optic nerve cut, in 6 fish from each group, showed that there was no difference between groups in the pattern of labelled cells. At 56 days there was visible clustering of labelled cells within the ventronasal retinal quadrant in all animals except those which had been maintained in stroboscopic light and which had had their lenses ablated. In these animals, cells were in the appropriate quadrant but no clustering was detectable.

At 70 days, comparison of fish kept in diurnal light with and without lens ablation showed no difference in the distribution of labelled cells, indicating that the presence of the lens is not necessary for regeneration nor for the degree of clustering seen at this stage of regeneration (Figures 21A and B).

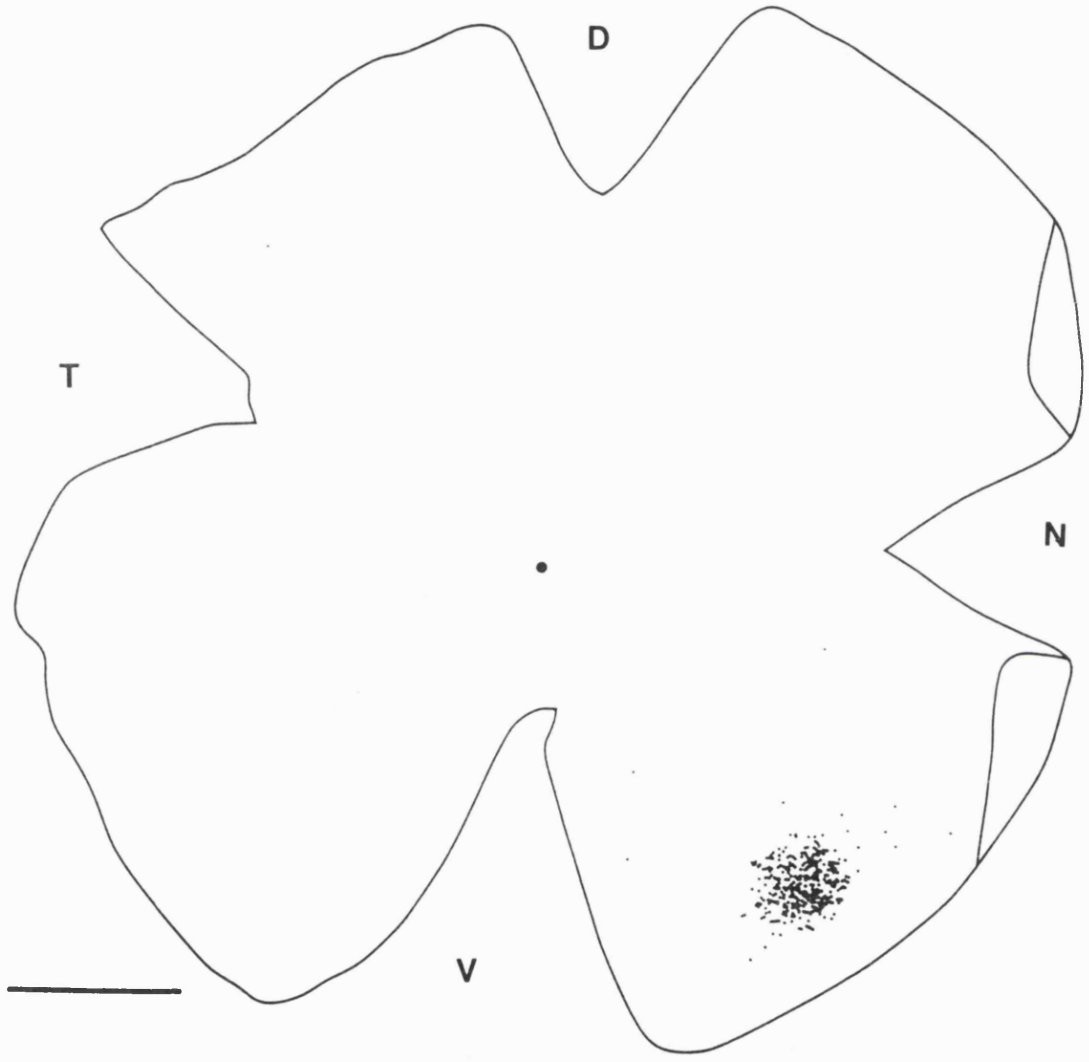

Figure 21A: Tracing of flat-mounted goldfish retina showing labelled RGC at 70 days of regeneration following WGA-HRP tectal injection. Fish, with lens intact, was kept under standard laboratory conditions. Bar represents 1mm.

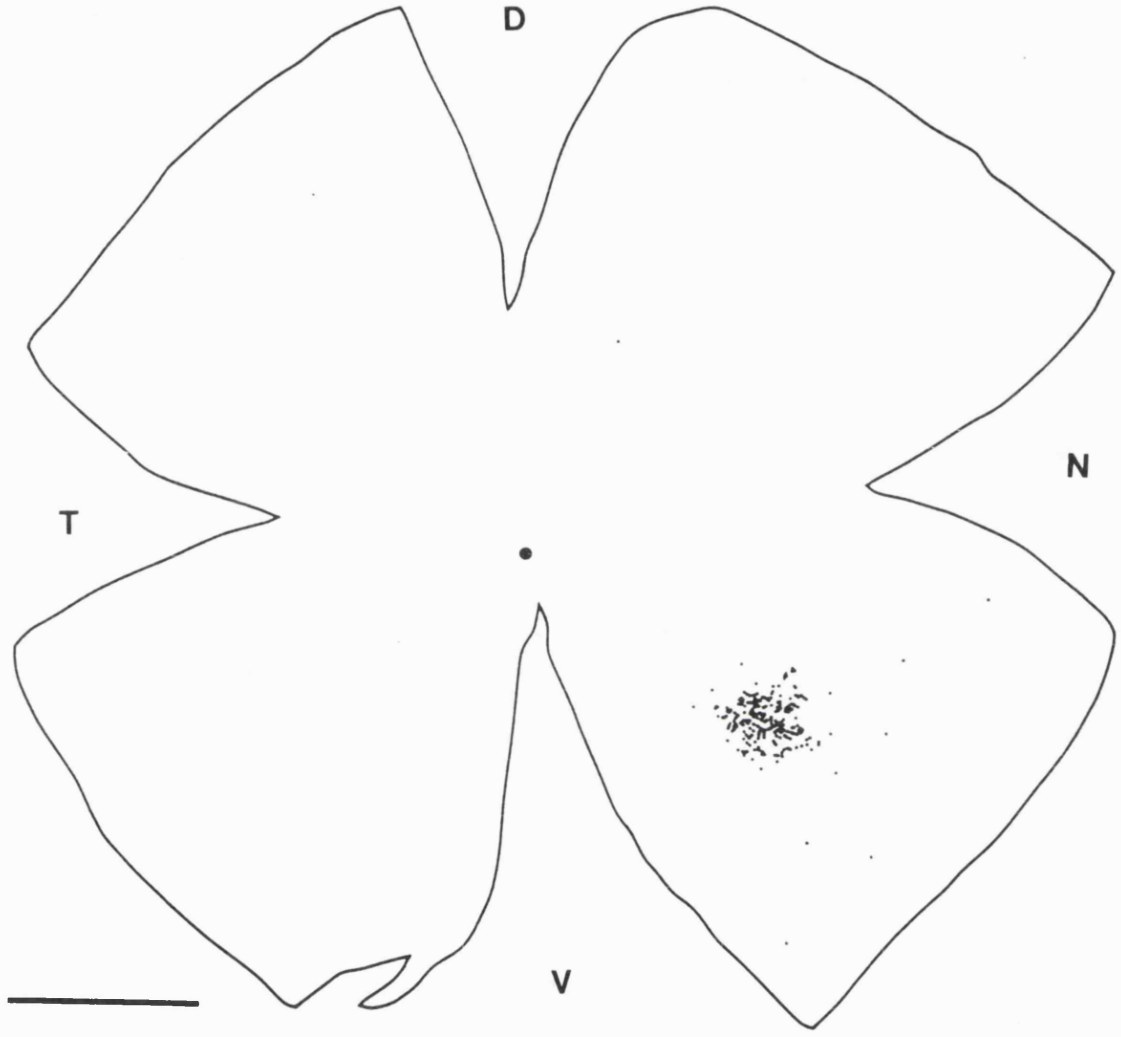

Figure 21B: Tracing of flat-mounted goldfish retina showing labelled RGC at 70 days of regeneration following WGA-HRP tectal injection. Fish, with lens removed, was kept under conditions of diurnal light. The presence of a lens is not necessary for map refinement to occur. Bar represents 1mm.

At 98 days, in the group of fish with the lens ablated which was kept in strobe light, there was still no clustering of labelled cells (Figure 22). The cells were widely scattered throughout the ventronasal quadrant. This was in contrast to the other groups where there were clear clusters. The distribution of filled cells in these strobe animals resembled the pattern seen at 35 days of regeneration in the group kept in standard laboratory conditions.

Examples of retinae from 91 days of regeneration under conditions of diurnal, constant and stroboscopic light are shown in Figures 23 A, B and C. In all these examples the lenses had been removed at the time of optic nerve cut.

Quantitative results were obtained for 26 fish successfully injected between 70 and 98 days after optic nerve cut. Only 4 of the fish kept in constant light survived to be analysed at this stage of regeneration. As before, the nearest neighbour distance was measured for each labelled RGC, and a value for the area index was obtained for each retina.

The mean nearest neighbour distance was greatest in the strobe light/lens ablated group (Figure 24). Highly significant differences were found, using one-tailed Mann-Whitney U tests, between the strobe light/lens ablated group and each of the other groups: constant light/lens ablated ($p=0.003$), diurnal light/lens ablated ($p=0.0003$) and diurnal light/lens intact ($p=0.0003$). There was no significant difference between the mean nearest neighbour distance values for the other three non-strobe groups (Mann-Whitney U test). The mean value for the strobe light/lens ablated group was 88.6μm, that for the diurnal light/lens ablated group was 40.2μm and that for the constant light/lens ablated group was 39.6μm.

The number of labelled cells showed significant variation among groups (Kruskal-Wallis test; $p<0.02$). There was a greater number of labelled cells in the constant light/lens ablated group compared to the other three groups (Figure 24). Two-tailed Mann-Whitney U tests showed that constant light/lens ablated retinae contained more labelled RGC than the

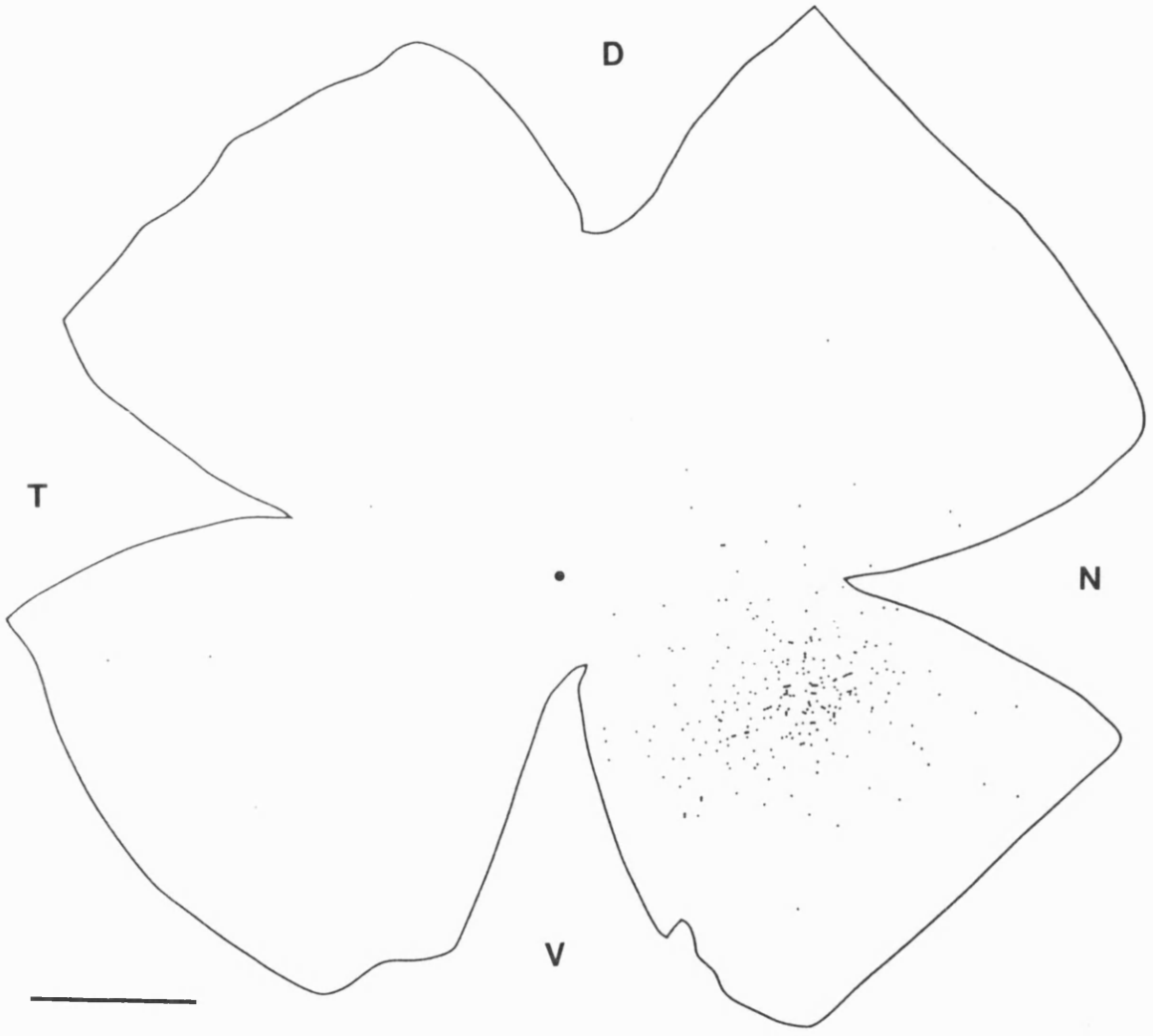

Figure 22: Tracing of flat-mounted goldfish retina showing labelled RGC at 98 days of regeneration. Fish, with lens removed, kept under stroboscopic light. Labelled cells are in the retinotopic quadrant, but there is no clustering. Bar represents 1mm.

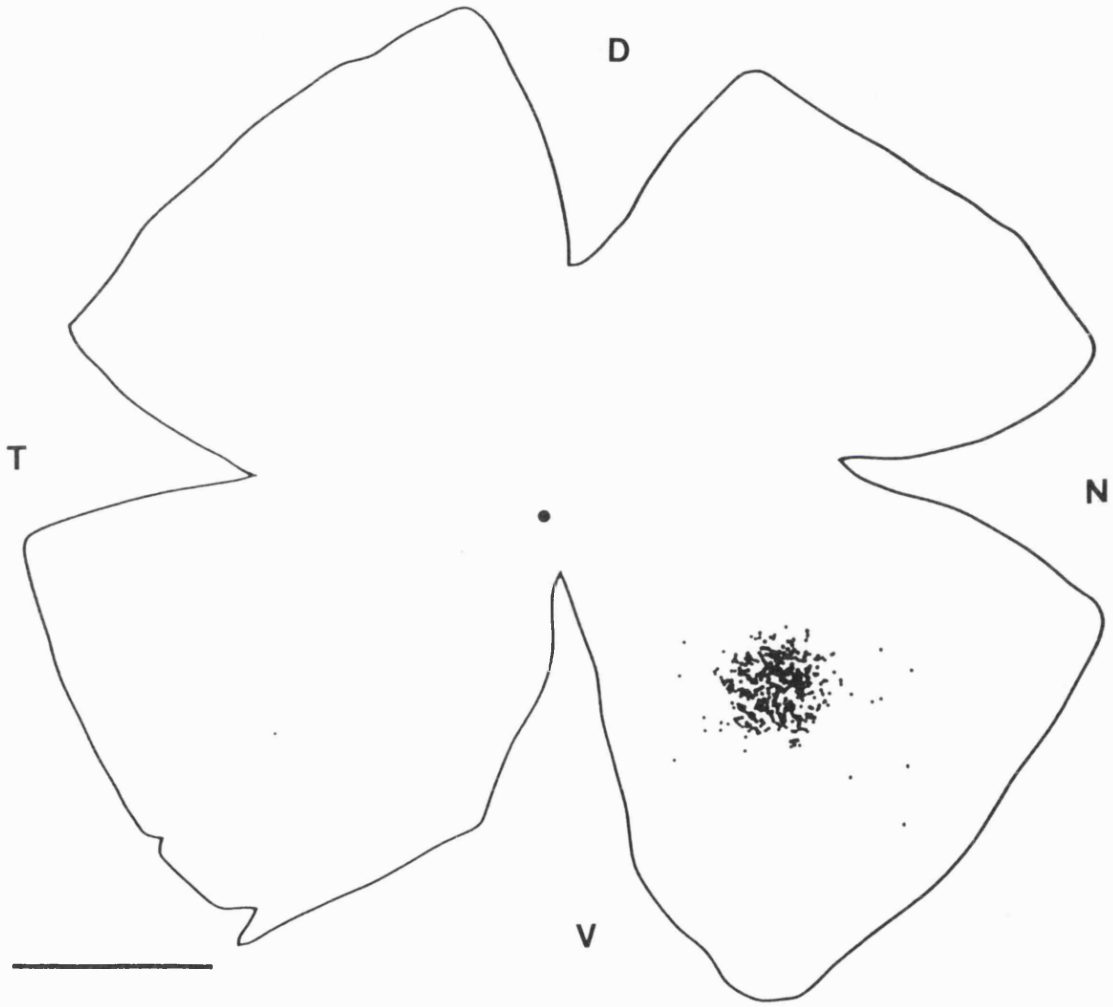

Figure 23A: Tracing of flat-mounted goldfish retina showing cluster of labelled RGC after 91 days of regeneration under diurnal light (lens out). Bar represents 1mm.

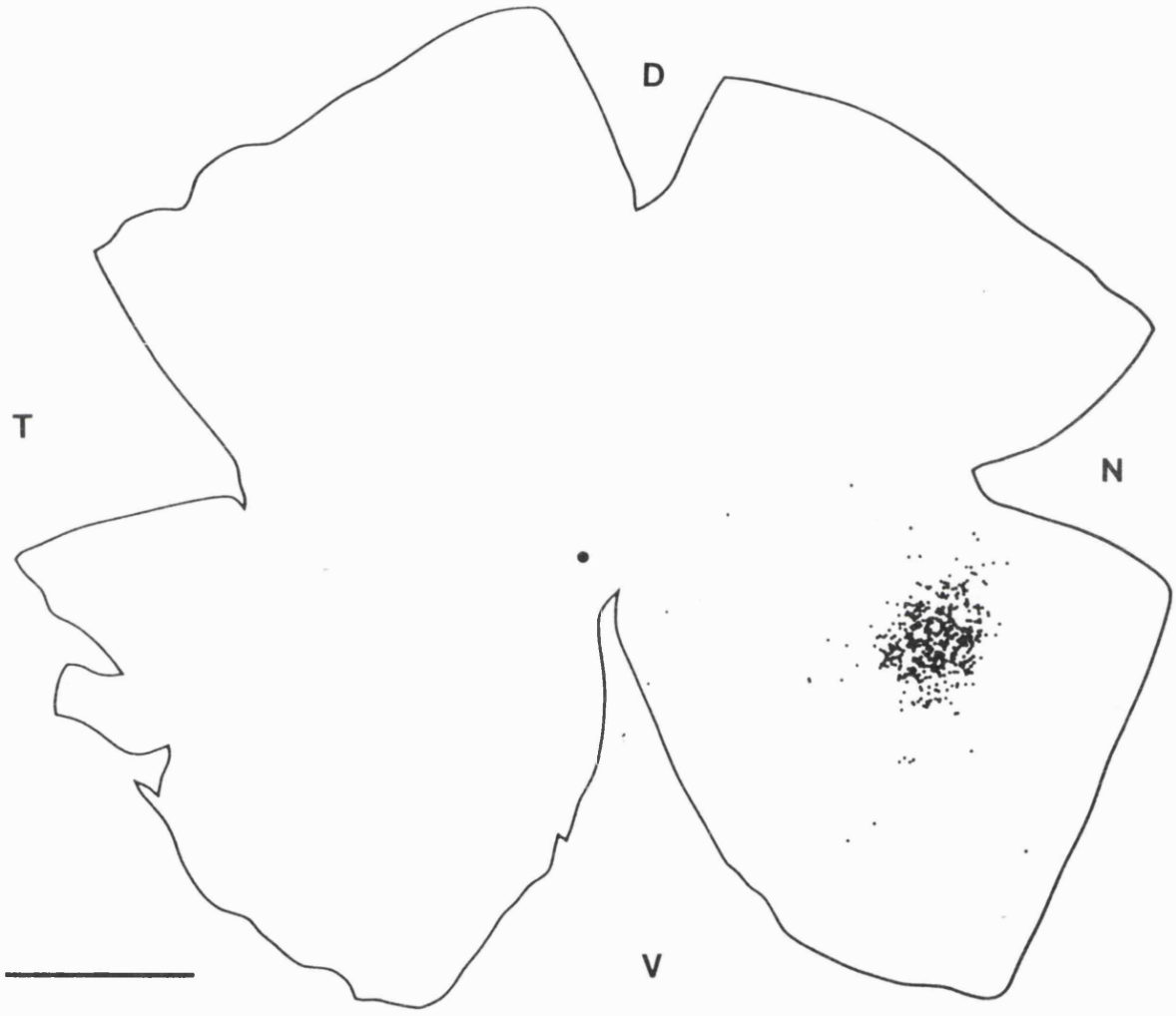

Figure 23B: Tracing of flat-mounted goldfish retina showing cluster of labelled RGC after 91 days of regeneration under constant light (lens out). Bar represents 1mm.

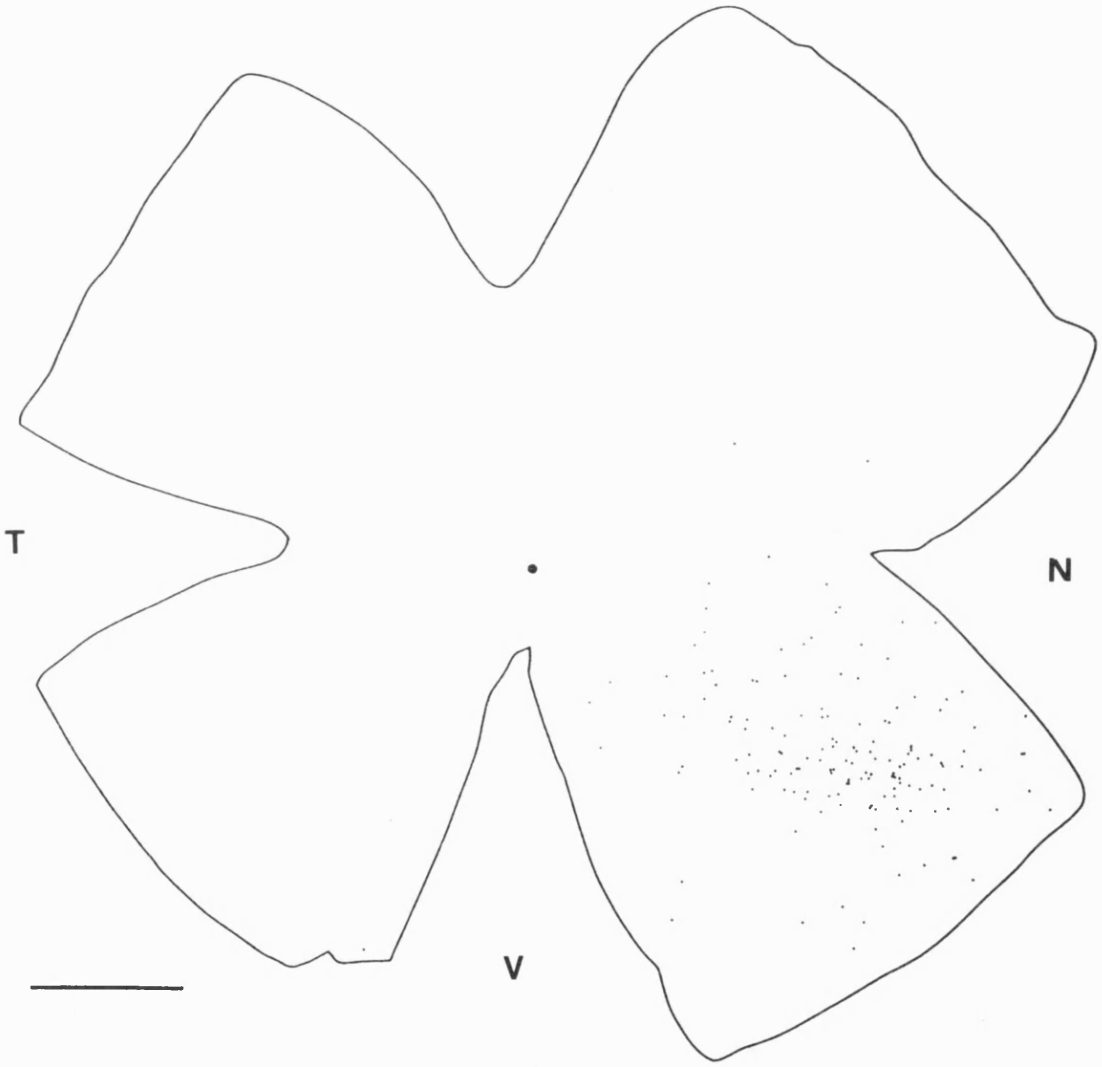

Figure 23C: Tracing of flat-mounted goldfish retina showing lack of clustering of labelled RGC after 91 days of regeneration under stroboscopic light (lens out). The lack of cluster formation is in contrast to Figure 23A. Bar represents 1mm.

other three groups: stroboscopic light/lens ablated ($p=0.006$), diurnal light/lens ablated ($p=0.008$) and diurnal light/lens intact ($p=0.042$).

The area index also showed significant variation between groups (Kruskal-Wallis test $p<0.002$; Figure 24). The area index was greater in the strobe light/lens ablated group compared to the other groups. Its closest rival was the constant light/lens ablated group (one-tailed U test $p=0.006$), while the greatest difference was between strobe light/lens ablated and the diurnal light groups ($p=0.0003$). There was no significant difference among the non-strobe light groups.

The retinae were also assessed by frequency distribution of the nearest neighbour distance values, and these distributions were compared between groups. To correct for differences in cell number and retinal area, all nearest neighbour distances were divided by the expected mean for a random distribution with the same average density as that particular retina. These normalised distances were pooled by experimental group, transformed logarithmically to increase resolution in the body of the distribution, and allocated to histogram bins (Figure 25). The frequency distribution histograms provide another way of analysing the scatter of labelled RGC within an experimental group of retinae, and this scatter can be assessed visually (Figure 25). The frequency distribution histograms are similar to the area indices in that variations in labelled cell number and retinal area are taken into account. They differ in that the former give a visual spread of nearest neighbour distances while in the latter a comparison is made of the observed distribution and an expected random distribution and a single number is generated.

Using data from regeneration under standard conditions, early regenerates were compared to the strobe light/lens ablated group to confirm the impression that there was a similar degree of clustering. Late diurnal light/lens intact and late diurnal light/lens ablated retinae yielded distributions which peaked around 0.1: that is, the commonest distances found were about one-tenth of those which would be expected if the cells had been randomly distributed over the entire retina. Late constant

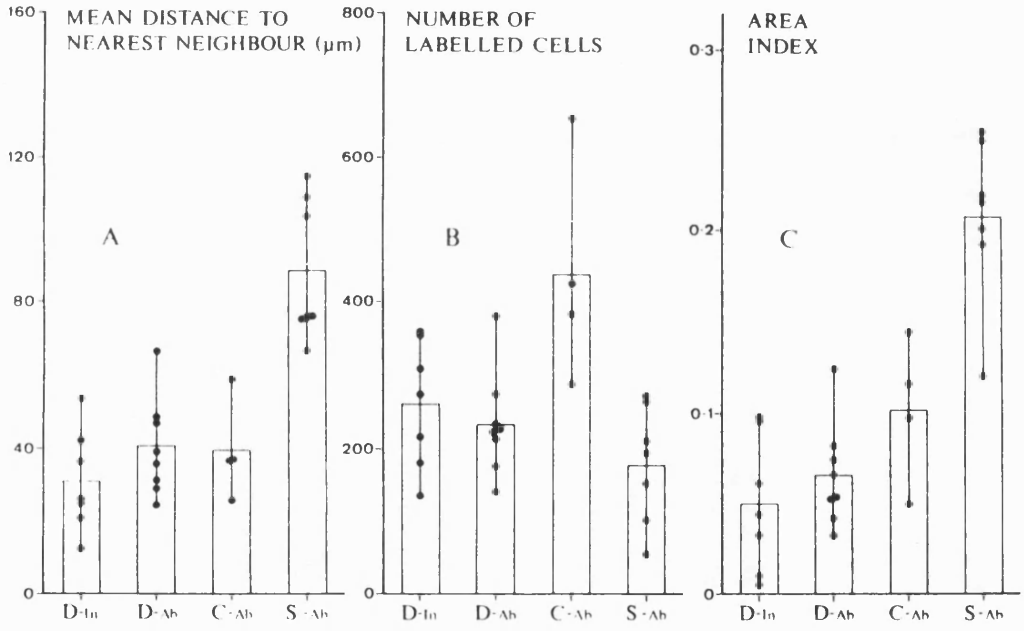

Figure 24A: Mean nearest neighbour distances of each labelled RGC in fish injected with WGA-HRP in the caudal tectum at days 70-98 of regeneration.
Figure 24B: Mean number of labelled RGC found in these retinae.
Figure 24C: Mean area indices for these retinae.
In all the histograms, fish in groups exposed to diurnal (D), constant (C) and stroboscopic (S) light are compared. Filled circles=individual retinae, histogram bars=group means. Lenses ablated except those in group D-In.

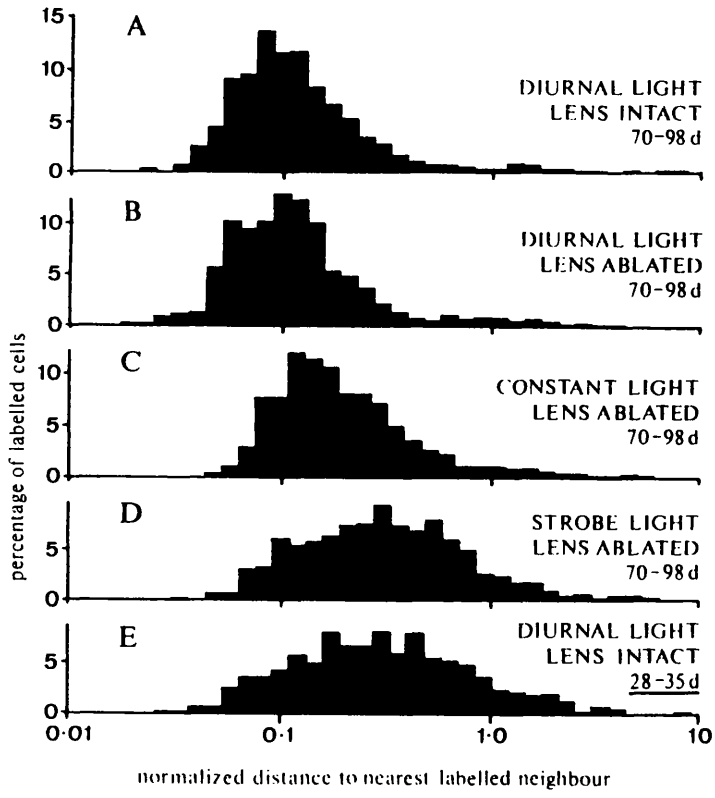

Figure 25: Frequency distributions of distance to nearest labelled neighbour for the four experimental groups (A-D) at days 70-98 days of regeneration, and for a group of lens-intact fish kept in diurnal light for 28-35 days of regeneration.

light/lens ablated retinae also yielded a distribution with a definite peak, though at a slightly greater distance. Late strobe light/lens ablated retinae yielded a different pattern of frequency distribution, with a poorly defined peak at about three times the usual distance. Also the distribution was much broader and with a different skew, compared to that obtained for late diurnal light animals. A similar distribution to the late strobe light/lens ablated fish was obtained from seven fish with lenses intact and kept in diurnal light labelled by caudal injections at 28-35 days of regeneration. Therefore, the degree of map precision seen in fish kept in strobe light after optic nerve cut and lens removal resembles, in its distribution of nearest neighbour distances as well as their mean value, that seen at much earlier stages of regeneration when the fish are kept in standard conditions.

4:5: The effect of different lighting conditions on cell number

Normal fish and fish with regenerating projections were kept in constant and stroboscopic light for 69-97 days. The optic nerve was then cut and packed with HRP. Two days later the retinae were developed to reveal labelled RGC. Sample cell counts were made in the ventronasal quadrants of these retinae, corresponding to the area filled after caudomedial tectal injections. Numbers of RGC in the whole retina were calculated using the total areas of each retina.

Table 3: comparison of cell numbers in retinae labelled with HRP in fish exposed to continuous or stroboscopic light, with and without optic nerve cut.

lighting conditions	normal vs regen	time	cell counts in 4 sample areas in the ventronasal quadrant	no. in whole retina
Strobe	Normal	70days	800+805+916+811=3332	75040
Strobe	Normal	96days	669+664+631+574=2538	67806
Strobe	Normal	84days	762+749+964+877=3352	83909
Strobe	Normal	69days	896+859+762+920=3437	79099
Strobe	Regen	97days	486+498+598+568=2150	76480
Strobe	Regen	69days	708+654+558+603=2523	77356
Strobe	Regen	70days	488+662+547+592=2289	57811
Strobe	Regen	84days	718+841+913+746=3218	68671
Continuous	Normal	69days	561+581+672+522=2336	52109
Continuous	Normal	97days	571+515+613+696=2395	63050
Continuous	Regen	97days	744+789+698+824=3055	66841
Continuous	Regen	70days	681+768+636+685=2770	53648
Strobe	Normal	83days	filling too variable	
Strobe	Normal	96days	poorly filled	

Only small differences in ganglion cell number were found between normal and regenerated retinae exposed to the same lighting conditions. However, eight retinae exposed to the random frequency strobe light used in the experiments on map refinement contained significantly more surviving ganglion cells than four exposed to constant light for similar periods of time ($p<0.01$, Mann Whitney U test*). Thus strobe light seems to be associated with less cell death than constant light, while it causes a greater effect on map precision.

* Carried out on numbers of RGC in the whole retina

Chapter 5: DISCUSSION

5:1: Anterograde HRP tracing of the retinotectal projection in the normal goldfish

Anterograde neuronal tracing with horseradish peroxidase (HRP) from the optic nerve or from the tectum labelled cohorts of axons which could be traced from fascicle to termination site. Optic nerve lesions resulted in larger numbers of labelled axons most of which terminated in rings on the surface of the tectum, while tectal lesions usually labelled arcs of tectal terminals at most. However, the two types of lesion yielded the same basic distribution of axons. While the tectal stab lesions often also resulted in the labelling of axons outside the retinotectal projection, the pattern seen after optic nerve lesions allowed the deduction that the projection did not terminate more than 200μm below the tectal surface. For this reason the extra labelled tectal axons could be ignored when labelled retinotectal axons were being drawn. Thus the tracing method was internally consistent and identified retinotectal axons.

As described in the Results section, in most cases the axons were seen to terminate at a distance from the fascicle in which they ran and this relationship was described graphically (Figure 8). Axons from rostral fascicles terminated at a considerable distance caudal to the fascicle, and that distance was greater the more rostral the fascicle. The rostral axons did not arise from the fascicles at all but directly from the rostral tectal margin, therefore they were not included in the graph. The graph (Figure 8) describes a constant relationship between the distance from fascicle to termination site of a group of axons near the rostrocaudal tectal axis and the distance from the fascicle to the caudal tectal rim. It is known that the goldfish tectum grows throughout life by the addition of cells around the caudal tectal margin (Meyer, 1978). Therefore the distance from the fascicle to the termination site can be inferred to be related to tectal growth. These results show that it is unusual for a retinal ganglion cell (RGC) axon that is destined to end on the caudal half-tectum to terminate directly beneath its fascicle. At a point near the caudal tectum the axons did in fact terminate

directly beneath their fascicle, and caudal to this the axons turned rostrally before terminating.

The findings were that the fascicles contained approximately half to two-thirds of the axons which formed the retinotopic map. The rest were seen to arise from the rostral tectal margin, to course caudally and to supply approximately the rostral half of the tectal surface. This combination of patterns of tectal innervation was well demonstrated when a localised lesion was made in the rostral half tectum (Figure 5B); the fascicle was seen to pass caudally and medially with axons leaving it along its trajectory to terminate, while another group of axons was seen arising from the injection site and passing directly backwards before terminating in the rostral half tectum.

It was surprising to find that the fascicles did not contain all the axons destined to terminate on the tectal surface, as had been assumed in the past (Attardi and Sperry, 1963; Horder, 1974; Meyer, 1980). Attardi and Sperry described fibres running in the superficial parallel layer from which they exited by dipping centrally into the underlying plexiform layer. They also described the sequential exit of the fibres from the parallel layer such that those axons arising from the retinal periphery left before those arising from central retina. While the results presented in this thesis provide support for the sequential peripheral-to-central exit of axons from the fascicles, anterograde and retrograde HRP studies show that the axons in the fascicles account for only about half of the retinotectal projection. It had previously been implicit that fibres terminate directly beneath their fascicle and that fascicles contain fibres which will terminate all over the tectum. It is perhaps not surprising that this is not the case since, although the fascicles are curved, they are neither concentric with the retinal map nor with each other. In the drawings which accompanied Attardi and Sperry's (1963) written findings, the trajectories of the fascicles are misrepresented since they appear to be branches of a peripheral trunk. However, photographs of wholemount preparations show that fascicles are a set of independent trunks which are bunched together rostrally and diverge caudally (Easter, 1985). At this stage in the argument this point is not important, but ultimately

any mechanism will have to account for the arrangement of the axons.

Interestingly, Meyer (1980) deduced, using autoradiographic methods, that the normal projection was not fully retinotopic on the tectal surface. He made point retinal lesions near the optic nerve head, thereby denervating a retinal sector, and observed that when the lesion was in the temporal retina there was always some light labelling not only in the *stratum opticum* (SO), as would be expected, but also in the *stratum fibrosum et griseum superficiale* (SFGS). When the lesion was on the nasal side of the optic nerve head, the edges of the denervated tectal sector were sharp with no significant labelling. These findings can now be explained. In the rostral tectum (temporal retinal lesion), according to the model arising from our studies, there will be a greater number of axons of passage in the rostral tectum especially in the SFGS. Theoretically, this layer should contain fibres running caudally both from the rostral tectal edge and from the fascicles. In the caudal tectum, there will be fewer axons of passage to blur the retinal sector seen in the autoradiographs.

As well as this "boundary" which has now been described between the rostral and caudal halves of the tecta, these studies also clearly showed that there is a boundary between the medial and lateral tectal halves such that the medial and lateral halves of the retinotectal projection are mirror images of each other. The optic nerve lesions showed this mirror image pattern effectively (Figures 2A and 2B), while the more localised tectal lesions showed the cut-off at the boundary with very few fibres crossing over (Figure 3).

Although the technique of neuronal tracing is static, certain inferences can be made about the dynamics of the system from knowledge of the patterns of retinal and tectal growth. As described in the Introduction, the goldfish retina grows by the addition of cells around its margin (Johns, 1977), and the tectum grows by the apposition of new cells along the dorsal, ventral and caudal rims (Meyer, 1978): therefore the modes of growth of the retina and tectum differ. If retinotopy is to be maintained, a systematic rearrangement of retinotectal synapses must occur: the pattern described

above is consistent with this. As discussed above, it is possible that the fibres which turned rostrally at the caudal tectum did so because they were unable to form synapses on the newly generated tectal cells. The negative intercept on the 'y' axis of the graph is a reflection of this rostral turning. The intercept on the 'x' axis reflected the point towards the caudal tectal pole where axons terminated directly beneath their fascicle. It is hypothesised that the caudal elongation of fibres from their fascicle to their termination site is a reflection of caudal migration of the terminal arborisations, that is that the lines of the axons are a historical record of their migratory routes. From HRP retrograde tracer studies it is known that the most peripheral fascicle which passes around the medial (or lateral) and then the caudal tectal margin is the youngest, since it contains axons with their origins in the peripheral (youngest) retina (see below). It is likely that each fascicle marks the position of the medial (or lateral) and caudal edges of the tectum at the time when that fascicle was developing, and that as further cells were added on to the caudal tectum the fascicle stayed where it was, thus appearing to be relatively more rostral. Fibres destined to terminate in the rostral tectum would not be spread out in the same way, because no tectal growth has been documented in this region. Therefore the 'fascicle equivalents' in this area of the tectum are bunched up along the rostral border. As more new RGC are added to the retina, there may be increasing "pressure" for space at the front of the tectum and axon terminals would move caudally in response. This in turn would create pressure on axon terminals further back, and those with their axons in the fascicles would have to migrate caudally with their fascicle of origin effectively staying in the same place. The gradient of the graph was close to 0.5 which could reflect the fact that, close to the mediolateral midline, axon terminals must migrate caudally in order to allow approximately half the number of new RGC to terminate on the rostral tectum.

Disparate modes of retinal and tectal growth were first demonstrated in the frog *Xenopus* (results repeated and summarised in Gaze, Keating, Ostberg and Chung, 1979) and it was proposed that synaptic plasticity must be a feature of this visual system. The normal goldfish retinotectal projection is therefore a valuable experimental model since it demonstrates features

which are widespread in nature, and it also allows easy examination of the axons' tectal paths.

The above discussion applies particularly to mapping along the rostrocaudal tectal axis. What may be the influences on axon migration away from this axis? The relative crowding of fascicles at the lateral and medial tectal borders means that it was more difficult to identify and trace individual axons in these areas. The new tectal area generated by each increment of growth will be less at the lateral and medial tectal borders because of the crescentic shape of the newly added tectum with the widest point of the crescent at the caudal tectal pole. At the lateral and medial tectal borders it is presumed that the pressures for space for terminal arborisations will be different from those at the caudal pole. There can be no caudal migration at those borders because a directly caudal trajectory would take the axon off the tectum. At this point the pressure for space is presumed to have caudal and centripetal vectors, therefore the axons are assumed to turn caudally and centripetally to find space to terminate. More rostrally along this tectal border no new tectum is being generated, therefore the pressure for space will in theory be greater; those axons which have already formed synapses will in theory have greater centripetal vectors than those further back on the tectum, which will have progressively greater caudal vectors. However, the rounded shape of the tectum dictates that the vectors should, further back on the lateral and medial borders, again become more centripetal. The terms "pressure for space" and "vectors" have been used as part of speculative inferences made from the above observations and as yet have no biological basis. It is known that, when part of the optic tectum is removed, retinotectal axons behave as though there is now increased pressure for space (Yoon, 1976; Cook, 1979). However, in this experimental system which must involve regeneration as well as development, the projections were characterised by electrophysiology and the routes taken by the axons were not studied.

What do the observations made from anterograde labelling lead us to infer about the mechanisms underlying the setting up of the retinotectal system during development? It must be stressed that these are inferences

as this method is purely descriptive of an unperturbed system. It is inferred that the pattern of RGC fibres seen on the tectum is established gradually as the retina and tectum continue to grow throughout adult life. Therefore models which seek to explain the formation of neuronal maps by extensive rearrangements of axon terminals so that they terminate next to their retinal neighbour are unnecessary. A model has been proposed by Willshaw and von der Malsburg (1976) which invokes such a mechanism, but in regeneration rather than in development. Their model is based on the fact that neighbouring RGC have correlated firing patterns (Arnett, 1978). Such correlations could form a means of signalling so that an axon terminal would recognise its retinal neighbour on the tectum and thus form a synapse next to it. Although it is feasible that such a mechanism could exist for the teleologically important possibility of damage to the optic nerve, the full capacity of such a system would be superfluous in the developing animal where there are strict limits to the tectal space available for synapse formation, and where half of the possible space has been made less available by the orientation of the brachia. The self-ordering process may have to act only within a quadrant of the cohort of recently arrived RGC axons (see below).

It is possible that the decision as to which brachium to take is of great importance in the developing animal since it is clear from the anterograde studies that the medial brachium supplies the dorsal tectum and the lateral brachium the ventral tectum, with very little overlap across the rostrocaudal tectal axis. There is evidence from compound eye studies for markers within the optic tracts (Straznicky, Gaze and Horder, 1979); all the fibres from the compound eye appeared to choose the "appropriate" brachium leaving the other brachium empty. It is also known that the gradual accretion of RGC axons within the optic nerve gives them an age related order (Bunt, 1982) so that the "active decisions" about the route to the tectum may be diminished. However, it is known that considerable rearrangement of RGC axons takes place at the level of the brachia (Scholes, 1979) which suggests that there may be specific choice points.

The chemospecificity theory of Sperry (1963) would appear to be

uneconomical. If retinal and tectal cells had a series of chemical labels allowing them to recognise and to synapse at the "correct" site, a large and ever increasing number of these labels would be necessary because of the continuing growth of the system throughout adult life. There is now good evidence that shifting of connections does occur. If the chemospecificity theory were extended to take account of this new evidence, it would have to propose that the chemospecific labels are in a constant state of flux to keep up with the changes. Attempts have been made to provide versions of the chemospecificity theory that might not require changing labels (Meyer and Sperry, 1976).

If the axons make a "decision" at the level of the brachia, a similar process may occur at the point where the axons reach the tectal margin. Axons either pass onto the rostral tectal margin or they pass along the medial (or lateral) tectal border. While the trajectory of those that take the latter course is not too dissimilar from the trajectory taken by the fibres in the brachium, those axons which pass onto the rostral margin must take an abrupt turn. This is circumstantial evidence for an active "decision making" process.

It would be interesting to see whether growth cone morphology differs in these regions. Such differences have been described in motoneurons in the lumbosacral region of the chick embryo (Tosney and Landmesser, 1985). Small groups of axons in the chick embryo spinal cord, for example, were labelled with HRP. The morphology of the growth cones was compared in "decision" regions, such as the origin of muscle nerves from the main nerve trunk, and in the "non-decision" regions, such as the spinal nerve and nerve trunk pathways. Motoneuron growth cones were larger, more lamellipodial and had more complex trajectories in the decision regions. In the cichlid fish, Maggs and Scholes (1986) correlated the known rearrangement of the RGC axons at the level of the optic tract with the glial environment which in turn was further characterised biochemically. Within the optic nerve, the glia expressed fish vimentin, a member of the intermediate filament family of cytoskeletal proteins, and did not express glial fibrillary acidic protein (GFAP). In contrast, glia elsewhere in the brain

and in particular in the optic tract expressed GFAP and did not express fish vimentin. Electron microscopy of the optic nerve and tract provided evidence for the swift passage of RGC axons in the optic nerve and the slower trajectory of these axons in the optic tract where branching occurred. There is evidence in the mammalian optic pathway also which correlates the morphology of the growth cones with apparent "decision" points (Bovolenta and Mason, 1987).

If these two "decisions" are taken, at the branching of the optic tract and at the tectal edge, and if each cohort of new cells is small, giving a single ring of axon terminals, say, on the tectal surface, then further resolution would involve only an ordering of the fibres within the quadrant, approximately, of the tectal circumference. This ordering could arise from the order within the fibres themselves, from cytochemical markers or from detection of neighbouring neuronal activity: or from a combination of these mechanisms with or without mechanisms so far undiscovered.

There is no free rostral tectal space as there is no neurogenesis here (Meyer, 1978). Therefore the requirements for the fibres terminating here may be different from those which pass along the medial tectal border. However, neither are there new tectal cells to accommodate those fibres destined to synapse along the medial border soon after they leave the brachia. If it is the case that new RGC axons are not able to synapse on new tectal cells (hence their rostral trajectory at the caudal pole), then this difference between rostral tectum where there are no new tectal cells and the caudal tectum where there are new tectal cells but they are not available for termination, may not be important from the point of view of a mechanism underlying the formation of the map. However, there must be a mechanism, possibly a process of continuous exploration, by which the newly generated tectum can be detected as otherwise there would be no caudal shift. The maturity of the tectal dendrites could be a factor in such a mechanism, since once mature tectal dendrites were available as termination sites RGC axons could move into the new territory. Subsequent evidence for continuous exploration is discussed in Chapter 6.

Is there another possible explanation for the order of the fascicles? Could the axons shift between fascicles? This is unlikely because the retina is growing continually so that the axons would have to withdraw frequently from one fascicle and regenerate into a new one. It would be more economical to leave the axon in place and to extend the terminal arbor to its retinotopic site. These anterograde studies are entirely consistent with a shift of the terminal arbors only, and shifting of the axons from fascicle to fascicle does not have to be invoked.

I have drawn upon the observed pattern of the retinotectal projection, a knowledge of the modes of growth of the retina and tectum and my observations on the age-related arrangement of the fascicles from retrograde tracer studies. I have concluded that the pattern of axons on the tectum is consistent with a systematic shifting of axon terminal arborisations so that the retinotopic nature of the map is preserved. Synaptic plasticity is thus a feature of normal growth and development in the optic system of the goldfish.

5:1:1: Concurrent work from other laboratories

At the time when the work described above was being carried out (1982-1983), Easter and Stuermer were undertaking very similar experiments. They too labelled RGC axons with HRP and studied the resulting anterograde and retrograde labelling patterns (Stuermer and Easter, 1984a; Easter and Stuermer, 1984; Stuermer, 1984). They applied a crystal of HRP to a lesion made in the retina near the optic nerve head or in the optic nerve itself. They too observed the axons in fascicles in the SO. Axons were seen to leave each fascicle at some point and to dip into a deeper tectal layer where they coursed for a variable distance before terminating. The distances of these extra-fascicular portions of the axons were related to the location of their fascicle of origin, such that these portions were longer, the more rostral the fascicle. Peripheral axons had scarcely any extrafascicular segment. Stuermer (1984) described fibres arising from the most peripheral fascicle turning rostrally near the caudal tectal pole. Stuermer and Easter did not describe fibres destined to synapse at the rostral pole as turning to run along the rostral tectal margin. It is implicit in

the discussion of this work that fascicles contain fibres from both nasal and temporal retina (Stuermer, 1984), whereas I found that the fascicles contained fibres largely of nasal retinal origin.

Easter and Stuermer (1984) agreed with the hypothesis of Cook, Rankin and Stevens (1983) that each fascicle marks the location of the caudal two-thirds of the tectal edge at the time the fascicle grew in. The factors responsible for the formation of discrete fascicles are not known. The fascicle becomes embedded in tectal tissue soon after arriving at the edge of the tectum, and it is possible that, once in place and bounded by radially arranged tectal cells, the axons within it are not free to move (Easter and Stuermer, 1984).

There is evidence that neuronal (and glial) proliferation in the tectum is controlled to some extent by the arrival at the tectum of the RGC axons (Stevenson and Yoon, 1978; Kollros, 1982; Raymond, Easter, Burnham and Powers, 1983). It was shown that permanent removal of optic input (by enucleation) resulted in a reduction in ^{3}H-thymidine uptake in the tectal germinal zone on the denervated compared to the intact side. Optic nerve crush initially had a similar effect, but on reinnervation of the tectum by regenerating optic fibres uptake was enhanced on the regenerating side compared to the intact side. Therefore, it is not only the pressure for space, which it is argued occurs on the tectum causing shifting of arborisations, that is a consequence of ingrowing axons: there is also evidence that the ingrowing fibres have a trophic action on the tectum causing cells to divide and thus ultimately provide more space for RGC arbors to synapse on. The possibility that axonal transport is a factor in the trophic action was investigated using injections of intraocular colchicine (Davis, 1990), but the effects of colchicine are more widespread, affecting cell division as well as axonal transport, so this question was not resolved.

It is not known why tectal growth occurs at the caudal rim. It has been argued that only this part of the tectum borders the ventricle and thus has a ventricular germinal zone at which neurogenesis can occur (Raymond and Easter, 1983).

5:2: Retrograde HRP tracing of the retinotectal projection in the normal goldfish

Almost every pattern of retrogradely filled retinal ganglion cells (RGC) could be seen to consist of an 'arc' and a 'bar'. The arc was a partial annulus of variable length and radius centred on the optic disc. The bar, which also varied in length and position, ran in an approximately temporonasal direction to join the arc. Although this may at first appear to be an artificial distinction, these components of the pattern of labelled RGC can be inferred to correspond to distinct aspects of the tectal axonal structure, as will be discussed.

The following is a summary of the explanation of the retrograde tracer studies outlined in the Results section. To generate a retinal arc a tectal lesion must interrupt a set of axons which arise from RGC of a single age (Johns, 1977), and which terminate, since the projection is retinotopic, in a partial annulus. To generate a retinal bar a tectal lesion must interrupt a set of axons which terminate in a column approximately parallel to the rostrocaudal axis of the tectum. Two corresponding types of axon path have been observed on the goldfish tectum in previous studies. These include the anterograde tracer studies described above. The *stratum opticum* (SO) is known to contain fascicles of optic axons (Attardi and Sperry, 1963) in an age-related arrangement (Stuermer and Easter, 1982), from which the axons peel off sequentially to enter the *stratum fibrosum et griseum superficiale* (SFGS) before terminating in a partial annulus. Therefore a lesion in the SO will generate a retinal arc. The SFGS contains a loose, grossly parallel array of unfasciculated axons running in an approximately rostrocaudal direction as shown by the anterograde tracer studies above. The SFGS also is the site of the terminal arborisations. By interrupting the axons in the SFGS, so that they are labelled as axons of passage, a retinal bar will be produced. The terminal arborisations themselves can be labelled by endocytosis of the tracer (Mesulam, 1982) therefore this part of the axon need not be damaged in order to be labelled. In this case, the lesion permits the tracer to pass into the SFGS where it can be taken up. These three components of the innervation of the tectum combine to give the

uninterrupted patterns of RGC seen after retrograde tracing.

The patterns of filled RGC confirm some aspects of the findings from the anterograde studies already described. The compact and ordered patterns show that the paths of tectal axons are orderly, at least on the medial tectum where the tectal lesions were made, and by implication on the lateral tectum as well. Some of the patterns (eg Figures 9A and 10A) show some isolated labelled cells at a distance from the main body of labelled cells. Although the vast majority of tectal axons have ordered paths these examples show there are a few exceptions.

In the discussion of the anterograde studies, inferences were made about the direction of terminal migration probably being different away from the rostrocaudal tectal axis because of the shape of the tectum and the mode of tectal growth leading to different pressures for space. In terms of the retrograde studies, this would correspond to a different trajectory away from the nasotemporal retinal axis of the 'bar'. As mentioned in the Results section, the trajectory of the bar can be altered by the cuts made in the retina in the flatmounting process. However, Figures 9A and 10A do add some support to the argument since the bars in these retinae are curved.

The findings from the retrograde transport of HRP in the goldfish optic system are complementary to the anterograde studies described above, and demonstrate a pattern of RGC axons consistent with a systematic shift of these axons having taken place. In general more inferences have to be made about the tectal arrangement of the retinotectal projection from the patterns of filled RGC. However, making these inferences is possible because the nature of HRP as a neuronal tracer is well characterised (Mesulam, 1982), because the patterns of growth of retina and tectum in the goldfish are understood (Johns, 1977; Meyer, 1978) and because reference can be made to the anterograde studies. Again the retrograde studies are descriptive of an unperturbed developing system at one point in time; the same reservations exist about the inferences which can be drawn from these as for the anterograde studies.

Retrograde tracing of neuronal connections using HRP has been used to study the arrangement of axons in the retina (Easter, Bratton and Scherer, 1984), optic nerve (Rusoff and Easter, 1980) and optic tract brachia (Easter, Rusoff and Kish, 1981) of the adult goldfish. The tectal paths of normal and regenerating axons have been compared (Cook, 1983; Stuermer and Easter, 1984b). Although inferences have to be made about the projection at a distance from the labelled cells being analysed, the advantages of this method lie in its reproducibility, the clear labelling of the RGC somata which can be plotted from retinal flatmounts and the greater resolution at light microscope level of axon populations from their somata compared to their axons on the tectal surface.

This method has also been used to examine the retinotectal projection in the frog, *Rana pipiens* (Constantine-Paton, Pitts and Reh, 1983) where a similar pattern of axons running rostrocaudally before terminating was described.

5:3: "Rules" for tectal innervation derived from anterograde and retrograde HRP studies

1. The fascicles of the SO are distributed over the entire tectum. This can be seen by direct observation and inferred since tectal lesions at all locations can yield arcs in the retina.
2. The fascicles have an age-related arrangement: rostral fascicles are the oldest and caudal the youngest. A rostral tectal lesion labels cells near the optic nerve head, which are therefore older RGC, while a caudal lesion labels cells nearer the retinal periphery.
3. Fascicles from the two tract brachia respect a boundary between the medial and lateral tectal halves, though retrograde studies suggest that there is some interdigitation along this axis.
4. In the normal goldfish the tectal paths are highly ordered, but with the greater resolution afforded by the retrograde method a few individual cells can be inferred to have erroneous paths.
5. The fascicles in the SO contain fibres destined to terminate in the

(approximately) caudal half tectum only. No arcs were seen in the temporal part of the retina, which corresponds to the rostral tectum.

6. Axons of cells in the temporal part of the retina run caudally from the rostral tectal margin. These axons have been seen in the anterograde studies.

7. The caudal migration of the axons is systematically related to their age. This has been most effectively demonstrated by the anterograde studies along the rostrocaudal tectal axis (Figure 8). Axons migrate further from the rostral (older) fascicles than from the caudal (younger) fascicles, where in fact the fibres can be seen to turn rostrally, a point which can be inferred from the graph also.

5:4: Assessment of the map during regeneration under standard laboratory conditions using retrograde transport of WGA-HRP

An anatomical assay of the map refinement which is known to occur after optic nerve regeneration has been presented. Retrograde transport of iontophoretically injected wheatgerm agglutinin conjugated to HRP (WGA-HRP) from two separate tectal sites resulted in patterns of labelled retinal ganglion cells (RGC) which became more compact and retinotopic as regeneration progressed. Initially the cells were widely distributed with little retinotopic bias. They were later found in the "correct" retinal quadrant and later a cluster became discernible. Finally, the cluster was compact and the number of scattered cells was few. However, the clusters of cells were not as compact as in normal animals. Occasionally, later in regeneration, after single injections of WGA-HRP, double clusters were seen in the retinotopic quadrant.

The wide distribution of very few labelled cells seen in some examples early in regeneration could have been due to an increase in the diffusion of the WGA-HRP from the injection site, at a time when there were few axon terminals available to take up the tracer. Staining of the injection sites at 22 days of regeneration in a separate group of 3 fish compared to 3 normal controls showed no evidence of a change in the injection site to support this explanation for the cell distributions seen early in regeneration. WGA-HRP, unlike unbound HRP, does not diffuse widely from injection sites, binding strongly to membranes near the pipette tip (Mesulam, 1982). In those cases where there were many labelled cells, despite the small injection size, it is possible that there was exuberant proliferation of early axon terminal branches with much overlapping, therefore many terminal structures were available to take up the tracer. The reason for the great variation in the number of labelled cells early in regeneration is not known.

It is known that in regeneration the first axons to reach the tectum do so at about day 14 after optic nerve cut, at 25^{0}C (Stuermer and Easter, 1984b). They enter the tectum at the rostral pole between the two brachia. Axons which are destined to synapse caudally will also pass through this

zone. Therefore the wide scatter of cells could be a reflection of the fact that cells from all retinal quadrants pass through the area early in regeneration and their terminals are available to take up the tracer.

The ventral bias which was seen in some retinae early in regeneration could have reflected a bias in the route of regenerating axons, such that most axons from the ventral retina pass through the medial brachium and are therefore more likely to be labelled from the rostromedial injection site. However, evidence from the area indices at these stages of regeneration does not support this hypothesis. The area indices from caudal injection sites at 24 days (6 days later) and at 28 days were as great a those from rostral sites, indicating that no such bias existed.

It is known that the paths of regenerating RGC axons remain disordered after the map is refined in the goldfish (Horder, 1974; Cook, 1983; Stuermer and Easter, 1984b). Even if there is no early bias in routes of axons, as shown in later experiments (Becker and Cook, 1987; 1988), the early diffuse WGA-HRP labelling in some retinae and the wide variety of numbers of axons could reflect a period of 'trial and error' synaptogenesis. These 'abnormal' routes would not be seen at later stages because this tracer detects terminals only. The period of trial and error may form part of the self-ordering that is thought to occur among the terminals of regenerating axons (Willshaw and von der Malsburg, 1976). The self-ordering would allow the map to become more refined, and this would be reflected in a fall in the value of the area index, the clusters becoming more compact. The regenerating system may rely on self-ordering more than the developing system does: the choices of sites for synapsis for the newly-arriving axons in the latter are in theory more limited. In the regenerating system, the axons may branch exuberantly thereby increasing the chances of finding a near neighbour on the tectum. Axon counts in the optic nerve, tract and tectum of animals after optic nerve cut compared to normal animals would suggest that there is an increase in axon branch number, as do direct observations of HRP-filled axons (Murray, 1976; Murray, 1982; Murray and Edwards, 1982; Fujisawa, Tani, Watanabe and Ibata, 1982).

It is possible to over-interpret the findings from the WGA-HRP studies, especially from the earlier stages of regeneration. The nature of the terminal structures at these stages has not been well-characterised, nor has their ability or inability to take up WGA-HRP. Therefore the possibility of a trial and error phase of synaptogenesis during early regeneration must remain a supposition.

Later in regeneration, clusters of labelled cells appeared in retinotopic sites and there was a gradual elimination of the scattered cells surrounding the clusters. This confirms the findings of other mapping methods, electrophysiology (Humphrey and Beazley, 1982; Northmore and Masino, 1984) and autoradiography (Meyer, 1980), that the map refinement seen as regeneration progresses is a consequence of the elimination of non-retinotopic terminal structures from a tectal site. Such elimination of non-retinotopic terminal structures and the maturation of retinotopic terminal arborisations would result in the labelling of more compact clusters of RGC after iontophoretic injection of WGA-HRP, which is in fact what is observed.

There was no further refinement of the map after 70 days according to this assay. However, when the clusters are so compact, a few scattered cells will increase the area index considerably. In order to investigate the possibility of any further refinement, much larger numbers of animals would have to be studied. However, for practical purposes, regeneration beyond 70 days in these standard laboratory conditions did not lead to an appreciable increase in map refinement.

Another feature of late regeneration was the appearance of compact but double clusters in 8 of the retinae, 5 after caudal and 3 after rostral injections. They were in the retinotopic quadrant, and in those following caudal injections they had a radial orientation while those after rostral injections all had different orientations. It is possible that these distributions arose because of completely overlapping terminal structures, therefore one injection resulted in the labelling of both sets. If so, it is likely that such overlap would have been extensive; it is unlikely that one injection of tracer detected the only area of overlap in a projection. Was this period of possible

overlap a stage in the process of continuing map refinement? The fact that they were seen in the mid- to late stages of regeneration, including at 524 days, argues against this idea. In addition, the clusters, though double, were relatively compact with only a few scattered cells, again suggesting that much of the retraction of non-retinotopic terminals had taken place. An alternative possibility is that the terminal arbors from the two clusters were segregated into adjoining fragments of a piece-wise continuous map, so that one injection of WGA-HRP was taken up by both 'fragments' of the map. It is interesting that these double clusters have only been seen in the retinae of regenerating systems. It is possible that when the mechanisms by which axons achieve retinotopy depend more heavily on self-ordering, as postulated above for regeneration, the incidence of these double clusters increases as does the piece-wise nature of the map. Why were these double clusters not seen more often? If they were due to an overlapping of terminal structures, it suggests that this was a relatively rare event. As such a projection would be less efficient, because the tectal cells would be doubly innervated over all, a mechanism to avoid overlapping would be advantageous. If the double clusters were due to formation of a piece-wise continuous map, their rarity in these studies might be due to greater areas of separation between the adjacent fragments in some animals, or to the small size or immaturity of terminal structures, which may also be small in number, in an adjacent fragment. The latter could occur if there were an imbalance in the sizes of the fragments. Indeed it is possible that the sizes of the fragments were continuously changing as the self-ordering mechanism continued to act.

The piece-wise continuous map in the regenerated retinotectal projection in the goldfish was concurrently described by Meyer, Sakurai and Schauwecker (1985). Using anterograde WGA-HRP tracing, they discovered that the regenerated map was usually in this form. The regenerated map resolved into patches of reaction product, and one injection yielded more than one patch. The patches were of variable size. In contrast, in normal goldfish, one injection yielded one patch. The time course of the appearance of the double clusters of retrogradely labelled RGC at 56-524 days of regeneration corresponds well with the appearance

of the patches at 59-158 days in their anterograde tracer study. Although these authors noted that the sum of the areas of the individual clumps was not different from the area of a normal patch, it is not clear how much the initial branching of axons which occurs after optic nerve section contributed to the separate patches. It appears that the resolution of this retrograde tracer study using WGA-HRP is not great enough to detect the fragments in most cases, possibly because the distances between the patches are usually too great.

In four cases, two rostral and two caudal, the two clusters were approximately equal in size and in packing density of labelled cells but in the remaining four cases one cluster was distinctly larger or denser than the other. As is the case for the basis of the double clusters, the basis for their different sizes in some instances is not known. It is possible that this finding represents a transient situation in a continuously changing map, with the process of self-ordering altering the sizes of map fragments all the time.

The 5 cluster pairs which followed caudal injections had a radial orientation. This suggests that regenerating fibres in the caudal tectum tended to have larger and more contiguous map fragments along the radial axis than any other axis. It is interesting to consider that this axis in the developing animal arises from the pattern of growth of the tectum, and therefore one could postulate that normally the putative self-ordering mechanisms would be less active along this axis in the caudal half-tectum.

In the 3 cluster pairs which followed rostral injections, the orientations of the lines joining the centres of the clusters were different in each case: dorsoventral, circumferential and nasotemporal. On the tectum, these directions correspond to lateromedial, circumferential and caudorostral respectively, that is, three completely different axes. In the developing animal, the mapping on the rostral half-tectum is possibly guided by self-ordering in the circumferential direction plus a more passive 'pressure for space' which has a generally rostrocaudal direction. The direction of the pressure would therefore cross all axes except in the case of those axons directly in the midline. It is difficult to make general points, which are

speculative, from small numbers of examples. However it is interesting to consider that the resolution of the regenerated map may be affected by the possibly different requirements for self-ordering along the different axes in the developing animal.

The hypothesis that the orientation of the clusters is related to the axes which are most 'active' in a putative self-ordering process could be tested by labelling much larger numbers of animals with regenerated maps to see whether the orientation of larger numbers of double clusters bears it out.

Previous quantitative studies of the regenerating map have been based mainly on electrophysiology. The recent finding that electrophysiological recordings are largely post-synaptic rather than pre-synaptic (Grant and Lettvin, 1991) has called these findings into doubt, since they are at least one step removed from the map being studied. However, they do show that the multi-unit receptive fields decrease in size as regeneration progresses (Cronly-Dillon, 1968; Horder, 1971; Humphrey and Beazley, 1982). The findings from electrophysiological studies are similar to those of the WGA-HRP in that the MURFs are large early in regeneration and get smaller as regeneration progresses but do not reach the size of the MURFs of the normal fish. The equivalent sizes of the receptive fields which would exist given the area indices obtained from the WGA-HRP studies were calculated using an equation derived from spherical trigonometry. The receptive field was assumed to be circular. For a goldfish retina subtending 185.3^0 (Easter, Johns and Baumann,1977) this relationship is :

$$\cos(\theta/2) = 1 - 1.046(I_A)$$

where θ is the angular subtense and I_A is the area index*. It is interesting that the equivalent MURFs are consistently larger, both in normal fish and throughout regeneration, than typical MURFs determined experimentally. It is possible that the electrophysiological MURFs correspond to the relatively compact areas of the WGA-HRP clusters which are seen from 35 days of

* See appendix 3 for derivation of the formula

regeneration onwards. These in turn may represent the more mature arbors. Therefore electrophysiological methods may select only the more mature, clustered axons and not the scattered cells seen around the clusters. Given that there is now evidence that electrophysiological recordings are largely post-synaptic, the different sizes of the MURFs obtained from the two methods may have another basis. The post-synaptic, electrophysiological MURF may appear to be smaller because signals have converged onto the post-synaptic cell or because the signal has been attenuated. The calculation of the MURF from the area index is obviously pre-synaptic because the RGC themselves are the basis of the calculation. WGA-HRP mapping is therefore an excellent method to quantify the pre-synaptic projection.

5:5: Assessment of the map during regeneration in stroboscopic light using retrograde transport of WGA-HRP

The results show that the visual environment during regeneration affected the resolution of the map. When the fish were exposed to stroboscopic light throughout regeneration, with the lens ablated, the pattern of retrograde labelling at three months was similar to the pattern seen transiently at a much earlier stage when the fish had been exposed to diurnal light. In contrast, lens ablation alone or exposure to constant light had little effect on the resolution of the map.

Could the effects of the stroboscopic light have been non-specific, for example by affecting the general condition of the fish? Inspection of the fish revealed no differences between those kept in stroboscopic light compared to those kept in diurnal light. In addition, their retinae contained as many labelled cells. More specifically, subsequent experiments have shown that removal of the lens is necessary for the full effect of the stroboscopic light on the regenerated map (Cook, 1987). A focussed image can apparently 'rescue' the precision of the regenerated map in stroboscopic light. The presence or absence of the lens during regeneration in diurnal light appears not to affect the map. These findings provide a strong argument for local activity correlations being important in the process of map refinement. In diurnal light, the asynchronous firing patterns in the retina, which may see a focussed or unfocussed image, may give sufficient information at the tectal level for the fibres to detect their nearest neighbours. In stroboscopic light, however, the lens must be removed for the lighting pattern to have full effect. Then the combination of synchronous firing and the unfocussed image may remove some or all of the nearest neighbour correlated firing patterns so that on the tectum a terminal arborisation cannot detect the terminal arborisation of its nearest retinal neighbour.

Comparison of the stroboscopic light/lens ablated and the constant light/lens ablated groups should reveal a possible effect of the loss of diurnal light which the fish in the former group experienced. There was very little effect of constant light on the resolution of the regenerated map, suggesting

that loss of diurnal light was not a factor greatly influencing the resolution of the map in stroboscopic light. However, constant and stroboscopic light are very different visual environments, and there may be factors operating other than the loss of diurnal light. It is known that constant light has an effect on receptor and ganglion cell loss in the goldfish (Marotte, Wye-Dvorak and Mark, 1979), and in the experiments described above a loss of RGC was detected. Labelled RGC counts using retrograde transport of HRP from the cut optic nerve showed that in animals exposed to the stroboscopic lighting conditions used in the experiments fewer RGC were lost than in those exposed to constant light. There was very little difference between the cell numbers in the normal and regenerate retinae kept in the same lighting conditions. Therefore stroboscopic light in regeneration has much less effect on RGC number than on the map, compared to constant light.

Although there were fewer RGC in the retinae exposed to constant light during regeneration, the number of labelled RGC was greatest in these retinae. It is possible that with fewer cells competing for synaptic space on the tectum, those remaining were able to form more extensive and bushy arborisations. This could be investigated by anterograde labelling of RGC after regeneration in different lighting conditions. Indeed further characterisation of the morphology of the terminal arborisations at different stages of regeneration and under different lighting conditions would be of interest, as would their site of synapsis in the layers of the tectum. It is possible that, for example, the greater number of labelled cells seen in the constant light group arose because there was a greater concentration of terminal arborisations in the layer where the greatest concentration of the tracer existed.

In previous experiments designed to show an effect of neural activity on the regenerating projection, tetrodotoxin (TTX) a neurotoxin which blocks voltage-dependent sodium channels, was used. As with the effects of stroboscopic light, non-specific effects of the toxin must be considered especially as TTX is by its very nature a toxic substance. However, general toxic effects must be distinguished from more specific cytotoxic effects. Edwards and Grafstein (1983) have shown that, in the doses which have

been used experimentally, TTX had an adverse effect on the number of axons in regenerating optic nerves, on fast axonal transport of protein and on the level of amino acid incorporation by RGC. However, a single injection of TTX at the time of optic nerve crush, or a schedule of repeated injections during the first 14 days of regeneration, before retinotectal synapse formation, retarded axon outgrowth but did not affect the precision of the electrophysiological map (Schmidt and Edwards, 1983). Thus it is possible that additional non-specific effects of the toxin were not important in these experiments.

It is unlikely that stroboscopic light would have the same non-specific effects as TTX. Tectal activity levels, as measured by uptake of [^{3}H]2-deoxyglucose, in stroboscopic light in particular appear to be very much like those in either normal light or total darkness, and much greater than those found after TTX block (Edwards and Grafstein, 1983). Therefore the finding that stroboscopic light impairs map refinement in much the same way as TTX strengthens the conclusion that this effect of TTX is independent of its other effects on regeneration and depends only on its ability to silence optic axons, and thereby to prevent locally correlated activity patterns reaching the tectum.

TTX has also been used in the normal goldfish retinotectal projection under circumstances of local blockade of nicotinic acetylcholine (ACh) receptors by α-bungarotoxin (Schmidt, 1985). Local blockade led to movement of optic axons into adjacent areas, and this movement was blocked by intraocular TTX. When α-bungarotoxin was applied to the whole tectum, normal maps were found. This experiment demonstrates the plastic nature of the normal retinotectal projection and is a further indication of the specific effects of TTX. However, it has subsequently been shown (Zottoli, Rhodes, Corrodi and Mufson, 1988) that the nicotinic ACh receptors are part of the isthmotectal projection and not the retinotectal projection.

Schmidt and Edwards (1983) showed that repeated intraocular injections of TTX after optic nerve cut in the goldfish yielded regenerated retinotectal maps which were normally organised but which contained

enlarged MURFs. In regenerates treated with TTX, the MURFs measured on average 27^0, whereas in control regenerates they measured 11-12^0. Four months after release from TTX blockade, there was no evidence of further map refinement. However, after repeat optic nerve crush and regeneration in the absence of TTX, a normal map was formed, suggesting that there had been no permanent effect of the initial TTX doses. The greatest effect on the map was seen when TTX was injected at days 14-34, the period during which regenerating axons reach the tectum, at temperatures of 19-23^{0}C, (McQuarrie and Grafstein, 1981) and then form synapses.

Schmidt and Eisele (1985) studied the effects of stroboscopic light on the regenerated optic projection in the goldfish. Intra-retinal recordings showed that the xenon flash of the stroboscopic light produced correlated activity in all RGC. The goldfish were approximately twice the length of the animals used in the experiments described above. They were kept individually in white or black featureless tanks, the lenses were intact and the strobe frequency was a constant 1Hz. The map was analysed using electrophysiological methods. The regenerated projection was grossly retinotopic but the MURFs were larger than normal, the effect of stroboscopic light being greatest at 14-34 days of regeneration. This is similar to the results described above in that the resolution of the map was impaired by stroboscopic light. It is interesting that with a constant strobe frequency and with the lenses intact the regenerated map was still impaired. Experiments comparing the effects of the lens in regeneration in stroboscopic light indicated that the lens could 'rescue' the precision of the map (Cook, 1987). The constant strobe frequency might theoretically allow a focussed image to be detected more easily compared to a random frequency. If it is the case that the MURFs are post-synaptic, then electrophysiology may not be the best method for determining the resolution of the map since the information relates to the tectal cells and does not isolate the RGC. The tanks in the two experiments were different in colour, grey in the experiments described above and white or black in Schmidt and Eisele's experiments, and fish were in groups in the former and isolated in the latter. It is possible that these factors overrode the presence of the lens and the constant flash frequency of the stroboscopic light.

It is also interesting that complete darkness should have been reported to have blocked the sharpening process (Schmidt and Eisele, 1985). The MURFs were not as large as those seen after TTX or stroboscopic light, but they were apparently larger than controls. The statistically correlated spontaneous activity which has been shown to occur in the dark in the goldfish retina (Arnett, 1978) may not have been sufficient to resolve the map in these experiments. However, an examination of the statistics used shows that individual recording points were used rather than the number of individual fish in the interpretation of the data. It is possible to speculate that with larger numbers of individual fish there would not have been a statistically significant difference.

The results of Schmidt and Eisele's experiments (1985) in which the effects of stroboscopic light and darkness on the regenerating projection were tested are similar to those involving TTX (Schmidt and Edwards, 1983) in that normal lighting conditions after 35 days of regeneration permitted little sharpening of the diffuse maps. Similarly, the sensitive period in both experiments was 20-35 days, the time of early synaptogenesis. Neither intraocular TTX nor stroboscopic light unsharpened the mature, non-regenerating map.

Meyer (1983) used autoradiographic mapping to detect the effects of intraocular TTX injections on the regenerating projection. Precise topography was absent following TTX injections at days 42-81 of regeneration, though interestingly survival to 104 days with no TTX for the final 24 days did yield a precise map. Thus there was some doubt about the ability of the imprecise regenerated map which had formed in stroboscopic light or under TTX blockade to become more precise subsequently.

Following removal of one tectal lobe in the adult goldfish, the displaced optic fibres regenerate to the remaining tectum where the two retinal projections form ocular dominance patches (Levine and Jacobson, 1975; Schmidt, 1978) after a period of complete overlap (Boss and Schmidt, 1984). Bilateral intraocular injections of TTX from day 18-75 postoperatively resulted in much less marked patches; and extending the injections until day

98 did not allow patches to develop, indicating that there was a block rather than a delay in patch formation, at least up to this time (Boss and Schmidt, 1984). It was thought that the subtle changes in innervation density which remained, despite the bilateral TTX injections, came about as a result of the inequality of the two projections in that one was intact and the other was regenerating. It would appear that the segregation of inputs, like the formation of a precise regenerated map, is activity-dependent. Subsequent work on the role of activity in 'stripe' formation in developing three-eyed frogs is discussed in Chapter 6.

Other workers have had negative results using stroboscopic light in regeneration. Chung, Gaze and Stirling (1973), for example, studied the retinotectal projection electrophysiologically in two frogs (*Xenopus laevis*) after optic nerve regeneration in stroboscopic light of frequency 0.2Hz. They reported the map to be normal though SURFs were measured and MURFs were not reported. It is possible that local correlations in activity may have been detected either because of the presence of the lens or because of the relatively long intervals between the flashes of stroboscopic light.

The effect of stroboscopic light on mammals has also been tested. Chalupa and Rhoades (1978) studied the visual response properties of tectal neurons in hamsters reared from birth in a diurnal cycle of darkness and 2Hz stroboscopic light, and found no difference in the distribution of receptive field sizes. These neurons were a mixed population and their fields may not have reflected minor changes in the pre-synaptic map. In addition, map refinement may have resulted from correlated activity which may have arisen during the daily dark period. There were, however, differences in the dynamic response properties such as a reduction in the incidence of directionally selective cells.

Pearson and Murphy (1983) studied tectal neurons in rabbits reared from birth in a diurnal cycle of darkness and stroboscopic light of frequency 4 Hz, and significant receptive field enlargement was found in all laminae. Pre-synaptic field sizes were not studied. It is difficult to study the role of the visual environment in mammals since there is evidence that spontaneous

RGC activity is locally correlated in the dark (Mastronarde, 1983), and therefore map refinement can potentially occur *in utero* or before eye-opening.

Such spontaneous neural activity was indeed demonstrated in the rat fetus from embryonic days 17 to 21 (Galli and Maffei, 1988), and was shown to be locally correlated (Maffei and Galli-Resta, 1990). Locally correlated spontaneous neural activity was also demonstrated in RGC of the developing cat and ferret retina (Meister, Wong, Baylor and Shatz, 1991). It is interesting that these patterns should develop prenatally before the development of photoreceptors which are obviously redundant *in utero*. The mechanisms are therefore established early for locally correlated neural activity to be able to act in the formation of a precise retinotopic map in the absence of photic stimuli and of photoreceptors.

The experiments described in this thesis provide evidence for the theory that locally correlated neural activity can be important for map refinement, since its obliteration by stroboscopic light appeared to arrest the process of map refinement at the diurnal light equivalent of 35 days after optic nerve crush, at 20^{0}C. This is about the time when synapses first become electrically detectable. Since synaptogenesis begins about 20 days before this (Stuermer and Easter, 1984b), the importance of an anatomical method to assess the early map is evident.

In spite of the probable masking of spatial information by the stroboscopic light in these experiments, a degree of retinotopy was seen. The filled cells were in the correct quadrant though there was no cluster formation. This argues that activity-independent mechanisms were responsible for the gross order of the map but could not resolve it fully, though one should be wary of creating an artificial divide between two putative mechanisms which are probably simultaneous if not synergistic in the developing projection. It is interesting that this system is capable of using fundamental biological properties of the cells themselves, namely electrical activity, to derive spatial information, and to use that information to order the map. This would seem teleologically to be a more economical

approach than, for example, the chemical markers put forward by Sperry (1963).

Chapter 6: DISCUSSION OF WORK RELEVANT TO THIS THESIS CARRIED OUT AFTER 1985

6:1: Shifting connections

Since the above experiments were carried out in 1982-1984, shifting connections have been further characterised in fish and frog retinotectal systems.

The modes of growth of goldfish retina and tectum had already been shown to be disparate in juvenile and adult goldfish (Johns, 1977; Meyer, 1978). Evidence for larval and early juvenile stages was available for the retina (Johns, 1982). Raymond (1986) demonstrated that, at all stages up to one year after hatching, retinal and tectal growth patterns were mismatched. Using ^{3}H-thymidine autoradiography, she showed that the retina grows by the addition of peripheral annuli and the tectum by the addition of caudal crescents of tissue. Therefore, in order to maintain a retinotopic map, the need for shifting connections is present from very early stages. An estimate of the rate of shift in the first two years of growth was given as 5μm per day. The anterograde studies presented above were criticised in one respect in Raymond's paper: the measurements given for the extra-fascicular segments of the RGC axon did not allow for histological shrinkage. If shrinkage were taken into account, the results from the two studies would be more in agreement, with an overall shift of approximately 1.5mm.

6:1:1: Shifting connections in fish with a *fovea*

An interesting 'problem' arises in those fish species which, unlike the goldfish (Johns and Easter, 1977), have specialised retinal areas designed to yield greater visual acuity. In almost all fish with such a region of high cell density, the specialised *area centralis* or, in some cases, the *fovea* is in the temporal retina. The fish *fovea* differs from the 'complete' *fovea* seen in various reptilian, avian and mammalian species: in the former all retinal layers except the axons are present, whereas only the photoreceptor layer is present in the latter. It has been observed that the *fovea* in these fish remains in the temporal retina during development. How is it 'kept' there

while the fish grows? If annular growth were to occur symmetrically, as it does in the goldfish, the *fovea* would be pushed centrally. Easter (1992) demonstrated that the patterns of retinal growth in many teleosts with a *fovea* were different from the pattern seen in the goldfish. He applied a fluorescent dye, dioctadecyl-tetramethyl-indocarbocyanine (Di-I) to various sites in the cross-section of the optic nerve, which in these species was ribbon-shaped. He observed the annular pattern of retrogradely labelled cell bodies in the retina, exploiting the fact that each new generation of RGC sends its axons into the optic nerve as a cohort. In seven of the fifteen teleost families studied, retinal growth was clearly asymmetric, with labelled annuli closer together on the temporal side and further apart nasally. This nasotemporal asymmetry would serve to keep the *fovea* in the temporal retina. In addition, this asymmetry was associated with a patent ventral embryonic fissure. The fact that it was patent allowed portions of the retina to 'slide' relative to one another during development. It was postulated that the sliding of the tissues may have contributed to the lack of fusion of the embryonic fissure. The contributions of cell proliferation, cell death and hypertrophy were not fully discussed in this paper.

Some species had a *fovea* and yet had symmetrical growth (Easter, 1992). However, the difference in cell density between peripheral and specialised retina was much smaller compared to the other species, therefore a much smaller discrepancy in retinal growth would be necessary.

6:1:2: Shifting connections in the frog *Rana*

The rapidity of the shifting connections in the frog, *Rana pipiens*, was demonstrated by Fraser and Hunt (1986), using electrophysiological methods and a double lesion technique. They plotted the projection of the optic nerve head to the tectum at two stages of development (late larval and metamorphic) separated by one week. Marker lesions were made at these two times, and the distance between the two lesions was measured. A shift of 275μm per week was detected in late larval life, and topographic postsynaptic units could be detected at all stages investigated. This implies that the axons shift while the map is functional, as predicted. Given that rates of tectal histogenesis and overall size increase fall somewhat as

metamorphosis approaches in *Rana* (Fraser and Hunt, 1986), the measurement represents a large absolute shift with little contribution from tectal hypertrophy or hyperplasia. The rapid shift, which had been documented previously (Reh and Constantine-Paton, 1984), could be one of several ways in which *Rana* differs from other species. Rate of shift of connections is greater in *Rana* than in *Xenopus* for example (150μm in 2 weeks, cited in Fraser and Hunt, 1986). The growth pattern of *Xenopus* retina is more asymmetric than that of the *Rana* retina, more cells being added to the ventral than to the dorsal retina. There is therefore less retinal mismatch, tectal cells being added medially, at the site of termination of the ventral retinal ganglion cells. If the degree of mismatch is less, there will be less need for shifting connections. The *Rana* visual system is larger than that of *Xenopus*, with a relatively slower rate of growth of tectum compared to retina. In addition, electrophysiological mapping in *Rana* showed that the innervating optic fibres delayed covering the whole surface of the tectum. In larvae, there were defects in the map with caudal and medial tectum being relatively spared (Fraser and Hunt, 1986). By metamorphosis, the maps were similar to those of the adult, therefore there must have been a large shift of axons into the 'spare' tectum in a relatively short time period.

The marker lesions, as well as showing a caudal shift, also demonstrated a medial shift, a finding which was complementary to the centripetal shift predicted by Hitchcock and Easter (1987) in postmetamorphic *Rana pipiens*. These authors used retrograde transport of HRP from tectal lesions and observed the patterns of labelled RGC, a method very similar to that described above. Evidence for a centripetal shift came from the radially directed bars of labelled RGC. This shift arises because of the disparity in the amounts of retinal and tectal growth.

6:1:3: Evidence for a centripetal shift

In Chapter 5, I discussed the possibility of different vectors in the growth of the shifting connections in the goldfish. It was difficult to draw conclusions from the tectal wholemounts because histological processing and the flattening of the specimens meant that distortions had probably occurred. Although there were some instances in the retrograde tracer

studies which could have been interpreted as evidence for a centripetal shift of axons, away from the rostrocaudal tectal axis, it was also possible to explain these on the basis of the wholemount procedure. There were no examples in our series which showed clear radially-directed bars as were seen by Hitchcock and Easter in *Rana* (1987). It is possible that these were seen in *Rana* because of the much greater discrepancy between the amounts of retinal and tectal growth at this stage of development compared to the goldfish. In the former, tectal histogenesis stops at metamorphosis, whereas tectal growth continues in adult life in the latter. As retinal growth continues in *Rana*, the RGC terminals are accommodated on the tectum by a relatively greater centripetal shift. This should not be necessary in the goldfish.

These studies on different species provide interesting examples of the ways in which 'problems' arising from different modes and rates of growth can be overcome. They also demonstrate that the need for plasticity of connections is widespread in nature.

6:1:4: Causative factors in shifting connections

What causes the shift of the terminals? Are the arbors being "drawn" into newly generated tectum? We know that this cannot be the case in postmetamorphic *Rana pipiens* because no new tectum is being generated. Or are the old arbors being "pushed" by newly arriving RGC arbors which are competing for space? This question was addressed in the cichlid fish, *Haplochromis burtoni* (Wilm and Fritzsch, 1991). Juvenile animals received enucleation of one eye and crush of the other optic nerve. An ipsilateral projection formed, consisting of regenerated fibres from the optic nerve crush. There was little contribution from newly formed RGC as the fish grew. The retinal and tectal areas increased, yet retrograde HRP labelling of the ipsilateral projection yielded labelled central RGC only. Newly born RGC were not represented on the ipsilateral tectum. Ipsilateral retinal terminals were restricted to the rostral and central tectum. There was no evidence for expansion or a shift into the caudal tectal areas throughout the period of growth. These findings are compatible with the hypothesis that newly arriving fibres induce older terminals to shift. They are against the

hypothesis that arbors are "drawn" into newly generated tectum.

6:1:5: Morphology of shifting arbors

The mechanism by which the arrival of new RGC arbors is detected by older arbors and translated into shift is not clear. Greater detail of the morphology of terminal arbors in the developing fish and frog has now been obtained. For example, using anterograde labelling with HRP, Fujisawa (1987) demonstrated RGC arbors in tectal wholemounts of larval *Xenopus* at various stages of development. He observed axonal sprouting when each retinal axon arrived at its topographically appropriate termination site. Later in development, each retinal axon divided and old branches, now inappropriately located due to tectal growth, were retracted.

Stuermer and Raymond (1989) used a similar anterograde HRP technique to examine the terminal arbors in larval goldfish. Arbors were already present in newly hatched fish and the projection was already retinotopic. The projection was retinotopic in that fibres from local regions of the retina projected to discrete patches of the tectum, with the smallest patch covering 3.5% of the surface area of the tectal neuropil. However, the site of the retinal lesion was not confirmed, therefore exact retinotopy could not be confirmed. The arbors in the larvae were elliptical in shape and growth cones were frequently seen. Arbors were larger in the adults compared to the larvae, however their average tectal coverage fell with age since the tectum expanded rapidly during development. Therefore the increase in map precision which occurs as development proceeds is partly due to a relative expansion of the tectum and not to retraction of the arbor branches. The authors did not address the mechanisms of shifting connections in this paper.

Observation of HRP-labelled RGC terminal arbors and growth cones (Reh and Constantine-Paton, 1984) in *Rana* where connections shift rapidly (Fraser and Hunt, 1986) showed that growth cones and synapses can co-exist on highly branched terminal arbors. Growth cones tended to be found caudally on the arbors, though some were found rostrally. If growth cones were to predominate caudally and branch retraction rostrally, then a caudal

shift of the terminal arbor could occur.

Both physiological and anatomical tracer techniques are limited in their ability to demonstrate a dynamic process. Assessments must be made at different stages of development. Such methods are then open to errors of over-interpretation because deductions are made about events which cannot be continuously observed.

O'Rourke and Fraser (1986) devised a method to overcome this problem. Defined halves of the developing *Xenopus* eyebud were labelled with fluorescent dextrans which fill the growing axons. The projection of labelled cells was followed for up to two weeks in individual animals which had received eyebud grafts from the labelled animals. An image-intensifying video camera was used allowing low light levels, which minimised the bleaching of the dye and possible phototoxic effects of the dye on the cells. Good dorsoventral ordering was seen early in development, at stages 40-50. In contrast, anteroposterior ordering of the projection was not seen until stage 48. After an initial overlap, the nasal fibres had grown over the temporal fibres to innervate caudal tectum. Therefore a shift in a population of axons had occurred. However, resolution was rarely good enough at this stage in the development of the technique to observe the terminals themselves.

To improve resolution, the fluorescent carbocyanine dye Di-I was microinjected into the retina to label as few as one RGC (O'Rourke and Fraser, 1990). A confocal microscope was used to observe the three-dimensional structure of the labelled axon(s). As before, the nasal and temporal RGC arbors initially overlapped on the tectum. In spite of subsequent remodelling, the dimensions and positions of the temporal arbors remained relatively stable. Nasal fibres grew caudally, as they extended caudal branches and retracted rostral branches. All the terminal arbors were dynamic, including those which underwent no net changes. It would be interesting to observe terminal arbors at later stages of development.

6:1:6: Conclusion

Subsequent evidence has therefore continued to confirm, or at least to be consistent with, the hypothesis of shifting connections. The molecular basis of the shift has not been elucidated. Anatomical evidence suggested a dynamic process involving growth cones and the branching of terminal arbors, and this has been backed up by direct observation using the confocal microscope. This retinotectal map cannot be considered in isolation. Its registration with other maps, which is discussed in section 6:5, may provide clues to the factors influencing the connections described above.

6:2: Activity and the map

Evidence that map refinement takes place following regeneration of the cut optic nerve, and that refinement can be impaired by altering the visual environment, has been presented. Since these experiments were carried out, in 1983-1985, similar questions on the role of the visual environment have been addressed in mammals, birds, amphibians and fish. Other mapping systems, including somatosensory and auditory, have been examined, and the role of marker molecules has been explored in more detail. Ultimately we may understand the relative contributions of marker molecule recognition and the recognition of correlated firing patterns in establishing and maintaining an ordered map.

6:2:1: Mammalian visual system

The visual environment and its effects on neuronal activity have been shown to be of importance in establishing neuronal connections in the developing mammalian visual system. In the mammalian retino-geniculocortical visual pathway, inputs from the two retinas are segregated in the dorsal lateral geniculate nucleus (LGN) and in the afferent layers of visual cortex, mainly layer IV. This segregation has been shown to be affected by patterns of activity in the visual system. When visual stimulation does not allow simultaneous delivery of similar patterns of neuronal activity to the same cortical cells through the left and right eye visual pathways, after one or more of the extraocular muscles has been cut for example, the two pathways lose the ability to drive those binocular cells. Instead the two inputs segregate onto two mutually exclusive sets of cortical neurons (reviewed by Fregnac and Imbert, 1984). In monocular deprivation studies, one eye of a neonate kitten or monkey is sutured shut for a period of time (weeks or months). Then the eye is opened and the ability of isolated cortical units to respond to either eye is tested. The deprived eye loses its ability to drive cortical units and all cortical neurons develop pronounced responses to the open eye (Hubel, Wiesel and LeVay, 1977). The degree of domination is related to the duration of the monocular deprivation, but even short exposures of 1-4 days during a critical period can give marked changes in eye dominance (Olson and Freeman, 1975).

A similar response is seen in the LGN. Those neurons receiving input from the deprived eye have smaller cell bodies relative to the corresponding cells receiving input from the non-deprived eye. These changes are seen only in the binocular areas of the LGN. The somal shrinkage could be due to the loss of ocular dominance termination space in the cortex, with or without competitive effects in the LGN itself (reviewed by Sherman and Spear, 1982).

Tetrodotoxin (TTX) has been used in a number of experiments on the developing mammalian visual system in order to eliminate spontaneous activity and thereby demonstrate its importance *in utero* or before the eye is capable of pattern vision. Left and right eye inputs did not segregate from each other in the cat fetal LGN following continuous intracranial TTX infusion during the fetal period (Shatz and Stryker, 1988); or into cortical layer IV ocular dominance columns following bilateral intraocular TTX injections in the post-natal period (Stryker and Harris, 1986). It is not only the segregation of left and right eye inputs in the LGN which is affected by impulse blockade. The receptive field characteristics of the LGN neurons are also affected such that they can be driven by both on- and off-centre ganglion cells (Dubin, Stark and Archer, 1986). This implies that there is a disruption of functional organisation at the level of RGC convergence onto the LGN neurons. It is possible that obliteration of normal electrical activity by TTX allows different ganglion cell types to synapse on one LGN neuron, whereas normally their asynchronous firing patterns would separate them.

Direct stimulation of central visual pathways using implanted electrodes has been used in kittens with bilateral retinal TTX blockade (Stryker and Strickland, 1984). Cortical neurons were sampled following stimulation with either synchronous or asynchronous impulses for a period of 4-6 weeks. After synchronous stimulation, only 2% of neurons sampled were monocularly driven. After asynchronous stimulation, 72% of neurons were monocularly driven. These results support the hypothesis that patterned electrical activity is important for the segregation of afferents in the mammalian visual cortex.

6:2:2: The role of activity in the developing sub-mammalian visual system

For the most part, studies of the role of activity during development have been carried out in mammals, and its role in regeneration has been investigated in fish and amphibians. However, the few experiments which have been carried out on developing fish and amphibia have emphasised the technical difficulties and the need for high resolution anatomical mapping.

As mentioned in the Introduction, Harris (1980) demonstrated that when the eye primordium of an axolotl embryo, *Ambystoma tigrinum*, is grafted to the embryo of the Californian newt, *Taricha torosa*, a species which "manufactures" TTX and which is insensitive to its toxic effects, action potentials are blocked in the transplanted eye. In spite of this and the competition from the host eye, the transplanted eye was able to make a retinotopic projection to the host tectum (Harris, 1980). In subsequent experiments, the eye primordium was transplanted to an ectopic site on an axolotl embryo and the axolotl embryo was parabiotically joined to a Californian newt (Harris, 1984). In this way the axolotl optic nerve could grow to the axolotl tectum in the absence of competition from host optic axons. Again, mapping showed normal topographic retinotectal projections in spite of the axons' abnormal routes due to their ectopic origin. These experiments show how an intriguing idea can be limited by the experimental method, since the resolution of the map as detected by HRP tracing was too low to demonstrate anything more precise than gross retinotopy. It would be of great interest to repeat Harris's experiments in amphibians using a mapping method of greater precision.

Normal maps were observed in dark-reared *Xenopus* (Keating, Grant, Dawes and Nanchahal, 1986), as shown by electrophysiological mapping at metamorphosis. The topographic precision of the projection was the same in light- and dark-reared animals, as determined by multi-unit receptive field size. In addition, the laminar distribution of terminals from different classes of retinal ganglion cells was the same in both groups of animals. Therefore, while a wide range of visual experience is not necessary for development of a retinotopic map in *Xenopus*, a role for activity has not been excluded since

in many species there is spontaneous activity in the retina in the dark (goldfish, Arnett, 1978; rabbit, Arnett and Spraker, 1981; cat, Mastronarde, 1983).

The effect of TTX has been investigated in the developing chick retinotectal projection during the refinement phase (Kobayashi, Nakamura and Yasuda, 1990). TTX was shown to increase the numbers of overshooting fibres and arborisations outside the terminal zone. The mapping method used in this experiment was the fluorescent dye, Di-I. This result argues for a role for neuronal activity in the refinement of the developing retinotectal projection in the chick.

In general the maturation of the lower vertebrate retinotectal system seems to be less dependent upon visual experience than does the mammalian geniculocortical system. Such a comparison is perhaps not helpful since the systems have so many different aspects; the mammalian system has a much higher order of complexity, with a greater number of relays and a greater degree of binocularity.

6:2:3: Subsequent experiments on the role of activity in the fish visual system

Stuermer (1988) showed, in the developing zebrafish, that the terminal arborisations are small and that a high degree of retinotopic order exists from the very beginning. The effects of neural activity on the developing system was investigated using intraocular injections of TTX (Stuermer, Rohrer and Munz, 1990). A precisely ordered retinotopic map was found, and terminal arborisation sizes were the same as controls. This is interesting given the results of Harris's experiments on the developing amphibian visual system described above. In those experiments, the methods were of insufficient resolution to determine the effects of TTX on map precision. However, if the effects of TTX were in fact similar to the effects on the developing zebrafish, there is less reason to repeat Harris's experiments using more precise methods. These results demonstrate the reduced effect of the blockade of neural activity in the fish compared to the mammalian visual system. In addition, it may be the case that in the

developing zebrafish and axolotl the degree of retinotopy is too poor to need refining.

Further tests of the effects of TTX on the regenerating goldfish optic nerve projection have been carried out. The ability of regenerating goldfish optic fibres to grow from an inappropriate to an appropriate region of optic tectum in the absence of neuronal activity in the optic nerve was tested by deflection of the fibres from a retinal quadrant to the wrong site on the ipsilateral tectum and injecting the eye with TTX (Meyer, 1987). The fibres on the 'host' tectum were eliminated by earlier enucleation of the other eye. The termination sites of the axons were determined autoradiographically. Under impulse blockade, fibres were still able to synapse at the 'correct' site even after taking abnormal routes across foreign tectum. Although lack of neuronal activity was not demonstrated electrophysiologically, the doses of intravitreal TTX were higher than doses which had previously been shown to abolish activity. In this experiment, half the fish received an intravitreal injection of 0.05μl of 1.2mM TTX three times per week and the other half 0.1μl of 0.3mM TTX. 0.1μl of 0.01mM TTX twice a week had been shown to be just below the threshold for impulse blockade (Meyer, 1982). Even so, the fibres were able to regenerate. This study is also complicated by the tendency of axons to expand and fill the available space (Schmidt, Cicerone and Easter, 1978). It is interesting that the control and TTX-treated animals showed the same degree of expansion. This experiment indicates that neuronal activity is not an important factor in expansion of the regenerated projection, and neither is it important for axons which have been deflected which can find the appropriate area of the tectum. The mapping method used in this experiment was quantitative autoradiography. The size of the regenerating projection (a quadrant) should be sufficient for self-ordering mechanisms, which it is proposed rely to some extent on firing patterns, to come into action. However, it would appear not to be the case.

It appears that impulse activity is not necessary for compression either. Meyer and Wolcott (1987) showed that, in the presence of TTX, compression and expansion will still occur after optic nerve crush. An intermediate stage when axons return to previously occupied sites before

compression occurs was not demonstrated in this paper, because early stages were not mapped. It is in part surprising that compression was not affected by TTX. The projection is a disordered one following optic nerve crush, and it seems reasonable to suppose that there will be much self-ordering taking place in order to achieve retinotopy in the smaller space. If a degree of that order were generated by half the projection synapsing in its former site, it is possible that self-ordering was initially important but became less so. It is also possible that the mapping methods (electrophysiology and autoradiography) were not precise enough.

More recently, Olson and Meyer (1994) have carried out optic nerve crush and removal of the posterior half of the contralateral tectum in the goldfish. In some animals, activity was blocked by intravitreal TTX injections. Mapping was by anterograde transport of WGA-HRP injected into a small region of nasal retina. The initial projection was unaffected by impulse blockade. Further compression under impulse blockade occurred, but the resulting degree of retinotopy was less than that seen on an intact tectum with impulse blockade. Interestingly, in the absence of TTX, fine retinotopy was not compressed even though the map as a whole was. Therefore, as discussed above, compression itself is generated by activity-independent mechanisms. And the activity-dependent map refinement is apparently not related to the ratio of numbers of terminal arbors versus available synaptic sites. "Competition" may not be an important factor after all, at least not in determining fine retinotopy.

In general these experiments argue that a neighbour-to-neighbour self-ordering system, based on local correlations in firing patterns, is not necessary to guide regenerating quarter-, half- and whole retinae in compression, expansion and in finding the appropriate area in an 'empty' tectum. It is surprising since the orderliness generated by successive cohorts of new, peripheral RGC in the developing system is absent.

6:2:4: 'Stripes' of terminal arbors in developing three-eyed frogs

It had been observed that if the axons from the two retinas in the goldfish are caused to converge on a single tectal lobe, the terminals

become segregated into eye-specific clumps (Levine and Jacobson, 1975; Cook and Pilgrim, 1981). Similar findings have been made in embryonically created three-eyed frogs, *Rana pipiens*, which are of interest because, although this is an artificial system, the capabilities of a developing as opposed to a regenerating system can be characterised. In these animals, the host eye and the third eye converge onto a single tectum from early developmental stages, and anterograde labelling reveals that the projections are separated into rostrocaudally-oriented stripes reminiscent of the ocular dominance stripes in mammalian visual cortex (Constantine-Paton and Law, 1978). The projection is laid down sequentially at the tectal margin, and using autoradiography the projections can be seen to overlap before segregating. The significance of the stripes is not known. However, it is possible that they reflect convergence of terminals from neighbouring zones of the same retina, and that stripe borders reflect separation from non-neighbouring zones of different retinae. This experimental system has been extensively studied to elucidate the molecular basis of synapse formation and maintenance, and its role in map formation (see below).

6:2:5: Long term potentiation in the hippocampus and the NMDA receptor subclass

Long term potentiation (LTP) was first described in the rabbit hippocampal formation by Bliss and colleagues (Bliss and Lomo, 1973) and has been extensively studied in the CA1 region of the hippocampus and in the dentate gyrus. The essence of LTP is a rapid and persistent synaptic enhancement, lasting weeks to months, in comparison to post-tetanic potentiation which lasts a few minutes. LTP was induced when pre-synaptic stimulation was paired with simultaneous post-synaptic depolarisation. The synaptic enhancement was manifest by an increase in the amplitude of the excitatory post-synaptic potential (EPSP) produced by an afferent input. As stated in the Introduction, Hebb (1949) proposed, as a learning mechanism, that co-existent firing at a synapse could lead to probable metabolic changes that in turn could lead to an increase in the efficiency of the afferent input at that synapse. It has been proposed that LTP could form the basis of such a mechanism.

LTP has been broken down into the stages induction, expression and maintenance (Brown, Chapman, Kairiss and Keenan, 1988). 'Induction' refers to the sequence of events that triggers the synaptic modification, 'expression' to the mechanisms that make up the afferent input that causes the enhancement and 'maintenance' to the factors that govern the duration of the enhancement. Induction appears to involve the excitatory amino acid *N*-methyl-D-aspartate (NMDA) receptors, since blockade of these receptors prevented induction but did not affect LTP that had already occurred (Cotman, Monaghan and Ganong, 1988). The basis of maintenance of LTP is not known but may involve changes in protein kinase(s) and subsequent structural changes.

Interest in NMDA receptors as part of the molecular basis for long term synaptic plasticity came from studies on the rat hippocampus (see, for example, Harris, Ganong and Cotman, 1984). Using NMDA receptor antagonists, the authors showed that the ion channels associated with NMDA receptors were not activated by a single pre-synaptic release but were activated during periods of intense synaptic activity. In the visual system, chronic intracortical administration of L-glutamate during a period of monocular vision in young kittens blocked the usual "takeover" of cortical neurons by the non-deprived eye (Shaw and Cynader, 1984). Whether this reflects a general role for NMDA receptors in affecting cortical cell excitability or a more specific function as detectors of correlated activity is not shown by this experiment. Recordings from the cortex showed that the infusion disrupted normal cortical patterns of response. It thus appears that the ability of the cortical neurons to respond effectively is important for plasticity.

The NMDA receptors are a subclass of the family of excitatory amino acid receptors. Agonists include NMDA and, *in vivo*, glutamate. They co-exist within the central nervous system with other excitatory amino acid receptors, "non-NMDA" receptors. The NMDA receptors have at least two components, regulated by a ligand and by voltage, and kinetics are relatively slow. Thus the initial depolarisation by ligand binding is unlikely to effect any further change in the receptors. However, summation of excitatory post-synaptic potentials (EPSPs) mediated by the neighbouring non-NMDA

receptors results in sufficient depolarisation of NMDA receptors (Kauer, Malenka and Nicoll, 1988) so that magnesium blockade of the latter ceases, and calcium influx can occur into the post-synaptic cell.

A possible role for calcium was identified when it was shown that a calcium chelator blocked the induction of LTP (Lynch, Larson, Kelso, Barrionuevo and Schottler, 1983) and that NMDA receptors have calcium channels. Experiments have so far investigated the role of calcium-sensitive protein kinases such as protein kinase C, protein kinase II and calpain 1 (Malenka, Kauer, Perkel, Mauk, Kelly, Nicoll and Waxham, 1989). Another potential messenger in the system is nitric oxide (for review, see Vincent and Hope, 1992).

Thus the mechanisms appear to exist, at least in the hippocampus, for the detection of coincident synaptic activity. Increased correlated activity would lead to increased NMDA receptor activation and increased synapse stabilisation. The influx of calcium could possibly trigger biochemical events resulting in synapse stabilisation.

6:2:6: Manipulation of NMDA receptors in three-eyed frogs

Chronic application of 2-amino-5-phosphonovaleric acid (APV), a specific NMDA antagonist, to the doubly innervated tecta of tadpoles or frogs with a supernumerary eye causes complete desegregation of the two retinal inputs (Cline, Debski and Constantine-Paton, 1987). The desegregation is not seen if the biologically inactive isomer of APV is used; and the effects of the active isomer are reversible. These results imply that NMDA receptors are specifically involved in the activity-dependent process of stripe maintenance. It had already been demonstrated that TTX with and without optic nerve crush also caused desegregation of eye-specific stripes implying that afferent activity is needed for occurrence and maintenance of eye-specific stripe segregation (Reh and Constantine-Paton, 1985). Interestingly, in the former experiment, extracellular recordings from the tecta of APV-treated animals were indistinguishable from normal animals, as was the morphology of the terminals. Therefore, NMDA blockade does not simply stop impulse transmission. The authors postulated that without active

NMDA receptors, co-activity of the RGC afferents would not be detected. Therefore synapses would no longer be stabilised and axon arbors would begin to migrate.

In contrast to the results of APV application, chronic application of non-toxic doses of NMDA to doubly innervated frog tecta produced a pronounced increase in eye-specific segregation (Cline, Debski and Constantine-Paton, 1987). Stripe boundaries became sharper, and there were fewer fusions and forks in the striped pattern. It appears that the continuous presence of an exogenous excitatory ligand for the NMDA receptor improves the ability of the system to distinguish correlated from non-correlated synaptic events. This result is in contrast to Shaw and Cynader's experiment (1984) in which the presence of the excitatory ligand (glutamate) prevented the usual shift in ocular dominance that occurs in monocular deprivation in the kitten.

The exact sites of the NMDA receptors are not known. Attempts have been made to localise them in *Rana pipiens* using autoradiography (McDonald, Cline, Constantine-Paton, Maragos, Johnston and Young, 1989). This method is relatively crude and cannot resolve the site of the receptors to the pre- or post-synaptic level. Excitatory amino acid receptors were found at high concentration in the pre-tectum. In the tectum, the highest density of labelled receptor binding was in the retinotectal synaptic zone, though binding was seen in other layers too. These results show that excitatory amino acid receptors are widespread in the frog retinotectal and pre-tectal systems and that they are present in the anatomically appropriate synaptic layer of the tectum. While their anatomical location provides circumstantial evidence for a role of NMDA receptors in synapse stabilisation, the evidence from the experiments involving the application of APV is more compelling. There is no conclusive evidence that the major action of APV is within the retinotectal synaptic zone though this would be the simplest conclusion.

The requirement for NMDA receptor activation for synapse stabilisation suggests that the associated rise in intracellular calcium ions might also be necessary for this process to occur. As mentioned above, the

transient influx of calcium ions into the tectal cell dendrites might activate calcium-sensitive protein kinases. The tecta of three-eyed tadpoles were exposed to a variety of protein kinase blockers for up to eight weeks. There was no desegregation of eye-specific stripes (Cline and Constantine-Paton, 1990). Therefore segregation can be maintained under conditions that significantly reduce protein phosphorylation as measured by ^{32}P incorporation into protein bands. It is possible that those protein kinases which escaped blockade were able to act in synapse stabilisation. Teleologically such a mechanism is important and it would not be surprising if there were a large safety factor built into the system. If, on the other hand, protein kinases are not involved in synapse stabilisation, how might calcium exert its effect? Although there was no desegregation of stripes, arbor morphology was affected. Treated retinal axon arbors covered about half the area covered by untreated arbors. Therefore protein kinases may well be involved in the growth of axon arbors. These enzymes are very widespread in nature and it is difficult to know how specific the effect of blockade is; or, for that matter, whether the observed effect has anything to do with calcium and NMDA receptors.

6:2:7: Arbor morphology and possible dynamics

Many of the pharmacological manipulations described above altered arbor morphology. APV-treated arbors were initially reported to be longer than untreated arbors in the doubly innervated tectum (Cline and Constantine-Paton, 1989). The stripes desegregated but there was no increase in the tangential area of the arbors. More detailed analysis led to different observations on arbor morphology (Cline and Constantine-Paton, 1990). APV-treated arbors were shorter and narrower than untreated arbors. Again, there was no significant difference in the tangential area of the treated arbors compared to the untreated, but there was a reduction in the number of terminal branches per arbor. The decrease in branch density was 35%.

At a concentration of $10^{-4}M$ in Elvax, a slow release polymer, NMDA treatment resulted in a 75% reduction in the number of axons crossing from a stripe to an interstripe zone. The arbors also had fewer branch tips.

Furthermore, NMDA treatment reduced the number of terminal branches by about 50%. In general, these arbors had a less elaborate branching pattern than the untreated or APV-treated arbors (Cline and Constantine-Paton, 1990). Both APV and NMDA reduced the rostrocaudal length of arbors in doubly innervated tecta, while NMDA did not alter arbor branch density in the normal tectum (Cline and Constantine-Paton, 1989).

How can these pharmacological experiments and the observations on arbor size and morphology be applied to a theory of synapse formation, stabilisation and maintenance in the retinotectal system? Chronic application of NMDA in three-eyed frogs decreases the NMDA sensitivity of the evoked tectal potential (Debski, Cline, McDonald and Constantine-Paton, 1991). Under these circumstances, acute application of NMDA decreased the evoked tectal potential to a lesser extent than it did in normal animals. Autoradiographic receptor counts suggested this was not simply a reflection of decreased receptor numbers in those animals which were treated chronically with NMDA.

Can this decrease in sensitivity of NMDA receptors be related to the observed change in stripe pattern and arbor morphology? As already mentioned, NMDA-treated three-eyed frogs have sharper stripes. Normally, correlated activity will converge onto a tectal cell and depolarise the cell sufficiently, via non-NMDA receptors, to open NMDA receptors to extracellular calcium ions. When the NMDA receptors are less sensitive, the chances of synapse stabilisation will be less. Therefore synapses will be stabilised only where activity is highly correlated since it is only in these areas that sufficient depolarisation will occur to allow NMDA channels to open. It is hypothesised that this will in turn lead to sharper stripes.

A model for the regulation of RGC terminal morphology by synaptic activity has been proposed (Cline and Constantine-Paton, 1990). Several aspects must be considered: branch initiation and retraction, synapse stabilisation and branch maintenance. Increased correlated activity would increase NMDA receptor activation, leading in turn to an increase in the synapse's lifetime. The greater the number of stabilised synapses that exist

within an arbor branch, the more likely it is that the branch will persist. In addition there is a decreased probability of branch initiation in that local region of the arbor.

How would this model explain the arbor morphology seen in three-eyed frogs treated with APV? If the APV is successful in blocking the NMDA receptors, there should be fewer surviving branches, and indeed APV treatment results in a 35% reduction in branch density. However, the model predicts that decreased receptor activation will lead to increased branch initiation. The latter factor must only partially counteract the former.

It is interesting that chronic NMDA treatment of two-eyed tadpoles did not change arbor density (Cline and Constantine-Paton, 1989). This does not exclude an action of NMDA at physiological concentrations on the arbors, but it may suggest that other factors such as the density of innervation are important. It is known that while the doubly-innervated tectum of a three-eyed frog has twice the number of RGC arbors, there is only a 40% increase in the tectal neuropil volume (Constantine-Paton and Ferrari-Eastman, 1987). Individual arbors from two-eyed tadpoles cover about the same tangential area as arbors from three-eyed tadpoles. Therefore there must be more overlap in the tangential plane among tectal arbors in three-eyed tadpoles. However, doubly innervated tecta support roughly the same number of synapses as the single tectum of the same animal (Constantine-Paton and Norden, 1986). Each arbor from doubly-innervated tecta has twice the branch number of an arbor in a normal tectum. Therefore there must be fewer synapses per branch on the arbor in a doubly innervated tectum, and, if a certain number of synapses is required for branch maintenance, then branches in a doubly innervated tectum will be more susceptible to branch retraction.

If greater overlap of arbors exists in doubly innervated tecta, the degree of correlated activity will be less in a unit of tectal volume. It is not known how much less and it is possible that the difference will be small. However, according to the model, decreased NMDA activation will lead to an increase in branch initiation. Consistent with this, branch density is

greater in doubly innervated compared to singly innervated tecta. The model also dictates that reduced NMDA receptor activation will lead to a decrease in synapse lifetime. It is proposed that the former factor outweighs the latter.

By breaking down synapse formation into its components, the model draws emphasis away from the dynamics of the arbor. In addition, these discussions have been conducted as if all arbors were uniform in their behaviour which is not the case. RGC have been classified on the basis of their firing pattern, morphology and the laminar distribution of their arbors. Eventually a model would have to take this extra degree of complexity into account. The possible significance of RGC subclasses is discussed further in section 6:2:9.

6:2:8: NMDA receptors and the goldfish visual system

Which neurotransmitters are present in the goldfish retinotectal system? It was already known that nicotinic acetylcholine (ACh) receptors are present in the layers of tectum that receive input from the retina. Using α-bungarotoxin, a specific post-synaptic blocker of nicotinic ACh receptors, Schmidt (1985) was able to show that retinotectal axons migrated out of the 'blocked' area. Subsequently, glutamatergic ligand binding sites have been characterised in the goldfish brain (Henley and Oswald, 1988). They were present in the optic tecta, as well as in cortex and cerebellum, though their numbers in the optic tecta were not decreased by enucleation of the eye. Alteration of the number of receptors might have been expected if NMDA receptors were part of the first relay of the primary visual system. Long term potentiation (LTP) was shown to occur in the goldfish optic tectum by Lewis and Teyler (1986). Subsequently, the role of NMDA receptors in the regenerating projection in the goldfish were investigated (Schmidt, 1990). The regenerating retinotectal projection was shown to have a greater capacity for LTP during the period when activity dependent sharpening of the map was occurring, though the mechanisms remain unknown. Specific blockers of the NMDA receptors blocked LTP, and chronic infusion of these blockers during regeneration prevented the sharpening of retinotopic

precision as detected by electrophysiological mapping. The infusion was set up at 21 days after optic nerve crush and lasted on average 15 days (that is, days 21-36 of regeneration), and fish were kept at 20°C. In the experiments described in this thesis, also in goldfish kept at 20°C, stroboscopic light prevented sharpening of the map, as detected by WGA-HRP labelling, beyond the area index seen at 32-39 days of regeneration in the control animals. Therefore the NMDA blockers were able to act earlier in regeneration to affect map sharpening than was stroboscopic light. According to the theory that correlated firing patterns amongst near-neighbours are important for map sharpening, stroboscopic light should cause synchronous depolarisation of the NMDA receptors throughout the tectum and thus widespread stabilisation of widely branching arbors. When arbor branches are not retracted, the map is less sharp. Since the agonists are able to act at the NMDA receptors in the presence of stroboscopic light, it is not surprising that NMDA blockade can have an earlier effect on the regenerating projection than can stroboscopic light.

6:2:9: Tectal lamination and its possible consequences for activity models

Until this point in the Discussion, a uniformity of RGC electrophysiological responses leading to locally correlated firing patterns has been assumed. In fact, these responses are far from uniform. RGC firing patterns in response to a variety of stimuli have been classified and will be discussed below. In addition, RGC terminal arbors are distributed non-uniformly in tectal laminae. Is it possible that semi-autonomous mapping of RGC classes could exist?

Retinal ganglion cells have been classified according to their responses to photic stimuli, though spots of light are not natural stimuli for the amphibian visual system, for example, in the way that a fly is. In the optic tectum of *Rana pipiens*, the responses of the terminations of five different types of RGC were found at four different depths in the tectal neuropil (Maturana, Lettvin, McCulloch and Pitts, 1960). Each class formed a topographic map in register with all other classes. Therefore, integration of the image was able to begin at the first visual relay. When degenerated optic fibres were stained after enucleation of one eye, they were seen in four

equally spaced sheets (*Rana catesbeiana*; Potter, 1972) confirming earlier studies of lamination. These layers correspond to what are now known as tectal laminae B, D, F and G. Witpaard and ter Keurs (1975) described four classes of terminations regularly distributed through the tectal depth, their classification being based on electrophysiological responses. Each class of termination had a different operation: contrast, slow ON-OFF, fast ON-OFF and OFF. The authors correlated these four classes with the four sheets of terminations seen after enucleation.

Further studies of the tectal lamination patterns in fish and frog have extended the classification. For example, HRP-labelled RGC axons in *Rana pipiens* (Hughes, 1990) were found in all of the superficial tectal layers, A-G, both as axons of passage and as terminations. From layer to layer, the RGC axons differed markedly in the diameter of their parent axons and in the morphology and horizontal extent of their terminal arbors. This extension of the anatomical classification is difficult to correlate with previous electrophysiological findings. Stirling and Merrill (1987) combined intracellular recording with HRP labelling of the larger, class IV or "dimming" detectors in *Rana pipiens*. They found these cells had large tectal arbors in layer 8, below layer G, having already given off smaller, pre-tectal arbors. Despite similarities in morphology, these cells were not functionally uniform, with a wide variation in receptive field size, and two types of response to reversal of square wave gratings. Although class IV cells of temporal retinal origin were seen to enter rostral tectum at the deep layer (8) where they synapse, this was not the case for nasal fibres which terminate caudally. These entered the tectum superficially and made a 'stepping descent' to their termination zone. Although the rostrocaudal dimensions of the growing fish and frog optic tectum have been well characterised in terms of hypertrophy and hyperplasia (Raymond and Easter, 1983), the change in laminar depth is less well understood. It is possible that the stepwise descent is a manifestation of the combination of changes in laminar depth and caudal migration of axon terminals with growth. Some axons ran superficially and had small descending branches which may have represented previous termination sites that existed during caudal migration. However, this is speculation. Interestingly, some of the class IV cells were

seen to have two termination sites. These could have represented the establishment of a second termination before retraction of the first, or an 'error' in termination site which had not been corrected. The significance of the double termination sites also remains speculative. Thus general aspects of morphology in this class of cell were similar, but there were differences in presumed axon paths and functional differences detected electrophysiologically.

There are no simple correlations to be made of the structure and function of the RGC terminal arbors in the optic tectum. For example, Stirling and Merrill (1987) observed seasonal changes in the responses of the class IV neurons, though the morphological basis of the changes was not clear. Therefore investigation of the possibility of semi-autonomous mapping of the different classes of RGC would be difficult.

In addition, the connections of the RGC terminal arbors onto tectal cells are far from clear. It is not known whether only one type of tectal cell receives retinal input, whether the tectal cells which are directly contacted are interneurons or whether they form the main efferent pathway, or whether multiple types of retinal cell converge onto one tectal cell. Incidental trans-neuronal transport of HRP via the synapses in the optic tectum of *Rana pipiens* has shown that at least three cell types receive a direct retinal input (Hughes and Hall, 1986).

Laminae are present in the goldfish tectum also (Kageyama and Meyer, 1988a). These laminae, and indeed the sublaminae, were re-established following optic nerve regeneration after optic nerve crush (Kageyama and Meyer, 1988b). Therefore if there is semi-autonomous mapping of the different RGC classes, a process which would be more active in the regenerating than in the developing system according to the model of Willshaw and von der Malsburg (1976), it would appear to be able to re-establish the map in all layers.

Simultaneous recordings from neighbouring RGC in goldfish in the dark showed that for most of the pairs of cells with overlapping receptive

fields the spontaneous discharges were statistically correlated (Arnett, 1978). Pairs of cells had one of two types of receptive field and these receptive fields were oppositely organised. Therefore, while a pair with one type of receptive field was likely to be firing together, a nearby pair with opposite receptive field type was likely to be silent. These recordings were made from isolated retinae. However, if the findings can be extrapolated to the developing or regenerating systems, and if the firing patterns are relayed faithfully to tectal cells, the anatomical and physiological bases for semi-autonomous mapping of different classes of RGC are probably present.

6:3: The possible molecular bases for chemospecificity

Following some of the earliest experiments on the fish and amphibian visual systems, Sperry (1963) invoked the theory of chemospecificity to explain the findings that regenerating RGC form a new retinotopic projection. Do marker molecules exist that are capable of directing axons in development and regeneration? If they exist in one species, can similar molecules be found in other species? What is their biochemical nature?

These questions have been addressed in elegant *in vitro* experiments, many of which involve axons of explanted RGC. The axons could be seen to grow preferentially on certain monolayers which were arranged either as a Y-junction or in stripes. Qualitative and quantitative analyses are possible in such experimental systems. It is possible to describe the axons and their growth cones, and also to measure rates of growth and retraction and the absolute numbers of axons choosing each monolayer. It is emphasised that these are *in vitro* systems and extrapolation to the *in vivo* situation must be carried out with caution.

Axons growing out from chick retinal explants, when given the choice between two cell monolayers consisting of either retinal or tectal cells, grew preferentially over tectal cells (Bonhoeffer and Huf, 1980). Telencephalon monolayers were no more attractive for retinal axons than retinal monolayers, and much less attractive than tectal monolayers. The preference for tectal cells was not absolute, but this experiment demonstrates that retinal axons can discriminate *in vitro* between neural cell types. It is interesting that the retinal axons should prefer their *in vivo* target. Cell sorting experiments suggested that the preference was specific to the outgrowing axons, since for the cell perikarya retina-retina adhesion was greater than retina-tectum adhesion. *In vivo*, the axons of RGC must first pass across the retina to reach the optic nerve head, and the nerve chiasm and tract must be negotiated before the tectum is reached. For a putative marker to be effective, if the *in vitro* results are extrapolated to the developing animal, it would have to exert its effect over a long distance or to exert its effect at a certain time in the development of the projection.

Given that some distance must be covered by the axon before the tectum is reached, how might the axons interact with each other *en route*, or when they have reached the tectum? Again, using chick embryo retinal explants, Bonhoeffer and Huf (1985) demonstrated that growth cones of axons from the temporal half of the chick retina grew preferentially along temporal axons, whereas growth cones from the nasal retina did not distinguish between nasal and temporal axons. Similar experiments using dorsal and ventral retinal explants failed to show any position-specific effects. Since dorsal and ventral axons are separated in the brachia, this finding is surprising. A substrate preference may underlie the 'choice' of brachium. *In vivo*, in the goldfish, temporal axons course over rostral tectum whereas nasal axons form age-related bundles. The organisation of nasal fibres may therefore be a more passive reflection of the age of the axons and the patterns of tectal growth. Temporal axons, in contrast, have to shift caudally as there is no histogenesis at the rostral tectal margin, and thus the process may be more active.

Part of Sperry's original chemospecificity hypothesis involved the presence of gradients within the system. This has persisted as an attractive hypothesis in establishing at least the boundaries of the map, allowing other mechanisms to act within their confines. If the gradient were fine enough then other mechanisms might not be necessary.

The presence of gradients was investigated using explants of embryonic chick temporal or nasal retina which grew onto tectal cells from various points along the rostrocaudal axis of the chick tectum (Bonhoeffer and Huf, 1982). Temporal axons, unlike nasal axons, preferred anterior tectum. Given a choice of monolayers from different tectal regions, the temporal axons always grew onto the cells from more anterior tectum. Cells from non-innervated tecta showed the same position-dependent differences as innervated tecta. This implied that the gradient was not simply a reflection of a gradient of density of innervation, with more axons terminating anteriorly at this stage of development. It also implied that previous innervation was not necessary to 'label' the tectal cells for the gradient to be recognised *in vitro*. The gradient appeared to be intrinsic to the tectum.

Although the question of a tectal gradient was not specifically addressed in Schmidt's experiments implying labelling of the tectum by retinal fibre innervation (Schmidt, 1978; section 1:6:3), this *in vitro* work provides further evidence that such a labelling process does not occur.

Interestingly, when given the choice of retinal and tectal cell monolayers, axons from nasal retina clearly preferred the tectal monolayer, whereas axons from temporal retina did not show such a preference (Bonhoeffer and Huf, 1982). Thus axons of nasal and temporal retina have different properties. It is possible that *in vivo* the nasal axons have only to detect the tectum, whereas the temporal retinal axons have to detect the anterior tectum.

The preference of growing axons for membrane-associated positional specificity was demonstrated using an *in vitro* assay of membrane fragments arranged in stripes (Walter, Kern-Veits, Huf, Stolze and Bonhoeffer, 1987). The authors showed, using 'carpets' of alternating membrane stripes from anterior and posterior chick tectum, that nasal retinal axons showed no preference, whereas temporal retinal axons preferred anterior tectal membranes. When the carpets were heat-treated, temporal retinal axons no longer showed a preference; and treatment of posterior membranes alone was sufficient to abolish the preference for anterior tectal membranes. This result is best explained by the presence of a factor in the posterior tectal membranes which repels temporal axons, and heat treatment appears to destroy this factor. These properties of the tectal membrane preparation were independent of tectal innervation by retinal axons and were seen only during the period of development of the retinotectal projection. The latter point is in favour of the membrane-associated factor(s) being relevant to the developing projection, as is the observation that the gradient which was demonstrated was in the appropriate direction. Again, no preference of dorsal or ventral axons for lateral or medial tectal membranes was demonstrated. Such a preference might be shown if the optic tract were investigated.

When purified membranes isolated from anterior and posterior thirds

of the embryonic chick tectum were added to retinal explants, it was found that temporal growth cones reacted strongly to posterior membranes by retracting and collapsing (Cox, Muller and Bonhoeffer, 1990). Similar preparations from the anterior third of the tectum showed much weaker activity, and nasal growth cones advanced in the presence of membrane preparations from either the posterior or anterior tectum. Heat treatment removed the ability of the posterior membrane preparations to induce growth cone collapse in temporal retinal axons, a result which parallels that described above for the membrane stripe preparation. It is possible that the factor inducing growth cone collapse and the repulsive factor in posterior membrane described in the stripe assay are identical.

A possible candidate for the repulsive factor has been characterised further (Stahl, Muller, von Boxberg, Cox and Bonhoeffer, 1990). Antibodies raised against tectal membranes abolished the repulsive factor of posterior tectal membranes, to which temporal retinal axons are sensitive. Analysis of tectal membranes by two-dimensional gel electrophoresis and immunoblotting revealed a 33 kilo Dalton glycoprotein that had a higher concentration in posterior than in anterior tectum. Furthermore, temporal retinal axons avoided the lanes containing the 33kd glycoprotein; and when vesicles without the 33kd glycoprotein were used, no avoidance reaction was observed. As well as being sensitive to heat, the activity of this molecule was also abolished by phospholipase C. Although the data were not shown, nasal axons showed a similar avoidance reaction in the presence of the 33kd glycoprotein. Thus there is no all-or-none response of axons from different retinal regions to this putative guidance molecule. *In vivo* the reaction to the glycoprotein of the nasal axons may be different: in the experimental system the molecule has been concentrated and incorporated into vesicles.

Interestingly, temporal fibres grow almost equally fast on purely anterior or purely posterior membranes (Walter, Muller and Bonhoeffer, 1990). This shows that when given a choice between anterior and posterior tectal cells, temporal axons will prefer the former probably because the latter contain higher concentrations of a repulsive factor. However, this factor is

not completely inhibitory.

When the putative guidance molecule was incorporated at different concentrations into membrane preparations (Baier and Bonhoeffer, 1992), gradients of different slope could be established and the responses of temporal axons observed. Continuous exposure to the repellent gradient impaired elongation of temporal fibres, while nasal fibres were not affected. Interestingly, there was a sharp cut-off in this behaviour at the mid-point of the nasal-temporal retinal axis. Also, temporal axons were able to grow down the gradient at a steady rate; elongation was only affected if the gradient was uphill. Differently shaped gradients brought about different responses from temporal axons. At a shallow enough gradient there was no response, and if the gradient was steep enough the temporal axons were unable to progress past this point. The mechanism by which the growth cone detects the repulsive factor, its concentration or its relative concentration is not known.

While most of the early *in vitro* experiments were carried out on cultures from chick embryo, similar results have been found in other classes of animal. For example, RGC from adult goldfish have been shown to recognise position-specific differences in membrane preparations from the tectum (Vielmetter and Stuermer, 1989). As in the chick embryo experiments, nasal axons showed no preference for stripes of rostral or caudal tectum, while temporal axons showed a preference for rostral membranes, their retinotopic target. Again temporal axons could grow on caudal tectum if no rostral tectum was present. Heat treatment of caudal tectum, designed to destroy a putative repulsive guidance molecule, was unsuccessful in that the membrane preparation lost its ability to sustain growth at all.

Other cross-species similarities have been demonstrated. While the membrane properties of the chick tectum were restricted to a period of embryonic development, it is interesting that similar properties were seen in the adult goldfish preparations. Unlike chick, retinal and tectal growth persist in the adult goldfish, therefore persistent guidance molecules may be

necessary. Part of the capacity for regeneration in the goldfish visual system may lie in the persistence of marker molecules into adult life.

The guidance molecules in adult fish and embryonic chick have similar properties. Adult fish axons respond to the repulsive component of chick membranes (Vielmetter, Walter and Stuermer, 1991). The guidance molecule on fish and embryonic chick membranes is abolished by treatment with phospholipase. The molecules are similar in distribution, in their effect on temporal axons and in their linkage to membranes.

Similar experiments comparing mouse and chick embryos have shown that when mouse retinal fibres grew on chick membrane preparations, or *vice versa*, the same preference for anterior tectal (chick) and anterior collicular (mouse) membranes was shown by temporal axons (Godement and Bonhoeffer, 1989).

Thus these properties and molecules are preserved across species, which is a teleological argument for their importance. Their expression in relation to development and growth of their respective visual systems is circumstantial evidence for their role in establishing the projection. However, demonstrating that these properties exist does not prove that they act in development. Experiments in the future should involve further biochemical characterisation and specific blockade of putative guidance molecules.

Another approach to the presence of marker or guidance molecules on the tectum is to 'trawl', using monoclonal antibodies, through molecules separated out from tissue extracts, in this case retinal or tectal extracts. Any monoclonal antibody showing a relevant binding pattern could then be characterised further, and the molecule which it recognises could be identified (Stirling, 1991). The recognition of a developmentally important molecule by a monoclonal antibody is not without difficulties. Small epitope changes could render the assay negative: this could be important in cross-species differences. Small quantitative changes in the marker molecule could be missed because the sensitivity of the assay is too low.

Examples of molecules which have been identified in this way are TOP (derived from 'toponymic') and TRAP (temporal axon protein). Both are present asymmetrically across the retina. The distributions of these molecules in time do not fit well for a role in axon guidance, nor have they been shown to affect axon growth. Since gradients have been shown to exist in the tectum, molecules should perhaps be sought here rather than in the retina. It has been possible to generate antibodies against the 33kd glycoprotein which has been shown to be a possible candidate for a guidance molecule. It would be interesting to know the effect of this antibody on development.

There is thus good evidence that the growth cones of retinal axons show regional specificities in their recognition of membrane-associated molecules and in their response to these molecules *in vitro*. While there are teleological arguments for the importance of such mechanisms, their role in development and regeneration remains unclear.

6:4: Binocular maps in the frog

The role of visual experience in the formation of binocular projections in frogs has been extensively studied. In the *Xenopus* tadpole there is no overlap of the visual fields, but after metamorphosis, when the eyes move rostrodorsally, there is eventually 170^0 of binocular overlap (Udin, 1985). The projection from the *nucleus isthmi* (NI) to the tectum forms part of the polysynaptic relay from the eye to the ipsilateral tectum (Grobstein, Comer, Hollyday and Archer, 1978), so there is a map of the left tectum's visual field on the right tectum, and *vice versa*. This map is in register with the retinotectal map from the contralateral eye, and this matching of maps forms part of the anatomical basis for binocular vision. In the adult, points receiving information from one locus of binocular visual space on the tecta are linked. As the eye position alters during metamorphosis, the points on the tecta will change and ipsilateral units will be found in the expanding binocular region of the tectum. Therefore to maintain correctly registered binocular vision throughout development, the intertectal link via the NI must also change. Electrophysiological mapping at different stages of development has shown that this is the case (Grant and Keating, 1986). At all stages the ipsilateral visuotectal projection arose only from the binocular portion of the tectal surface, and the maps were in register at all stages studied.

The pattern of growth of the NI in *Xenopus* has been studied (Udin and Fisher, 1985). Tritiated thymidine labelling showed that cells are generated throughout tadpole life in a ventrodorsal gradient. Thus the majority of NI cells are generated long before ocular migration occurs around metamorphosis. HRP labelling showed that tectoisthmotectal connections were also present long before ocular migration. Mapping of these connections in the ipsilateral projection showed that the oldest NI cells project near to the oldest tectal cells, but the youngest NI cells project to the rostromedial pole of the tectum well away from the youngest tectal cells (the oldest tectal cells are situated rostrolaterally). None of the patterns of growth of the three structures, retina, tectum or NI, matches completely. Therefore, there is a need for shifting connections in map systems other than the direct

retinotectal system.

The crossed isthmotectal projection has been studied in adult *Xenopus* at the ultrastructural level after labelling the NI with HRP (Udin, Fisher and Norden, 1990). Most isthmotectal axons were shown to synapse with post-synaptic elements which conformed to morphological criteria for dendrites. Retinotectal axons are known to influence isthmotectal map formation: when the retinotectal map is rotated the contralateral isthmotectal map rotates to come into register with it (Keating, Beazley, Feldman and Gaze, 1975), and visual input is necessary for this to occur (Keating and Feldman, 1975). The link appears to be indirect, via tectal cell dendrites. Such synapses are present in the tadpole before there is any binocular field (Udin, Fisher and Norden, 1992), and they may mediate registration of the binocular maps that depends on the visual environment.

In order to test the hypothesis that binocular visual experience is important for map registration, *Xenopus* tadpoles were raised in total darkness: though, of course, dark-rearing does not strictly test binocular aspects of visual experience. Their visual maps were assessed after metamorphosis by electrophysiological methods and by HRP histochemistry (Grant and Keating, 1989). Early visual deprivation by dark-rearing resulted in abnormalities in the ipsilateral visuotectal projection. Although perimetamorphic eye migration and direct retinotectal projections were not affected, dark-rearing led to poor registration of the retinotectal and isthmotectal maps. In addition, multi-unit receptive field size was increased in the ipsilateral visuotectal projection in dark-reared animals compared to age-matched controls (see also Keating and Kennard, 1987). It appears, therefore, that other developmental processes programme an initially ordered intertectal system but that visual experience is necessary for some aspects of the refinement of binocular visual registration.

It has been proposed that there is selective stabilisation of isthmotectal terminals by the correlation of retinal and isthmic activity, possibly by the convergence of retinotectal and isthmotectal synapses onto common postsynaptic dendrites. Both the retinotectal and crossed

isthmotectal axons are driven from the same locus of visual space and therefore may have similar patterns of neural activation. It is possible that the latter axons may have their firing patterns enhanced or attenuated in the polysynaptic pathway which includes tectal dendrites. It has been postulated that, as with the retinotectal projection in which neighbouring axons may synapse close to each other because coincident firing patterns probably reinforce the synapses, coincident activity can link the retino- and isthmotectal maps to yield the basis for binocular visual integration (Scherer and Udin, 1989).

A contribution to the stabilisation of synapses is thought to arise through activation of synapses involving excitatory amino acids. Retinotectal but not isthmotectal axons are at least in part glutamatergic. It has been proposed that correlated activity from the two eyes at each tectal location will stabilise the appropriate isthmotectal terminals through retinal activation of NMDA receptors on the tectal cell dendrites. Thus the tectal cells and in particular their dendrites would have a crucial role in aligning the maps. In order to test this hypothesis, NMDA and specific antagonists have been applied to the tectum during *Xenopus* development (Scherer and Udin, 1989). It had previously been shown that unilateral eye rotation in midlarval life caused the ipsilateral map to be reoriented to match the contralateral map (Keating, Beazley, Feldman and Gaze, 1975). Each ganglion cell in the rotated eye projects to its normal tectal target and therefore relays abnormal visual input. When the isthmotectal projection is confronted by the rotated retinotectal projection, axons are re-routed till they synapse in areas where there are matching receptive fields (for review, see Udin, 1985). When APV, a specific NMDA antagonist, was applied to this system, ipsilateral maps did not come into register with rotated contralateral maps in developing *Xenopus*. NMDA, however, did allow the two maps to come into register. It therefore appears that NMDA receptors can play a role in the experience-dependent alignment of binocular maps in the developing *Xenopus* tectum, since when these receptors are blocked a result very similar to that seen in dark-rearing is seen. Although APV prevented map alignment, the isthmotectal map was ordered, again implying that mechanisms other than NMDA-mediated receptor activity are important for the formation of these

maps.

There is a critical period from late tadpole to early juvenile stages during which visual input is important for the matching of the visual maps from the two eyes on the same tectum (Udin and Scherer, 1990). At eight months after metamorphosis, past the normal end of the critical period, the left eye was rotated by 90^0. NMDA in Elvax was applied to the right tectum on the same day. Control animals had no implant or Elvax alone. While there were signs of reorganisation within the binocular maps of control animals, with parts of the ipsilateral maps being disorganised, these animals could not use binocular activity cues to realign the map. However, in NMDA-treated animals the ipsilateral units had shifted to be in register with the contralateral map. The plasticity which is present in this experimental system may therefore end because the number of NMDA receptors is reduced at the end of the normal critical period. Exogenous NMDA may cause up-regulation of NMDA receptors permitting activity from the same locus in the binocular field to be recognised so that isthmotectal neurons can re-synapse on the 'correct' tectal dendrites.

The critical period corresponds to the time at which eye position changes with head growth at metamorphosis. Surgical rotation of the eye in larval *Xenopus* led to reorientation of the isthmotectal projection but this was not seen 3 months or more after metamorphosis, and, in the period from metamorphic stage 60 until 3 months post-metamorphosis, altered intertectal connections were only seen after progressively smaller degrees of eye rotation (Keating and Grant, 1992). The critical period therefore corresponds to a normal developmental requirement for inter-tectal plasticity.

This plasticity is not strictly age-dependent. Dark-rearing from early embryonic stages through the critical period, or even for just the critical period itself, prolonged the time during which inter-tectal plasticity was observed (Grant, Dawes and Keating, 1992). Thus the requirement for visual experience to lead to a reorientation of the intertectal projection in this experimental system exists for only the critical period.

Dark-rearing and NMDA receptor blockade by APV have similar effects on the developing visual projection in *Xenopus* as assessed by rotation experiments. The directness of the link is not known. For example, it is not known how active NMDA receptors are in dark-reared animals, if they are active at all. APV is considered a means of blocking the activity-dependent stabilisation of developing intertectal terminals, a process considered to be based on similarities of firing patterns among the binocularly driven tectal cells. Blockade of this process leads to a lack of realignment of the intertectal map after eye rotation. This is not surprising since the polarity of the whole map has to be altered rather than the self-order of the axons within the map. However, it is surprising that the plasticity of the binocular maps can be prolonged by the application of NMDA after the critical period, again because rotation of the intertectal map is involved. And why, in the absence of exogenous NMDA, do the maps stay out of register in the postmetamorphic animal? It is possible that the effect of exogenous NMDA is much more widespread than has been appreciated, either within the maps being studied or in other mapping systems linked to the visual system but so far unidentified.

This experimental system is interesting in that it provides further evidence for shifting of connections in order to incorporate the changes which occur during the growth and maturation of *Xenopus*, and because it provides further evidence for the importance of the visual environment in the normal formation of the intertectal relay. NMDA and its specific antagonist APV have been shown to have an effect in this system. Their site of action is likely to be the tectal cell dendrite. However, the precise way in which the maps "use" NMDA receptors to come into register with each other is far from clear.

6:5: The interaction of maps of different sensory modalities

The role of experience in modulating the maps in other systems has been extensively studied. I have shown that visual experience in regeneration in the goldfish can affect the precision of the resulting map. In birds and mammals, where a similar regeneration model does not exist, the developing animal must be studied. The barn owl, *Tyto alba*, has been used as an experimental model, in particular for its auditory, visual and motor maps in the midbrain.

Visual, auditory and motor maps are integrated in the barn owl superior colliculus, a midbrain structure equivalent to the optic tectum in the goldfish (Knudsen, 1982). This enables the animal to turn its head and ears towards a subject of interest. The eye itself remains in line with the head and does not move independently of the orbit. The contralateral visual space is represented on the superior colliculus in an arrangement similar to that of the goldfish visual system. The barn owl has a large area of binocular visual field. In the portion of the map on the superior colliculus representing the zone of binocular vision, 50% of the superficial layer units and 100% of the deep layer units were found to be binocularly driven (Knudsen, 1982). In addition, the frontal binocular region of space was greatly expanded. Superficial and deep tectal units also responded to auditory stimuli, most receptive fields containing a 'best area' where a sound source was most effective at driving the unit. Auditory space mapped topographically onto the tectum. The visual and auditory maps were in register, especially anteriorly and dorsally on the tectum. Posteriorly and ventrally the maps were found to be progressively misaligned. The auditory map represents larger areas of space than the visual map therefore a compromise may have to be reached between maps in perfect register and a tendency for the maps to spread over the tectal space available. While a visual map results from the point-to-point projection of the retina onto the tectum, the auditory map results from the neural responses to differences in the intensity and the timing of sound at the two ears.

Owls were raised with one ear occluded. Normal auditory cues were

therefore disrupted. Mapping these animals as adults demonstrated that auditory and visual maps were aligned as long as the earplug was in place. When the ear plug was removed, best auditory areas and visual receptive fields were misaligned, indicating that a change in auditory spatial tuning had occurred during the period of occlusion. When occlusion of one ear was carried out in the adult owl, even after one year the auditory best areas and visual receptive fields were misaligned and were aligned only when the earplug was removed. Therefore there appears to be a sensitive period in the young barn owl when abnormal auditory cues will modify the auditory spatial tuning of tectal units (Knudsen, 1985).

When the earplug was removed from a juvenile bird which had been raised with monaural occlusion, the visual and auditory maps became aligned over a period of weeks. This did not occur in the adult (Knudsen, 1985). These results indicate that there is also a critical period during which restoration of the normal auditory cues will cause modification of map alignment.

Interestingly, neuronal tracing with HRP showed that neurons in the auditory pathway linking the superior and inferior colliculi were anatomically the same as in the control animals. This suggests that the experience-dependent map realignment must have occurred at an earlier level in the pathway (Knudsen, 1985). However, this was demonstrated in a single animal. If this finding is repeated, experience-dependent changes could have occurred in earlier sites of binaural interaction and all subsequent processing steps could be as normal.

Not only do abnormal auditory cues lead to abnormal auditory 'best areas' on the tectum. There is also evidence that abnormal visual cues lead to abnormal auditory best areas. Auditory maps were compared in normal owls and those raised with both eyelids sutured closed (Knudsen, 1988). In spite of normal hearing, the latter group developed auditory maps which were abnormal in topography and precision. Exposure of a baby owl to a displaced visual field by applying binocular prisms resulted in a shift of sound localisation in the direction of the visual displacement (Knudsen and

Knudsen, 1989). Therefore when a young owl receives discordant information from visual and auditory cues, the visual cues appear to be dominant in the process of map alignment. Again, this developmental plasticity diminishes with age (Knudsen and Knudsen, 1990).

Characterisation of the motor map in the blind-reared owl showed that it was also abnormal in its topography. The abnormalities, however, were different from those demonstrated in the auditory map. Therefore early blindness affects the two maps in different ways (Knudsen, Esterly and du Lac, 1991).

Similar experiments have been performed on the ferret superior colliculus which also contains topographically aligned visual and auditory maps (King, Hutchings, Moore and Blakemore, 1988). In ferrets raised with abnormal binaural cues, the auditory and visual maps are approximately in register in the adult. If, in the young animal, one eye is deviated laterally, there is a compensatory shift in the auditory map. Early eye rotation totally disrupts the auditory map in the adult. Thus the ferret's superior collicular maps show similarities to those in the owl. Again there is a critical period in that eye rotation at a slightly later phase does not change the topography of the auditory map.

The alteration of map precision and alignment in response to visual and auditory experience is widespread, from fish (regeneration) to mammals (development), though in the latter the period of sensitivity is restricted.

6:6: Map refinement

There is evidence from the data presented in this thesis that map refinement occurs during re-establishment of the retinotectal projection after optic nerve cut. Clusters of filled RGC became smaller and scattered cells were eliminated in those animals which were kept in diurnal light. How might map refinement be achieved?

Cell death: Axons which have not terminated in the correct place may be eliminated and the RGC may die. This possibility would be unlikely if counts of the RGC remained the same. The contribution from continuing neurogenesis at the retinal periphery is likely to be negligible under standard laboratory conditions at periods of up to eight weeks after optic nerve cut. At periods of, say, up to 5 years after optic nerve cut, there may be a larger contribution from newly generated RGC; though if the map has not resolved further, the question is less relevant. Data from the experiments described above on cell counts in retinae up to 500 days after optic nerve cut, with the fish being kept in standard conditions, showed that there was little difference in RGC numbers between the normal and regenerated retinae.

Branch elimination at the tectal level: In order to increase the chances of finding its nearest neighbour, an axon may extend branches in several directions on the tectum. The growth cone that finds its nearest neighbour would be favoured and other branches would be eliminated. Extensive branching could be demonstrated by anterograde tracing with HRP. In the case of retrograde labelling, for example with WGA-HRP, more extensive branching would increase the chances of a particular RGC being labelled after an iontophoretic injection. This would be reflected by the total number of labelled cells reducing as map refinement takes place. Other changes in uptake properties of the terminals could obscure this. The data presented showed that the total number of labelled cells did not fall significantly with time after optic nerve cut.

Branch elimination in the brachia: The regenerating optic nerve may again increase its chances of finding its correct termination site by sending a branch down each brachium. Labelling RGC from a whole brachial lesion in an animal with a regenerated optic nerve would demonstrate whether RGC have axons in the 'correct' brachium or not. We know that in the normal goldfish axons from dorsal retina are almost all confined to the medial brachium. Double labelling would show whether one RGC could be doubly labelled from different brachia. This would be evidence for separate branches in the brachia.

Reducing the overlap of terminal arborisations: The precision of the map could be increased by retraction of the terminal branches of the arborisations themselves, as opposed to branches of the axons. Action potentials in a RGC axon would have a more precise effect spatially. If extensive overlap of terminal arbors did occur, it could be demonstrated directly by anterograde HRP tracing. Retrograde WGA-HRP labelling in the tectum would again be expected to result in a greater total number of labelled cells early in regeneration compared to later stages.

Although cell loss is a feature of regeneration and map refinement in some species of frog, cell counts have shown that there is little change after regeneration in the goldfish. Cell death is a feature of the developing mammalian visual system (O'Leary, Fawcett and Cowan, 1986). Initially there are diffuse projections with excess axons: later these are refined by collateral loss or cell death.

The role of cell death in the projection of the isthmooptic nucleus (ION) in the developing chick visual system and its reliance on neural activity has been investigated. The ION projects to the contralateral retina, the axons reaching the retina at embryonic day 9 (E9). The axons reach their target cells in the amacrine sublayer of the retina between E12 and E13. The ION then takes on a laminar appearance; and between E12 and E16 about 55% of the ION neurons die. Initially, a small proportion of the ION projects to the 'wrong', ipsilateral retina, and a much larger proportion to topographically inappropriate parts of the contralateral retina. These cells

are the ones that die (Catsicas, Thanos and Clarke, 1987). It was proposed that the elimination of these inappropriately targeted cells by cell death was a means of refining the map by the elimination of targeting errors. The process of cell death was shown to be affected by intraocular injection of TTX. By eliminating neural activity in the contralateral retina, the destination of the ION cells, it was shown that both neuronal death and ION lamination were affected, the effect on lamination being seen at a lower dose of TTX (Pequignot and Clarke, 1992a). Retrograde tracing, using the fluorescent dyes Di-I and rhodamine isothiocyanate, showed that the axons that initially make targeting errors were the ones that had survived in the presence of TTX (Pequignot and Clarke, 1992b). It was concluded that electrical activity plays an important role in the elimination of axonal targeting errors in the chick embryo's isthmooptic system.

6:6:1: Branch retraction in the brachia

It has been shown (Becker and Cook, 1987) that, in the initial stages of regeneration, ganglion cells can be labelled from the inappropriate brachium just as easily as from the appropriate one. This was first demonstrated by retrograde HRP labelling of ganglion cells from a cut brachium. As regeneration progressed, the proportion of labelled ganglion cells in the inappropriate half retina had fallen to approximately 25%, still considerably more than the 1.5% seen in the normal fish. This process of brachial refinement could be achieved in a number of ways. Axons could regenerate at different rates and those which grew more slowly could be more selective in their choice of brachium. If this were so, the total number of labelled cells should increase during refinement. This was not the case.

In theory, there could be a more selective contribution from newly generated, that is, marginal RGC. However, labelling showed no dorsoventral marginal RGC bias during regeneration, and, as discussed above, the numerical contribution is likely to be small.

A more likely explanation of these findings is that axons send more than one branch to the optic tectum, with some of these branches taking the inappropriate brachium. Becker and Cook hypothesised that if one of these

branches established a mature terminal arbor in the correct place, this branch would survive and the other ineffective branches would be retracted. Since taking the correct brachium would increase the chances of the correct termination site being found, branches which take the correct brachium are more likely to remain intact, leading to the pattern of brachial refinement which was seen.

Axon counts on the tectum have suggested substantial axon proliferation (Murray and Edwards, 1982). Anterograde transport of HRP by optic axons was used to characterise the retinal projection in the SFGS. Optic nerve crush resulted in a marked decrease in SFGS thickness. Three weeks after optic nerve crush massive numbers of regenerating optic axons were seen in the SFGS. The area occupied by regenerating axons and the number of terminals in the tectum only approached the normal level three months after optic nerve cut. Thus there is evidence that map refinement in part results from a pruning of exuberant tectal innervation. However, these studies relied on counts of neuronal profiles which can be confounded by axon tortuosity and branching.

Axon counts in electron micrographs showed that after optic nerve crush the number of axons in the nerve distal to the lesion increased fourfold during the first three months of regeneration (Murray, 1982). The number of axons then returned towards pre-operative levels. While regenerating axons in the nerve gave rise to, on average, four branches, if the profile counts are accurate, the area occupied by regenerating axons in the tectum increased twenty times (Murray and Edwards, 1982). If the axon counts are accurate this implies that collaterals may arise distally as the optic nerve regenerates.

The implications of the results of Becker and Cook (1987) are interesting. In the introduction to this thesis, evidence was put forward that although the chemospecificity hypothesis cannot explain all the features of the goldfish retinotectal system, there is significant evidence that chemospecificity operates at the level of the optic tract. Now it appears that in the regenerating system axons show no initial preference for their usual

brachium. It is possible that a mechanism which is important during early development when axon numbers are small becomes less so later in development. In regeneration it is also possible that refinement of the map at the tectal level leads to brachial refinement, a reversal of the mechanisms thought to occur during development. It is an intriguing thought that such mechanisms may operate during development also. In this case the process would be more gradual, since at the stages studied (late larval and adult) the only axons which would be involved would be a relatively small proportion of the total, that is, those recently generated at the retinal margin. To detect whether such a mechanism does make a significant contribution during development much younger animals need to be studied.

Stuermer and Raymond (1989) studied the developing retinotectal projection in larval goldfish. They showed that, in young larval tecta, there are short side branches on the axon shaft proximal to the terminal arborisation. In older larvae, these side branches were not seen. It appears that the axonal branching in the optic tract, which is a feature of the regenerating system, is not a feature of the developing system.

Double labelling has been used to study axon collaterals in the regenerating optic tract of the goldfish (Becker and Cook, 1988). Separate retrogradely transported fluorescent dyes were introduced at each of the brachia, at different stages of optic nerve regeneration. In normal animals, as expected, the label Diamidino Yellow applied to the lateral brachium was confined to dorsal retina, and the label Fast Blue applied to the medial brachium was confined to the ventral retina. Double labelled cells were not found, implying that individual RGC did not have axons in both brachia. In fish labelled early in regeneration, both dyes were spread over the entire retina and cells were singly and doubly labelled. Later in regeneration each dye came to dominate the appropriate retinal region and doubly labelled cells became harder to find. At 60 days after optic nerve cut and subsequently, doubly labelled cells were rare and were confined to the area where dorsal and ventral retina meet. This implies that axon collaterals are gradually eliminated from the inappropriate brachium during optic nerve regeneration given that the axon's ability to take up the label does not alter.

The presence of doubly labelled cells at the boundary between dorsal and ventral retina even late in regeneration is interesting. These cells are destined to terminate in a tectal zone equidistant from the two brachia. Therefore entering one or other brachium or eliminating one or other brachial collateral would confer no advantage. If it is the case that competitive interactions are responsible for collateral retraction, the 'correct' branch's termination being favoured over its more tardy axon collateral, then it appears that two axon branch terminals can be maintained under some circumstances. Although this method gives information about the retina and optic tract, if the collaterals are maintained as far as the optic tectum, there may be some overlapping terminals from the same RGC in this boundary zone.

While there is a body of evidence that neuronal activity is important for map refinement in regeneration at the level of the tectum, is activity important for the brachial refinement discussed above? Cook and Becker (1988) showed that neither TTX nor stroboscopic light had any effect on brachial refinement at any of the stages studied after optic nerve cut. Therefore, unlike map refinement assessed at the tectal level, brachial refinement does not appear to depend on normal neural activity. And given what is known about tectal map refinement, brachial refinement can continue in the absence of a precise retinotopic map. This should not be surprising as the two processes are occurring simultaneously.

Hartlieb and Stuermer (1987) found similar results in their studies of the effects of TTX on the loss of collaterals among goldfish RGC axons. Using anterograde HRP tracing of regenerating axons, they too showed that collaterals in the incorrect brachium were preferentially lost and that this process carried on in spite of TTX impulse blockade. However, they showed that the reduction of misrouted axons was delayed compared to controls which had been injected with Ringer solution.

So while initially regenerating axons seem to show no brachial preference and are equally likely to send a collateral down either brachium, which goes against chemospecificity being a dominant factor in these

circumstances, the other mechanism which has been invoked to explain map refinement does not appear to be operating either. It is possible that chemospecificity operates more distally in the system, for example at the tectal level, so that the axon collateral detects it is on the correct tectal half rather than finding its exact termination site, leading to retraction of the inappropriate collateral.

6:6:2: Map refinement at the tectal level

Since the completion of the work presented in this thesis, further studies on the role of the visual environment in regeneration of the goldfish retinotectal system have been carried out. For example, as already mentioned, Cook (1987) has shown that a sharp retinal image increases map precision during optic nerve regeneration in stroboscopic light. By leaving the lens in place, the precision of the map after regeneration under conditions of stroboscopic light was preserved. This argues for a specific effect of the stroboscopic light on the RGC. In the presence of the lens, how is spatial information extracted which is sufficient to sharpen the map even while the fish is exposed to stroboscopic light? The supposition is that even within the instantaneous focussed image short bursts of locally correlated activity can occur based on the brightness variations of the image.

Much seems to have been invested in evolutionary terms in mechanisms which allow precise maps to be formed in apparently adverse conditions, for example in the dark, conditions which may correspond to fetal stages. Stroboscopic light can mask the intrinsic differences which appear to allow the map to be established in the dark if necessary. The lens has the ability to override this blockade of the intrinsic system. It would appear that safety mechanisms are available to ensure that precise maps are formed, and that relatively crude measures such as application of TTX or stroboscopic light plus lens removal are necessary to abolish them.

Having established that stroboscopic light plus lens ablation can impair refinement of the visual map after optic nerve regeneration, is there a critical period during which the effect is maximum? A critical period has been identified in studies of the developing mammalian visual system in

terms of eyelid suture and its effect on the formation of ocular dominance columns (Hubel and Wiesel, 1970). Fish with optic nerve cuts and ablated lenses were exposed to varying sequences of diurnal and stroboscopic light in 21 day blocks up to a total of 84 days after optic nerve cut (Cook, 1988). At this stage, map precision was assessed using retrograde transport of WGA-HRP. Exposure to stroboscopic light instead of diurnal light for 42 of the 84 days, whatever the permutation of 21 day blocks, resulted in a map that had impaired precision compared to maps in animals exposed to diurnal light for the whole 84 day period of regeneration. However, that degree of impairment was greatest in those animals that had been exposed to diurnal light for 42 days followed by stroboscopic light for a further 42 days. When compared to animals described in Chapter 4 of this thesis, the degree of map refinement was no less at the end of the 84 day period than it would have been at the day 42 stage. Therefore, being in stroboscopic light for the second 42 day period of regeneration did not further impair or indeed improve the degree of map refinement already attained.

In those animals that formed a map of high precision having been in stroboscopic light for 21 days, diurnal light for 42 days followed by a further 21 days in stroboscopic light, the final period of stroboscopic light did not 'undo' the degree of precision that had already been achieved. Two further groups of animals were compared in these experiments, one that had been exposed to stroboscopic light for the first 42 days followed by diurnal light and the other that had been exposed to diurnal light for the first 21 days, then stroboscopic light for 42 days and then a final period of diurnal light. Therefore both were exposed to stroboscopic light for the second period of 21 days. The area indices in these two groups were in fact smaller than those in the other groups. It therefore appears that map refinement, as defined by the WGA-HRP method, was able to 'catch up' during subsequent periods of diurnal light. This argues against a critical period and argues for greater 'flexibility' among the RGC axons than had been suggested by previous experiments. For example, Schmidt and Edwards (1983) had shown, using electrophysiological mapping, that the enlarged MURFs which had formed following the presence of TTX for the first 27 days after optic nerve cut persisted long after the effects of the blockade would have worn

off. No recovery from TTX was seen unless the optic nerve was recrushed, when the MURF size was appropriate to the usual regenerated map. In addition, Schmidt and Eisele (1985) found that periods of normal visual exposure following stroboscopic light exposure for the first 35 days post-optic nerve cut resulted in very little sharpening of the map. Again the method of mapping was electrophysiological, and in both cases the numbers of animals was small. It is possible that the electrode samples only a few of the terminal arborisations and their post-synaptic connections, whereas WGA-HRP is taken up more reliably by the regenerating arborisations.

These results are interesting since there was no obvious reason in the goldfish visual system for a critical period to exist. There was a large body of evidence that the retinotectal projection is capable of great feats of plasticity and that connections are being modified throughout life. The experiments of Cook demonstrating that there need not be a strict time limit to map refinement are much more consistent with what was already known about the goldfish retinotectal projection.

Chapter 7: CONCLUSION

In this thesis, I have demonstrated a pattern of optic axons on the normal goldfish tectum that is consistent with a systematic shift of RGC terminal arborisations having occurred during development. I have also shown that the precision of the normal and regenerating retinotectal map can be assessed by the terminal uptake of iontophoretically applied WGA-HRP and that the precision so demonstrated can be analysed statistically. The degree of resolution of this method allowed the effects of a modified visual environment on the regenerating map to be measured. Specifically, the precision of the regenerating map in conditions of stroboscopic light and absence of the lens was less than in control animals. I have argued that stroboscopic light, by obliterating local correlations in intra-retinal activity, obscured the information 'required' by the regenerating RGC axons in order to restore a precise map.

Since the work described in this thesis was carried out, there has been much work on the formation of maps and their integration with other maps. The possible molecular bases of chemospecificity have been extensively investigated, as has the pharmacology of the Hebbian synapse. The citation lists in Appendix 5 in part demonstrate the value of the work described here. The discussion of work carried out after 1985 illustrates that the techniques of anterograde and retrograde HRP and WGA-HRP labelling have been used extensively, and that the results described have been confirmed and used as a basis for subsequent experiments.

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APPENDICES

Appendix 1: Pre-soak solution for development of retinae and tecta previously exposed to HRP

100mg *o*-catechol

50mg *p*-phenylenediamine

in 100ml Tris buffer, pH 7.3, stirred vigorously for 10 minutes. Four ml of this solution are made up to 100ml with Tris buffer.

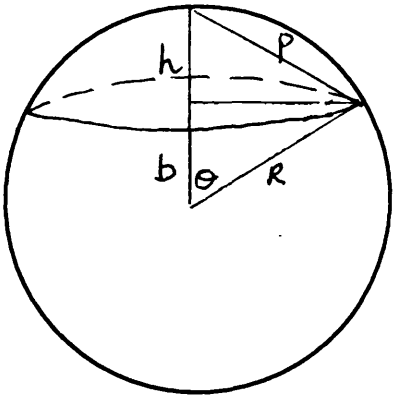
Appendix 2: Developer solution for retinae and tecta previously exposed to HRP

100mg *o*-catechol

50mg *p*-phenylenediamine

in 100ml of Tris buffer at 4^{0}C, stirred vigorously for 10 minutes. Add 5 drops 100 vol hydrogen peroxide (30% w/v).

Appendix 3: Derivation of spherical trigonometry formula



$S = 2\pi Rh$

$S = 2\pi R\ (R - b)$

$S = 2\pi R\ (R - R\cos\theta)$

$S = 2\pi R^2\ (1 - \cos\theta)$

For two circular fields of subtense $2\theta_1$, $2\theta_2$

$S_1/S_2 = 1 - \cos\theta_1/1 - \cos\theta_2$

For goldfish, retinal field subtends 185.3 ± 4.1^0 (Easter, Johns and Baumann, 1977). Let this field be $2\theta_2$.

$\cos\theta_1 = 1 + S_1/S_2\ (\cos\theta_2 - 1)$

$\cos\theta_1 = 1 + S_1/S_2\ (-1.046)$

$= 1 - S_1/S_2 \times 1.046$

$2\theta = 2\cos^{-1}\ (1 - S_1/S_2 \times 1.046)$

where S_1/S_2 = fraction of retinal area receiving image of field, eg area index

Appendix 4: Published papers

Please see enclosed papers in pocket at back of thesis

Appendix 5: Citations of published papers

*Citations of Cook, Rankin and Stevens, 1983 as of 6 July 1994**

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USE OF A LECTIN-PEROXIDASE CONJUGATE (WGA-HRP) TO ASSESS THE RETINOTOPIC PRECISION OF GOLDFISH OPTIC TERMINALS

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Key words: wheat-germ agglutinin – horseradish peroxidase – iontophoresis – topographic projection – regeneration – goldfish

The lectin wheat-germ agglutinin (WGA), conjugated to horseradish peroxidase (HRP), is an effective tracer for goldfish optic axons. Under suitable conditions, WGA-HRP injected iontophoretically into the normal goldfish tectum is taken up by retinotopic optic terminals (but not by axons of passage) and labels a small, discoid patch of retinal ganglion cells. Under identical conditions, unconjugated HRP is mainly taken up by injured axons of passage. WGA-HRP injected into the tectum after regeneration of the contralateral optic nerve again labels a small patch of ganglion cells, demonstrating the extent to which regenerated terminals are also retinotopic.

The terminal arborizations of developing or regenerating optic axons become organized within the tectum as a precise topographic projection of the retinal surface. The underlying mechanisms have been sought for 40 years [10]: nevertheless, techniques for assessing the precision of topography under different experimental conditions remain limited in accuracy and application. Most workers describe the retinotectal projection by the relationship between a rectangular grid of tectal electrode positions and a corresponding array of visual receptive field centres, using a presynaptic, multi-unit recording technique [11]. This method can reveal gross topographic errors; but its resolution is limited by the spacing of the tectal grid, usually 200 μm or more. Smaller errors have occasionally been studied by estimating the diameters of multi-unit receptive fields [7, 9]; and the degree of scatter of several single-unit fields recorded in a single tectal penetration has been used as a more exact measure [15].

However, electrophysiological methods require both eye and nerve to be functionally normal. They cannot be used to assess the effects of surgery which disrupts the optics of the eye, of agents which modify retinal activity, or of conduction

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blockers, unless normal function can be shown to be quickly restored. These are unfortunate limitations in view of a recent resurgence of interest in the possibility that activity-dependent mechanisms contribute to retinotopy [2, 5, 14–16]; anatomical methods would clearly be more appropriate. Retrograde tracing with horseradish peroxidase (HRP), which can be used to determine the tectal paths of optic axons [4], does not permit direct distinctions between axon terminals and axons of passage, both of which take up HRP after injury. Meyer [13, 14] resorted to autoradiography, studying the projections of acutely lesioned retinae labelled with [^{3}H]-proline and estimating their precision from the sharpness of the reconstructed boundary between labelled and unlabelled tectal zones.

In this paper we describe a new and effective method for studying the spatial arrangement of normal and regenerated optic axon terminals within the goldfish tectum. We use the non-toxic lectin wheat-germ agglutinin (WGA), which binds to plasma membranes, is avidly ingested by axon terminals, and is transported to their somata: conjugation to HRP makes it a convenient and sensitive axonal tracer [12]. We find that the spread of WGA-HRP from a site of iontophoretic injection is considerably less than that of unconjugated HRP. It can also be a virtually specific tracer for axon terminals, presumably because its membrane-binding enhances their endocytotic uptake mechanism: small quantities can be injected which suffice for detectable uptake by terminals but not by axons of passage. These properties make WGA-HRP a powerful mapping tool.

We inject WGA-HRP (Sigma) into goldfish anaesthetized with MS-222 (Sandoz), from micropipettes of tip diameter 15–25 μm filled with a 0.1% solution of WGA-HRP in 0.2 M KCl, buffered to pH 7.9 with 0.05 M Tris. (Ten μl aliquots of this, stored at $-10°$C in small polythene embedding capsules, retain their activity for several weeks and can be thawed as required.) With the pipette tip in the stratum fibrosum et griseum superficiale of the tectum, 100–200 μm beneath the pia mater (see below), we pass 600 nA (positive) continuously for 10 min. After closure of a reflected cranial flap the fish is revived and maintained for two days. The remaining protocol is identical to that used by us for HRP [4]: in short, the fish is reanaesthetized; the contralateral retina is removed intact, flattened, and fixed in glutaraldehyde; and the transported WGA-HRP in retinal ganglion cells is revealed by means of a modified Hanker–Yates reaction. Each retina is flat-mounted and can be scanned systematically for labelled ganglion cells, which are plotted on a photomontage of the retina with the aid of a drawing tube.

We have employed this method, with variations discussed below, to label small, discoid patches of ganglion cells in a total of 57 normal retinae, represented here by Figs. 1A and 2A. Some experimental parameters were found to be more critical than others. The *concentration of WGA-HRP* was chosen for economy as well as for effective control of delivery. Lower concentrations would require compensatory increases in current magnitude or duration. Higher concentrations would lead to increases in the diffusion component of tracer delivery, which varies appreciably

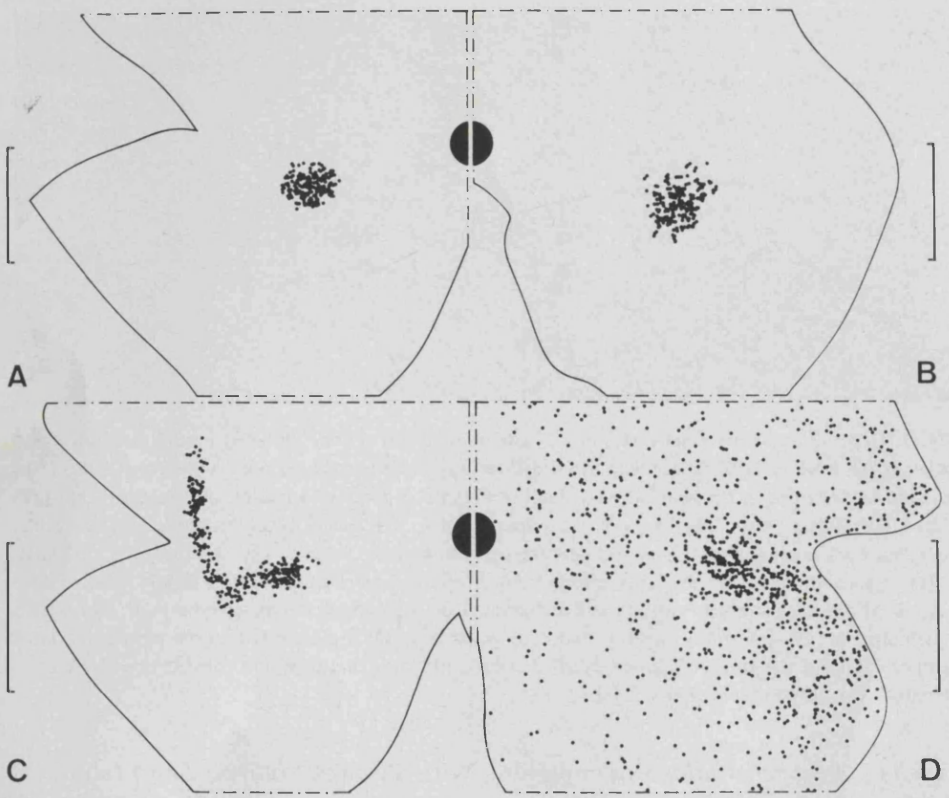

Fig. 1. Tracings of the ventronasal portions of 4 flat-mounted goldfish retinae containing cells labelled with WGA-HRP or HRP from the caudomedial tectum. A small dot marks the site of every ganglion cell showing peroxidase reaction product. Large divided spots correspond to the positions of the optic discs. Scale bars = 1 mm. A: left retina following iontophoretic injection of WGA-HRP in a normal fish. The patch of filled cells is compact and well-defined, showing that the optic terminals at the injection site were retinotopic. Axons of passage crossing the tectum are not revealed. B: right retina following an injection of WGA-HRP, 88 days after right optic nerve section. The patch is slightly less regular but still shows clearly that the regenerated terminals were retinotopic. C: left retina following direct application of HRP solution to a tectal lesion in a normal fish. This method, in contrast, fills axons of passage more heavily than intact terminals. Axon groups of uniform age, arising from cells in a partial annulus, crossed the tectum together: older axons intersected the same site as they ran caudally to terminate (see ref. 6). D: right retina following direct application of HRP solution to a tectal lesion, 80 days after right optic nerve section. Regenerated axons from the entire nasal retina evidently passed through the lesion site. Only those from a restricted area (denser cluster) can have been filled through retinotopic terminals (see ref. 4).

(unlike the current-dependent component) with *pipette tip size*. With the standard concentration (0.1%) and range of tip sizes (15–25 μm) no retinal label was detected unless current was passed; so tip variation was not critical. Larger tips were avoided to minimize damage: smaller ones would have restricted current flow too much. The

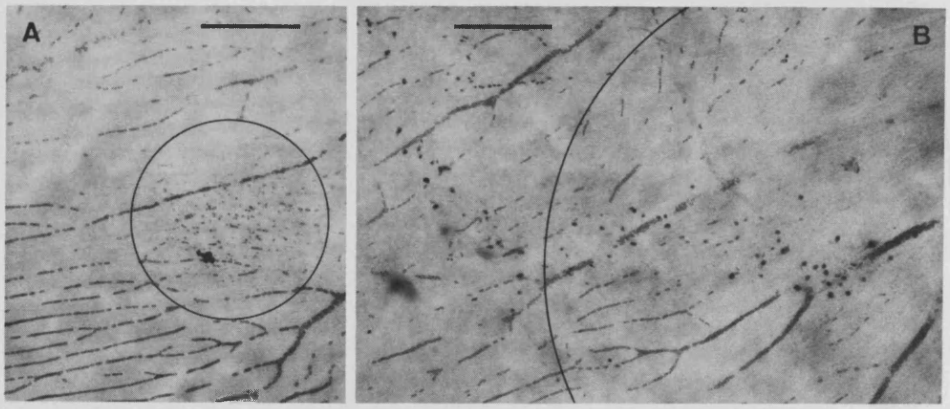

Fig. 2. Cells in the ventronasal quadrants of two normal left retinae, labelled iontophoretically with either WGA-HRP or HRP from the caudomedial tectum. Scale bars = 0.2 mm. A: WGA-HRP (0.1%), injected as described in the text, labels a small, well-defined patch of ganglion cells (circled) that projected to the injection site. Erythrocytes in capillaries and a small cluster of pigment cells are also visible. Systematic scanning at higher magnification reveals no scattered labelled cells (see Fig. 1A). B: HRP (4%), injected in the same way, labels a broad, poorly defined patch of cells rather lightly (circled: label scarcely visible at this magnification); and a characteristic, asymmetrical, 'axon of passage' distribution much more heavily (compare with Fig. 1C). The patch is found to be centred on the right-hand, most temporal limit of the group of heavily labelled cells, confirming that this limit consists of cells that terminated very near the injection site [4].

depth of tectal penetration was important. With the pipette mounted on a calibrated micromanipulator, penetration was measured from the point of first contact with the pia. The most repeatable results were obtained at depths of 100–120 μm in caudal tectum, 120–150 μm in mid-tectum, and 150–200 μm in rostral tectum, where additional correction for tectal curvature was made as necessary. Changes in the *magnitude and duration of the applied current* had complementary effects over the range of times tested (5–40 min). The chosen permutation kept experiments short and currents well within the limit set by the pipette resistance (about 12 MΩ) and the voltage compliance of our current generator (50 V). Increases in total applied charge above the standard value led to approximately proportional increases in the *area* of the patch of labelled cells. Decreases, on the other hand, were reflected chiefly in poorer labelling, patch area being reduced only slightly before the identification of labelled cells became difficult. Intermittent application of current (0.3 Hz square wave [8]) seemed only to reduce the consistency of labelling between fish, which was normally remarkably good. Even using different pipettes on different occasions, the range of patch sizes could be kept within limits corresponding to a two-fold variation in charge. Moreover, a single pipette could make at least 15 consecutive injections without any detectable loss of efficacy. The *time allowed for transport* of WGA-HRP to the retina was not critical within the range 2–8 days.

For comparison, we labelled another 13 normal retinae using HRP (Sigma Type

VI or Boehringer Grade I) in place of WGA-HRP. Higher concentrations were needed; and, even at the threshold of detection for terminal uptake, the patches of labelled cells were broad and poorly defined. Superimposed on each patch was a distinctive, asymmetrical pattern of heavier labelling (Fig. 2B) just like that seen after direct application of HRP to a tectal lesion (Fig. 1C) and representing uptake by injured axons [4]. Iontophoretic injection alone evidently does not prevent this form of uptake.

Finally, we used WGA-HRP to label 7 retinae with regenerated tectal projections, following section of the optic nerve by standard methods [4]. In 6 of these, regenerated for 54–89 days (at 25 ± 3°C) after nerve section, the labelled cells formed discrete patches much like those in normal retinae, though somewhat less regular in outline. An example is shown in Fig. 1B. In a seventh (88 days after nerve section) they formed two loosely linked, irregular aggregates in the same retinal region; but in no case were WGA-HRP-labelled cells scattered across the retina in the way that HRP-labelled cells always are following regeneration (ref. 4 and Fig. 1D). A more detailed study is in progress; but this simple contrast between the two tracers strengthens the previous conclusion that widely-scattered HRP-labelled cells are filled through axons of passage misrouted in regeneration, rather than through misplaced terminals [4].

It seems certain that, in normal retinae, the patches of WGA-HRP-labelled cells do reflect uptake by axon terminals. There is ample evidence for this mode of uptake in other systems [12] and there seems to be no alternative here, since uptake by normal axons of passage is known to lead to an entirely different spatial distribution. Though the same arguments apply to patches seen after regeneration, care in interpretation is needed. For example, it should not be assumed that an abnormal retinal distribution of labelled cells would reflect a numerically equivalent abnormality in the distribution of tectal synapses. In a normal retina, each labelled cell has presumably taken up tracer from many synaptic sites. During regeneration, the same cell might pass as unlabelled even though it has again formed synapses, either because it has too few, or because they are so much more widely distributed that too few lie within range of the injection site. The method may therefore detect only those terminals which are both bushy and relatively compact. Moreover, there is another axonal structure, the growth cone, which can take up tracers by endocytosis [1,3]: it would therefore be unwise to assume that cells labelled by this method in an experimental retina, especially at earlier stages in regeneration, would necessarily constitute proof of arborization or synaptogenesis.

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Topographic refinement of the regenerating retinotectal projection of the goldfish in standard laboratory conditions: a quantitative WGA-HRP study

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Summary. The topographic precision of the regenerating retinotectal projection of the goldfish was studied between 18 and 524 days (at 20° C) after optic nerve cut, using retrograde transport of wheatgerm agglutinin conjugated to horseradish peroxidase (WGA-HRP) from one of two standardized tectal injection sites. All labelled ganglion cells in each flat-mounted retina were plotted individually, and their degree of dispersion was assessed by a statistical method based on distance to nearest neighbour. Labelled cells in normal fish were clustered tightly, covering on average only 1.3% of the retina. Early in regeneration (18–28 days) they were widely dispersed, covering up to 75.2%, and they did not begin to form recognizable clusters at appropriate sites until about 35 days after nerve cut. Between 18 and 70 days, the proportion of retina covered by labelled cells fell dramatically, halving about every 14 days. Between 70 and 524 days, no further reduction could be demonstrated: overall, clusters remained significantly larger than normal, though a few individual retinae were virtually normal. Several others, labelled from similar single injections between 56 and 524 days after nerve cut, showed pairs of cell clusters; a sign that persistent errors in topography are common. The very wide initial scatter of labelled cells reflects a striking lack of 'goal-directedness' in regenerative axon growth. Extensive branching in the optic nerve, tract and tectum, for which there is already evidence, must contribute to this. Though uptake of some WGA-HRP by non-synaptic growth cones cannot be ruled out, other evidence for mislocated functional synapses at early stages encourages us to favour 'trial and error' synapse formation as the likely basis of map refinement.

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Key words: Retinotectal projection – Regeneration – Topography – Goldfish

Introduction

In submammalian vertebrates, retinal ganglion cells of all classes send axons through the optic nerve and tract to the contralateral optic tectum, where their terminal arbors form a precise topographic representation of the retinal surface. In fish and amphibia the cut or crushed optic nerve regenerates to re-establish this 'retinotopic' tectal map. The visuomotor responses reappearing after optic nerve section and surgical rotation of the eye were studied systematically by Sperry (1948), who concluded that regenerating optic axons must form new arbors at their original tectal sites. Later, silver staining of the regenerating projection in goldfish showed that axons from an isolated half-retina tend to reinnervate the appropriate half-tectum (Attardi and Sperry 1963). However, these methods were too crude to allow any assessment of the precision of the regenerating map.

Electrophysiological recording from the terminal arbors in the tectum has become the usual way of assessing the progress of map refinement. Large errors of termination may be seen as distortions and discontinuities of the map. Gaze and Jacobson (1963) described abnormal map patterns at various times after optic nerve section in the frog *Rana temporaria* and suggested that they might represent sequential stages in regeneration. However, in the newt (Cronly-Dillon 1968), goldfish (Horder 1971) and frog *Hyla moorei* (Humphrey and Beazley 1982), the regenerated projection is essentially retinotopic from the time at which it can first be recorded. In any case,

map distortions are hard to measure and their visibility depends on the spacing of the sampling grid.

More detailed information can be obtained by estimating the size of the presynaptic multi-unit receptive field (MURF) at each recording site. Early in regeneration the MURF is considerably larger than the receptive field of a single presynaptic unit but it diminishes as regeneration proceeds (Cronly-Dillon 1968; Horder 1971; Humphrey and Beazley 1982; Adamson et al. 1984; Northmore and Masino 1984; but see Schmidt and Edwards 1983). However, this method also has limitations. The most important is that responses from regenerating axons are too small and fatigue too easily to be used for field measurement until long after synaptogenesis has begun (Schmidt et al. 1983). In addition, however, presynaptic responses are not always readily distinguished from postsynaptic ones (Chung et al. 1975; O'Benar 1976); and field margins are rarely sharply defined, so estimates of their size must be subject to observer bias unless made 'blind' or automatically (Northmore and Masino 1984).

More recently, anatomical methods have also been employed. Using autoradiography, Meyer (1980) showed that small retinal lesions made at the time of an intraocular injection of ^{3}H-proline caused corresponding gaps in the tectal distribution of labelled protein both in normal goldfish and late in regeneration, which must imply that the label is largely confined in both circumstances to retinotopic terminals. In contrast, no gaps were seen after similar small lesions early in regeneration (40–41 days): much larger lesions were needed then to cause detectable tectal deficits. This shows that the early projection is diffuse, though not necessarily that there are non-retinotopic terminal arbors: any such interpretation would rest on the dubious assumption that the label was confined to these arbors even during the early stages of regeneration, when axonal transport is abnormal (Grafstein and Murray 1969).

Because a sound understanding of the sequence of tectal reinnervation is essential for current work on possible mechanisms of refinement, we have re-examined it using another anatomical method, retrograde transport of wheatgerm agglutinin conjugated to horseradish peroxidase (WGA-HRP; Cook and Rankin 1984). When WGA-HRP is injected iontophoretically into the goldfish tectum, either in a normal fish or late in regeneration, it labels a small cluster of ganglion cells in the contralateral retina. Because the lectin WGA binds strongly to neuronal membranes (Mesulam 1982), the concentration of free WGA-HRP can be kept low enough to avoid detectable uptake by damaged or intact axons of passage (Cook and Rankin 1984). These properties of WGA-HRP provide a method of assessing the precision of the retinotectal map that differs enough from other methods to be a valuable source of experimental confirmation.

WGA-HRP tracing also permits quantification. The distance from each labelled retinal ganglion cell to its nearest labelled neighbour can be used as the basis for a statistical estimate of the fraction of retina containing cells labelled from a tectal injection site. This is an anatomical analogue of the electrophysiological MURF at the same site and can be used as a measure of retinotopy in the same way.

In this paper, we describe the use of WGA-HRP to assess the progress of map refinement during regeneration of the cut optic nerve under standard conditions. In the following paper (Cook and Rankin 1986) we use the same method to assess the consequences of changing the activity pattern of retinal ganglion cells by varying the lighting conditions. Some of these findings have been presented in abstract form (Rankin and Cook 1984).

Methods

All observations were made on goldfish (*Carassius auratus*; Cura and Sons, Hemel Hempstead, Herts) 55–65 mm long from snout to tail base. For all surgery, anaesthesia was induced by immersion in a 0.1% solution of MS-222 (Sandoz) and maintained by perfusion of the gills with a 0.012% solution. Right optic nerves were cut cleanly in mid-orbit with iridectomy scissors, sparing the ophthalmic artery. Fish that bled excessively or had any anatomical abnormality were excluded from the series. After recovery, a total of 53 fish were studied after various periods of maintenance at a constant temperature of 20 ± 0.3° C in diurnal lighting (12 h dark/12 h light, at approximately 600 lux from a 'Northlight' fluorescent tube).

Two standard tectal sites were chosen for iontophoretic injection, one rostral and one caudal. The rostral site lay on a rostrocaudal line that bisected the accessible dorsomedial part of the tectum and was located one-fifth of the way back from the visible rostral limit. The caudal site lay on the same line but two-thirds of the way back from the rostral limit (Fig. 1A). At intervals between 18 and 524 days after nerve section, different fish received injections of WGA-HRP into one or other site as described previously (Cook and Rankin 1984). In brief, a micropipette of external tip diameter 15–25 μm was filled with 0.1% WGA-HRP (Sigma) in 0.2 M KCl buffered to pH 7.9 with 0.05 M Tris. The tip was then lowered 150 μm below the pia mater rostrally, or 125 μm caudally (where the tectum is thinner), into the synaptic layer. In this experiment we passed 2 mA (positive) for 6 min, twice as much charge as in previous experiments, to ensure satisfactory labelling of the regenerating ganglion cells. One normal fish was injected with each group of regenerates as a control, at the same site and using the same micropipette.

Two days later the complete retina was dissected free, processed by a modified Hanker-Yates reaction (Cook 1983), and flat-mounted. A photomontage was constructed at an overall magnification of 112 diameters, on to which individual labelled cells, identified by inspection at 400 diameters, were plotted with the aid of a drawing tube. Only cells containing the characteristic reddish-brown granules of WGA-HRP reaction product (Fig. 1D)

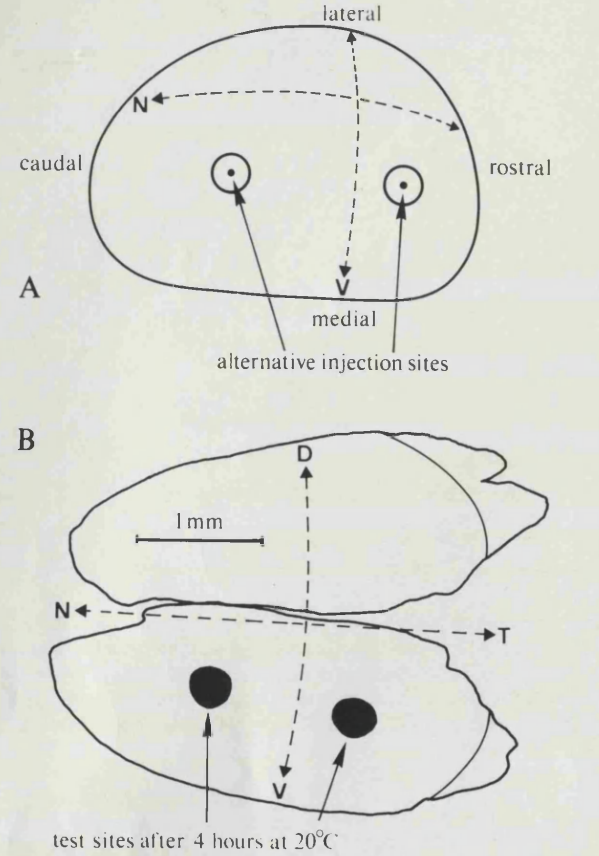

Fig. 1. A Diagram of the left optic tectum from above, showing rostral and caudal WGA-HRP injection sites. V and N mark the projections of the ventral and nasal retinal poles: the dorsal and temporal poles project to the most lateral and rostral regions which are hidden from above. **B** Tracing of a flat-mounted left tectum in the same orientation, 22 days after right optic nerve cut, showing the limits of two test injection sites. The retinal projection is marked as in **A**. The preparation has split longitudinally, and shows remnants of the tract brachia on the right. **C** The caudal injection site from **B**. Scale bar = 100 μm. **D** Two ganglion cells (arrows) labelled with WGA-HRP through regenerated axons, from the retina of Fig. 5A. Many unlabelled cells are also visible in the ganglion cell layer. Scale bar = 25 μm

and lying within (or just sclerad to) the ganglion cell layer were plotted. We took the presence of six resolvable granules as a lower limit because, though cells with fewer granules could be identified both inside and outside clusters, isolated cells labelled this lightly were likely to be overlooked. The total area of each flat-mounted retina was measured by tracing its outline (defined by the marginal vein) on a digitizing tablet.

To establish that injection sites were of uniform size, three normal fish and three fish 22 days after nerve cut received standard injections at both rostral and caudal sites. Their tecta were removed after 3–4 h at 20° C and processed in the same way as the retinae. The outlines of the tecta and of the individual injection sites (Figs. 1B and C) were drawn at 30 diameters, and their areas measured on the digitizing tablet.

We then required an objective and quantitative estimate of the degree of clustering of the labelled retinal ganglion cells. It was impractical simply to draw an envelope around a cluster: its edge was rarely well defined except in normal fish. We therefore adopted a statistical approach based on nearest neighbour distance measurements (Clark and Evans 1954). This provides estimates of cell dispersion that are not only empirically useful but also probably theoretically appropriate to map refinement, because the idea that neuronal 'neighbourliness' might be used as an information source is central to the hypothesis of self-organization of topographic projections, proposed by Willshaw and Malsburg (1976) and now receiving widespread experimental support (see Cook and Rankin 1986).

After plotting all the labelled ganglion cells, we measured the distance on the photomontage from each one to its nearest neighbour (using dividers and a calibrated inspection lens) and found the mean. Measuring errors were insignificant beside other sources of variation, and we were careful to choose positions for the injection sites that would minimize the number of labelled cells separated from their nearest neighbour by the cuts made during dissection. Clark and Evans derived an expression for the mean nearest neighbour distance to be *expected* if the individual members of a population were distributed randomly in two dimensions:

$$r_E = 1/(2\sqrt{D})$$

where D is the number of individuals in unit area (the average density of the population). The ratio of the mean nearest neighbour distance actually *observed* (r_A) to this *expected* mean (r_E) is called the dispersion index and provides a measure of deviation from randomness within the population. In our case, the average population density is the observed number of labelled cells divided by the total retinal area, and a dispersion index of unity would indicate that labelled cells were uniformly and randomly distributed over the entire retina. Smaller dispersion indices indicate increasing degrees of labelled cell clustering in the retina, and therefore of order among the tectal arbors. Indices greater than one would signify dispersion in a regular pattern but do not arise in the present context.

In practice, we present our data in terms of the *square* of the dispersion index which we term the *area index*, because this provides a direct estimate of the ratio between the area of the cluster and the total retinal area. In formal terms, the area index actually represents the fraction of the total retinal area that would be needed to enclose all the labelled cells, given that they were to be redispersed randomly inside a closed boundary while keeping their observed *mean* distance to nearest neighbour. For a typical discoid cluster, this hypothetical boundary can usefully be represented as a circle of appropriate area, as in Figs. 2–8, but its shape and position are not actually defined. Like the dispersion index, the area index would be zero if all members of the population were superimposed, or unity if they were distributed randomly.

To aid comparison with previous electrophysiological data on the regenerating projection, we have also derived, by spherical trigonometry, the relationship between the area index (I_A) and the angular subtense (θ) of its corresponding receptive field, assumed for this purpose to be circular. For a goldfish retina subtending 185.3° (Easter et al. 1977) this relationship is:

$$\cos(\theta/2) = 1 - 1.046(I_A)$$

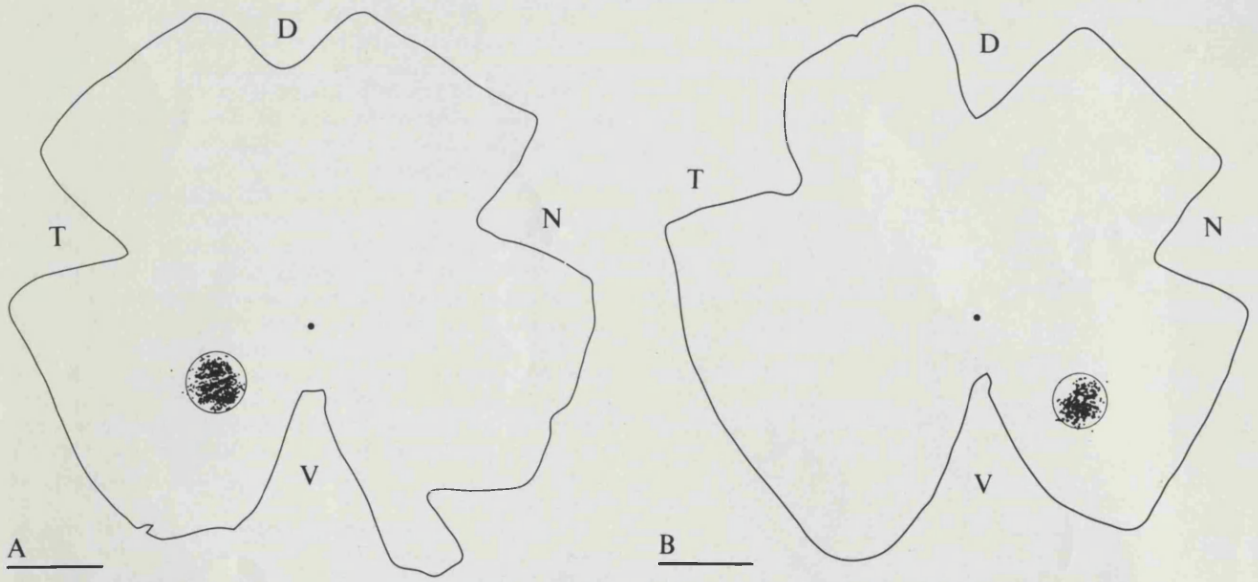

Fig. 2A and B. Flat-mounted right retinae from two normal goldfish. Each dot marks the position of a single retinal ganglion cell labelled with WGA-HRP from an iontophoretic tectal injection. The circle round each cell cluster is positioned by eye but its area, as a fraction of the total retinal area, exactly equals the area index derived from individual nearest neighbour distance measurements on the labelled cells. The ventral incision (V) followed the line of the choroid fissure. D, N and T show the directions of the dorsal, nasal and temporal poles. A large, central dot marks the centre of the optic disc. Scale bars = 1 mm. **A** Rostral injection site. Area index = 0.013. **B** Caudal injection site. Area index = 0.011

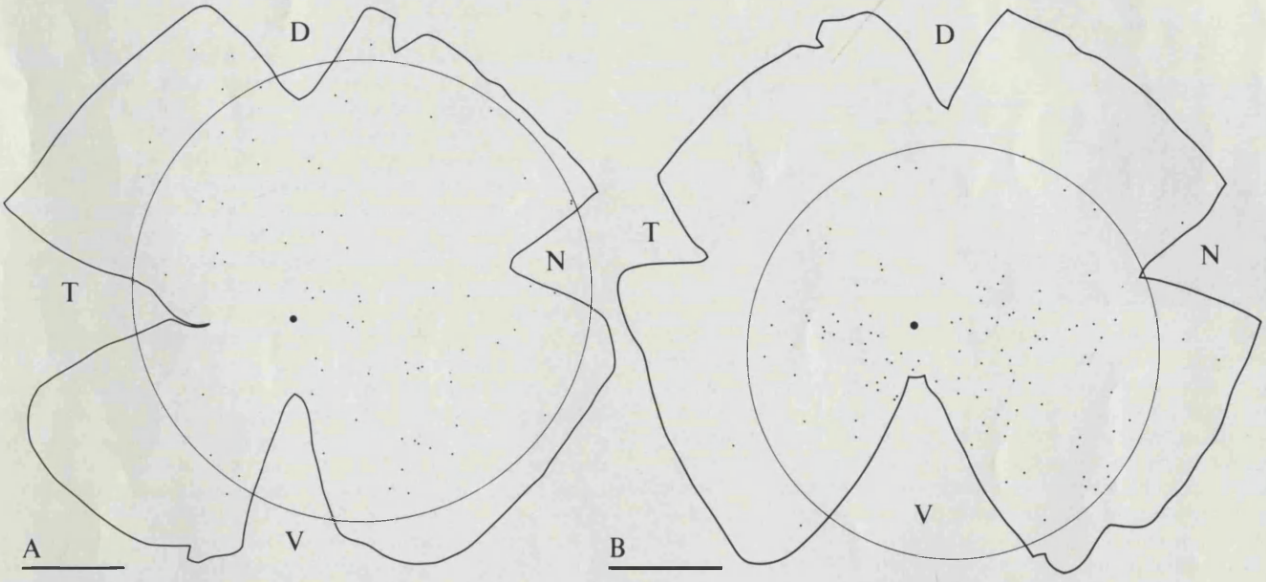

Fig. 3A and B. Flat-mounted right retinae from two goldfish 18 days after optic nerve cut, following *rostral* tectal injections. For conventions see Fig. 2. **A** Labelled cells were distributed over a large fraction of this retina, without any apparent focus. Area index = 0.752. **B** In this more typical case the distribution shows a ventral bias but no retinotopic temporal bias. Area index = 0.541

Results

Retrograde labelling

Iontophoretic injections of WGA-HRP into the tecta of goldfish with either normal or regenerated retinotectal projections resulted in the labelling of retinal ganglion cell somata. Labelled cells in normal and late regenerate retinae were of similar appearance, containing many small reddish-brown granules of reaction product in their cytoplasm (Fig. 1D).

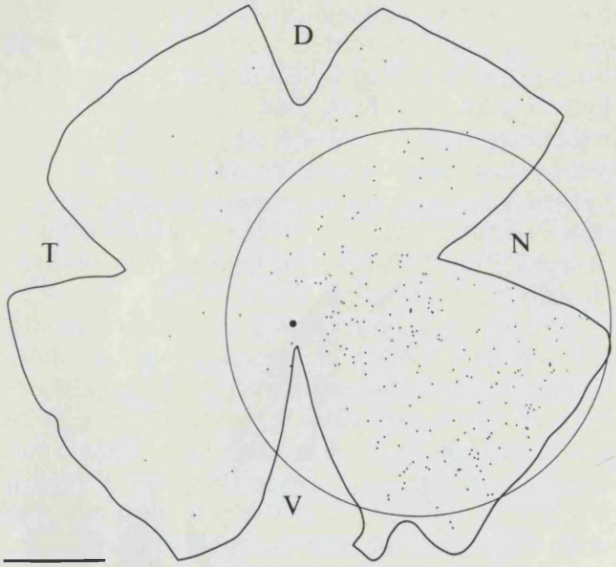

Fig. 4. Flat-mounted right retina from a goldfish 24 days after optic nerve cut, following a *caudal* tectal injection. For conventions see Fig. 2. More labelled cells were in the retinotopic ventronasal quadrant than in any other but they covered just as large a fraction of the retina as after a rostral injection (see Fig. 3B). Area index = 0.538

Labelled cells in early regenerates had a more swollen appearance, this being a characteristic response to axotomy (Murray and Grafstein 1969), and the granules were usually rather less dense. Trans-synaptic labelling of cells in the inner nuclear layer was never seen.

Retinal distributions

The distribution of labelled cells depended on whether the projection was normal or regenerated; if regenerated, on the time after optic nerve cut at which the injection was made; and on the injection site, which was at one of two standard positions, rostral or caudal, on the dorsomedial tectum (see Methods and Fig. 1). In eleven normal fish, each injection labelled a compact cluster of between 223 and 719 ganglion cells in either the ventrotemporal quadrant (rostral site; Fig. 2A) or the ventronasal quadrant (caudal site; Fig. 2B) of the retina. There was some variation in the size of the cluster and in its position within the quadrant which can largely be attributed to variation in the size and position of the injection.

Even at a very early stage of regeneration, after only 18 days, rostral injections in five fish produced clearly labelled ganglion cells; though their numbers varied widely, between ten and 802 per retina. In two cases these cells were distributed throughout the whole retina (Fig. 3A), two more showed a slight general bias towards the ventral half (Fig. 3B), and one showed a more obvious bias to the ventrotemporal quadrant. Caudal injections in five fish after 24 days yielded similar results: three retinae contained fewer than ten labelled cells, while the other two, containing 241 and 998 cells, showed a distinct ventronasal bias (Fig. 4). Injections were also made at both sites, in three fish at each site, after 28 days of regeneration. Most of the 50–1366 labelled cells in

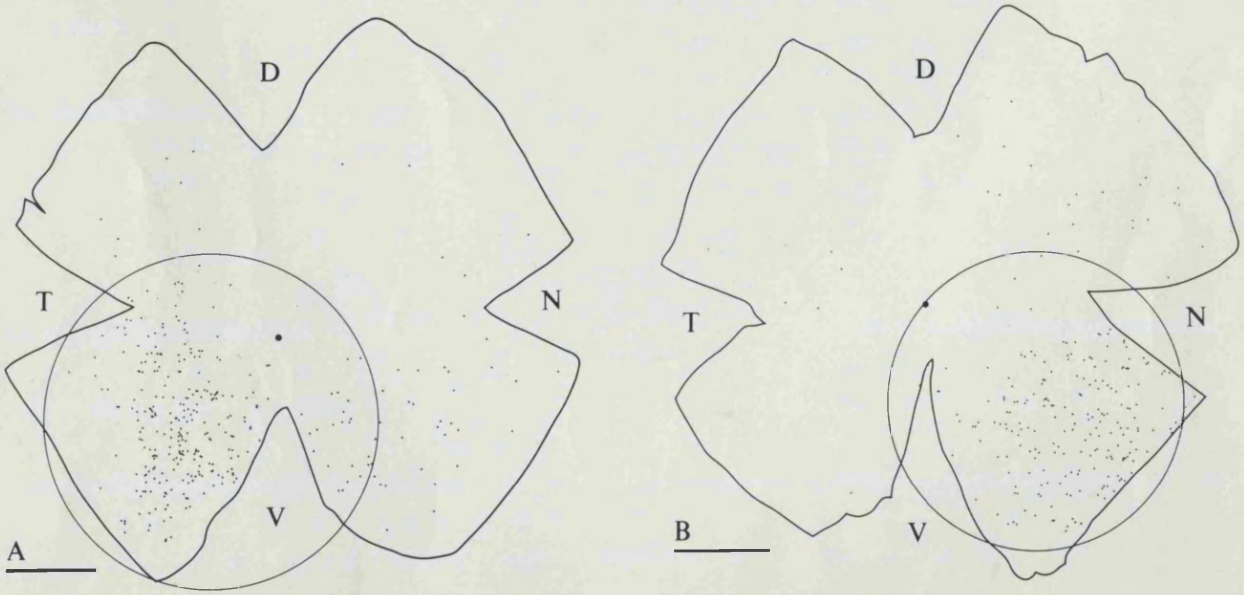

Fig. 5A and B. Flat-mounted right retinae from two goldfish 28 days after optic nerve cut. For conventions see Fig. 2. **A** *Rostral* injection. Gross retinotopy is obvious but there is still no focus of labelled cells. Area index = 0.418. **B** *Caudal* injection. The pattern is almost exactly complementary to that seen in **A**. Area index = 0.337

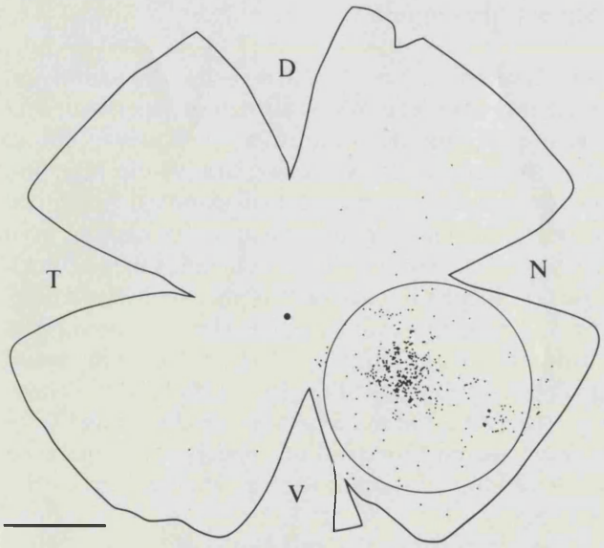

Fig. 6. Flat-mounted right retina from a goldfish 49 days after optic nerve cut, following a *caudal* tectal injection. For conventions see Fig. 2. Labelled cells were still found thoughout the retinotopic ventronasal quadrant, but a cluster was forming at its centre. As a result, the area index has fallen to 0.167

each retina were in the retinotopically correct quadrant, but there were still no signs of cluster formation (Fig. 5A and B).

Between 35 and 56 days after nerve cut, caudal injections labelled 37–777 cells, and in nine out of twelve fish some of these cells formed recognizable clusters at rather variable locations within the ventronasal quadrant (Fig. 6). By 70 days, six rostral and four caudal injections all produced distinct retinotopic clusters (179–456 labelled cells; Fig. 7A and B) though scattered cells remained in every case but one. Little overall change was then seen up to 524 days in a further 12 fish. Of the 22 retinae studied between 70 and 524 days, a total of five appeared virtually normal (Fig. 8).

In eight cases, however, a single injection yielded two separate clusters of labelled cells. All these occurred in the middle to late stages of regeneration, between 56 and 524 days: it would not have been possible to resolve them earlier. Five of them followed caudal injections, and in every one of these a line joining the centres of the two clusters in the ventronasal quadrant had an approximately radial orientation (Fig. 9A). Three followed rostral injections, and in these the lines joining the centres of the clusters had different orientations; dorsoventral, circumferential, or nasotemporal (Fig. 9B). In four cases (two rostral and two caudal) the two clusters were approximately equal in size and in packing density of labelled cells but in the remaining four cases one cluster was distinctly larger or denser than the other.

Although the number of labelled cells varied widely from retina to retina, there was no overall correlation between cell number and regeneration time.

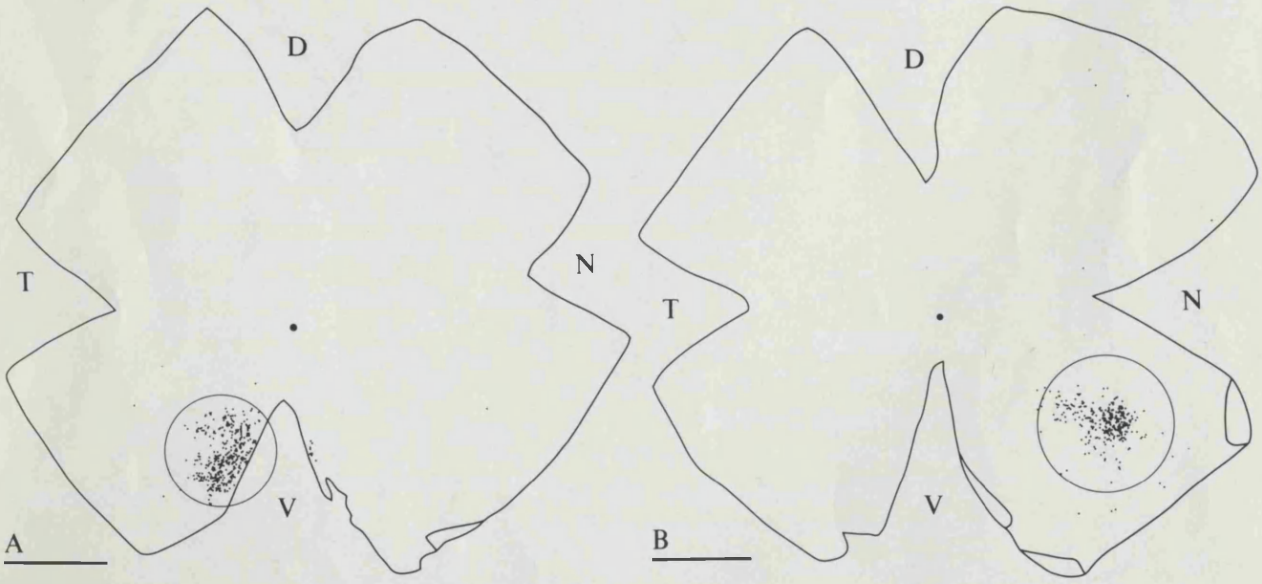

Fig. 7A and B. Flat-mounted right retinae from two goldfish 70 days after optic nerve cut. For conventions see Fig. 2. Most labelled cells lay within a cluster by this time, though scattered individuals remained in every case but one. **A** *Rostral* injection. Area index = 0.044. **B** *Caudal* injection. Area index = 0.061

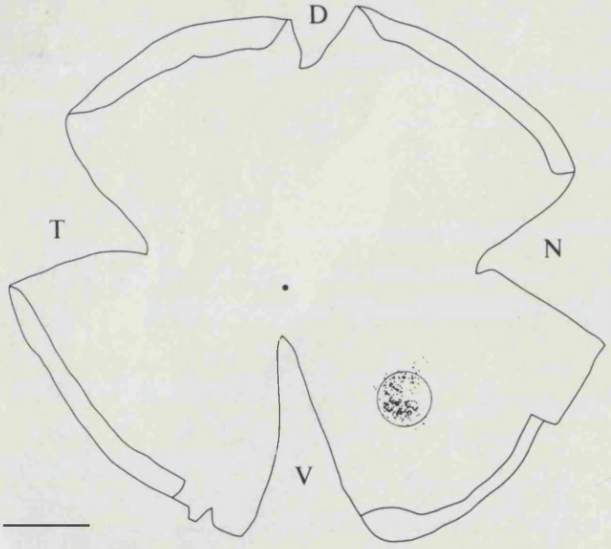

Fig. 8. Flat-mounted right retina from a goldfish 524 days after optic nerve cut, following a *caudal* tectal injection. For conventions see Fig. 2. (The retinal margins have become folded: this was always allowed for in measuring the total area.) This retina is typical of five that appeared virtually normal, out of 22 studied between 70 and 524 days after nerve cut. Area index = 0.010

Injection sites

The great scatter of labelled cells at early stages raised the possibility that the effective injection site might have been larger then than in normals or late regenerates. Standard iontophoretic injections were therefore made at both rostral and caudal sites, in three normal fish and three fish 22 days after nerve cut, and the tecta were removed after 3–4 h and processed in the same way as the retinae. All twelve injection sites were discoid and well circumscribed (Fig. 1B and C), their mean diameter (with its standard error) being 294 ± 5 μm; and all occupied very similar fractions of the total tectal area (mean 0.0093; standard deviation 0.0011) in normals and regenerates, both rostrally and caudally. The size of the observed site after 3–4 h at 20° C is probably a good approximation to that of the effective injection site. Reactive expansion can occur after the injection, but does not begin for at least 2 h even at 37° C (reviewed by Mesulam 1982); and the temperature coefficient (Q_{10}) of the process is unlikely to be less than 1.5. Moreover, if expansion had in fact begun, the effective sites would all have been *smaller* than the measured areas.

Measurement of dispersion

To quantify our labelled cell distributions, we chose to use a measure of dispersion, based on distance to nearest neighbour, that we term the area index (I_A; see Methods). In general, for a constant injection site size, the smaller the area index the greater the degree of map refinement. Area index values fell dramati-

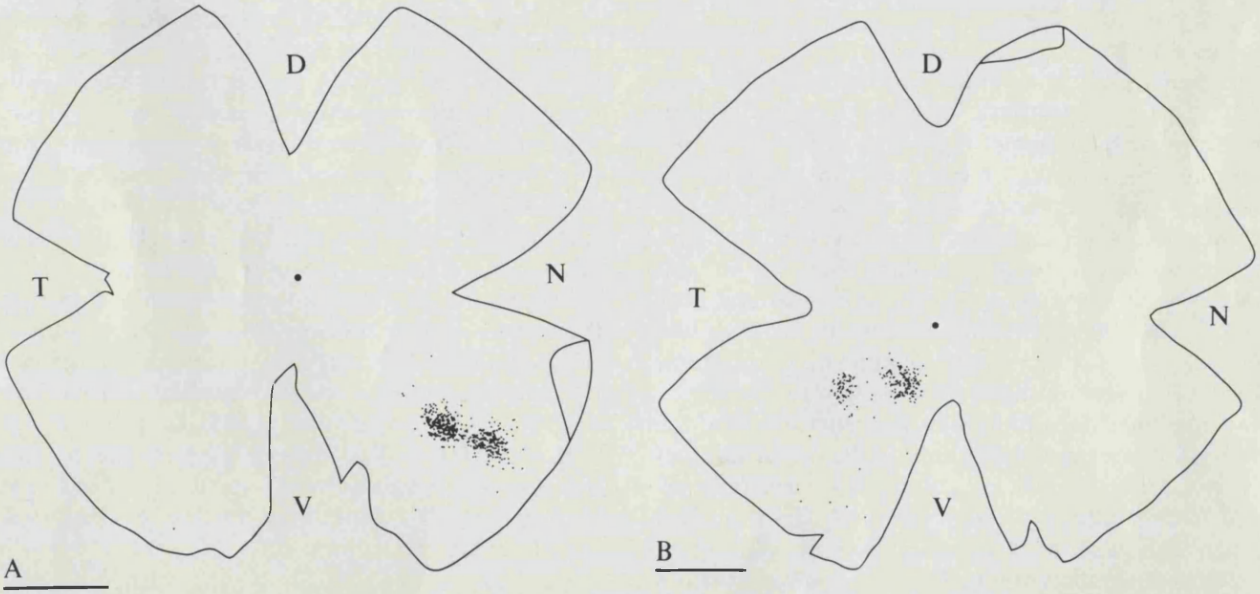

Fig. 9A and B. Flat-mounted right retinae from two goldfish 125 and 70 days after optic nerve cut. For conventions see Fig. 2. These are typical of eight in which a single tectal injection yielded two separate clusters of labelled cells, showing that the refined projection was not entirely retinotopic. Nevertheless, the area indices were in the normal range for these stages. **A** *Rostral* injection after 125 days. Area index = 0.046. **B** *Caudal* injection after 70 days. Area index = 0.043

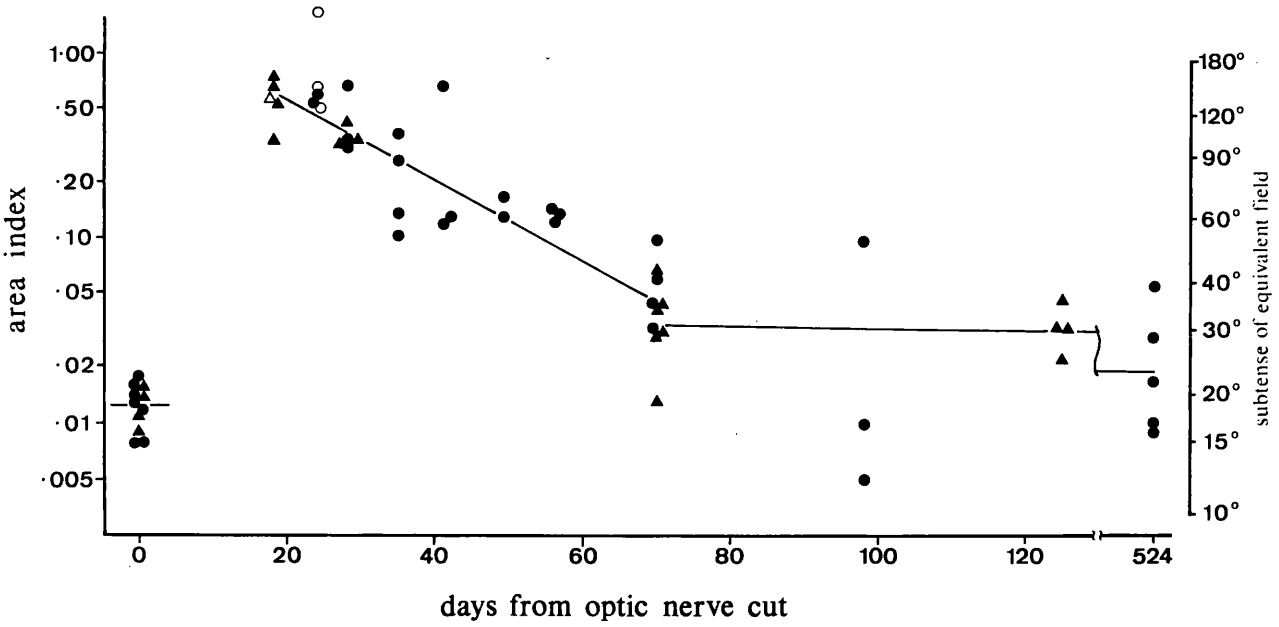

Fig. 10. Area indices derived from measurements of distance to nearest neighbour on 19,662 labelled ganglion cells, in 50 retinae after optic nerve regeneration and eleven normal retinae, plotted on a logarithmic scale against time from optic nerve cut. *Triangles* were from rostral injections. *Circles* were from caudal injections. *Open symbols* denote indices derived from fewer than 30 labelled cells: these were omitted from the calculations for the two *regression lines*, for which coefficients, constants and significance values are given in the text. A short line through the normal indices (at 0 days) shows the mean value of log (I_A). Note that an axis discontinuity between 125 and 524 days causes the shift in the regression line. The ordinate on the right shows calculated angular subtenses for circular receptive fields with equivalent retinal image areas (for equation see Methods)

cally during regeneration, in both the rostral and the caudal series, up to 70 days from nerve cut. Figure 10 shows a graph of these values, on a logarithmic scale, against time in days from nerve section. On the left of the figure, indices from eleven normal controls (one injected with each group of regenerates) are included for comparison. The variance of the area index also fell sharply with time, making it impossible to perform a valid linear regression analysis on the untransformed data. However, the variance of $\log(I_A)$ was approximately constant, as Fig. 10 shows, and a regression of $\log(I_A)$ on time in days from nerve cut fitted the data well up to and including 70 days. Regression coefficients and constants determined separately for the rostral and caudal series were not significantly different. Figure 10 therefore shows only the regression line for the combined logarithmically transformed data, for which the regression coefficient (with standard error) is -0.0219 ± 0.0019 and the constant is $+0.1805 \pm 0.2106$. Indices derived from nearest neighbour measurements on fewer than 30 labelled cells (open symbols in Fig. 10) were omitted from the calculations. The change in $\log(I_A)$ with time is highly significant ($P < 0.001$). Because of the logarithmic transformation, the constant slope of the regression line actually represents an exponential decline in the area index which was halved, on average, every 13.75 days throughout the period from 18 to 70 days.

From 70 to 524 days, much less change was seen. Area index values fell slightly in both rostral and caudal series but, because the fall was small in relation to the variance, the regression coefficients were not significant, either separately or combined. Again Fig. 10 shows the regression line of log (I_A) on time for the combined data, for which the coefficient (with standard error) is -0.0006 ± 0.0004 and the constant is -1.4312 ± 0.3315. A highly significant difference remained, however, between the area indices of the 22 late regenerates (70–524 days) and the eleven normal controls ($P = 0.0008$, one-tailed Mann-Whitney U test). The mean area index for the late regenerates (with standard error) was 0.0373 ± 0.0055, while that for the controls was only 0.0126 ± 0.0010. The mean value of $\log(I_A)$ for the late regenerates (with standard error) was -1.539 ± 0.073, while that for the controls was -1.913 ± 0.037.

Discussion

We have described an anatomical assay of the refinement of the goldfish retinotectal projection that

follows regeneration of a cut optic nerve. Retrograde transport of WGA-HRP, after iontophoretic injection at one of two tectal sites, yielded distributions of labelled ganglion cells in the contralateral retina that changed systematically, as regeneration proceeded, from a wide scatter with little or no retinotopic bias to a small cluster (or sometimes two) in the region normally projecting to the injection site.

In normal fish, and late in regeneration, uptake of WGA-HRP injected by the methods used here is confined to the terminal arbors of optic axons (Cook and Rankin 1984) and seems to depend on adsorptive endocytosis at the synaptic bouton (reviewed by Mesulam 1982). Early in regeneration, since synaptogenesis is known to begin almost as soon as axons reach the tectum (Schmidt et al. 1983; Stuermer and Easter 1984), synaptic uptake may also be presumed to occur. However, growth cones are known to take up HRP by endocytosis, unlike axon shafts (Bunge 1977; Chu-Wang and Oppenheim 1980). If they take up WGA-HRP as well, cell distributions seen early in regeneration might reflect both forms of uptake as discussed previously (Cook and Rankin 1984). In weighing up the significance of this possibility it is important to note that the implied distinction between synapses and growth cones may itself be artifical and misleading: Scalia and Matsumoto (1985) have shown directly, for regenerating optic axons in the frog *Rana pipiens*, that structures with growth cone morphology can themselves be presynaptic elements. In the following sections, wherever possible, we shall use the non-committal phrase 'terminal structures' when referring to uptake sites early in regeneration.

Distributions early in regeneration

The most striking aspect of our results is the wide scatter of labelled cells seen early in regeneration. For this there are several potential explanations. One, which we can swiftly dismiss, is that denervation might have caused some transient change in the tectum that allowed injected tracers to diffuse widely. We could find no difference betwen injection sites in normal tecta and in tecta 22 days after nerve cut. Moreover, because WGA-HRP (unlike native HRP) forms sharply defined sites, binds strongly to membranes near the pipette tip, and does not diffuse freely in the extracellular space (Mesulam 1982), we cannot even attribute the widespread, afocal retinal labelling to enhanced uptake from outside the observed injection site.

The remaining explanations relate the retinal scatter to the paths of regenerating axons through the tectum, about which two basic facts are known. One is that axons enter at the front of the tectum, first arriving in small numbers about 14 days (at 20° C) after nerve cut, and innervating only a marginal crescent that spans the attachment of the two brachia and encloses the rostral pole (Stuermer and Easter 1984). Even axons that will eventually terminate caudally pass through this zone, so we must consider whether the scatter seen after early rostral injections could simply reflect the transient presence there of terminal structures from all retinal quadrants. We might allow a bias towards ventral retina, such as we saw in three out of five cases after 18 days of regeneration, because the injections were rostromedial and most regenerating ventral axons would have reached the tectum, as usual, through the medial brachium (Stuermer and Easter 1984). However, strong evidence against this simple interpretation is the fact that the first area indices obtained from *caudal* injection sites six days later, and even those obtained there after 28 days, were just as great as those from rostral sites and cannot be explained in this way.

The other basic fact about the tectal paths of regenerated axons, which offers a more promising explanation, is that they remain disordered even after the map has been refined (Meyer 1980; Fujisawa et al. 1982; Cook 1983; Cook, Pilgrim, and Horder 1983; Stuermer and Easter 1984). The trajectory of an axon when map refinement is complete must reflect the trajectory of at least one of its terminal structures at earlier stages, for reasons discussed by Cook, Rankin, and Stevens (1983) and Easter and Stuermer (1984) in the context of normal development: thus we should expect the pattern of WGA-HRP uptake through terminal structures early in regeneration to have much in common with the pattern of HRP uptake through damaged axons of passage later. Both can readily be explained as manifestations of the same phenomenon, a striking lack of 'goal-directedness' in regenerative axon growth, which is consistent with the view that axons find exactly appropriate termination sites only by searching as they grow for other, retinotopically related, axons sharing some very restricted 'local sign' (Cook 1983). Much the best candidate for such a 'local sign' being the correlated firing of retinotopically related axons (see Cook and Rankin 1986), the recognition process now seems likely to be mediated through convergent synapse formation with common target neurons (Willshaw and Malsburg 1976; Schmidt and Edwards 1983). Thus the early diffuse WGA-HRP uptake, and the tortuous final paths, could both be reflections of an early phase of 'trial and error' synaptogenesis, through which a refined

final map gradually emerges from a coarse initial one, by selective elimination of the least effective, least well correlated inputs (Changeux and Danchin 1976).

We have to be circumspect in interpreting the early pattern of WGA-HRP labelling as actual evidence for such a phase: to arrive at such an interpretation from our evidence alone, we should need to make two assumptions. First, that the electrophysiologically and morphologically normal regenerated synapses that have previously been found in the rostral tectum after 20 days, and more caudally soon after (Schmidt et al. 1983; Stuermer and Easter 1984), are able to take up WGA-HRP. This, though not yet tested, is not in serious doubt. Secondly, that synaptic uptake sites are not heavily outnumbered by non-synaptic sites throughout the period up to 35 days. If this assumption were false, the few synapses present could all be retinotopic without contributing significantly to the pattern: but if true, they could not, or their contribution would be seen as a cluster. There seems to be no convincing evidence yet on this point.

There is, however, other evidence to support such an interpretation. Adamson et al. (1984) measured presynaptic multi-unit and single-unit receptive field sizes (MURFs and SURFs) electrophysiologically after nerve crush in the frog *Rana pipiens*, not only in the (crossed) retinotectal projection but also in the secondary projection which transmits visual information from one tectum to the other. Like earlier workers (see below) they found enlarged MURFs in the retinotectal projection early in regeneration, though SURFs remained small. However, in the secondary intertectal projection both MURFs and SURFs were enlarged, strongly suggesting that the primary projection made many mislocated functional synapses. Moreover, Hayes and Meyer (1984) have shown directly, by HRP electron microscopy, that regenerating goldfish optic axons are capable of forming such synapses.

'Trial and error' synaptogenesis might be an efficient strategy for refining the map during normal development, when new axons from the retinal margin need explore only the one-dimensional margin of the tectum to find retinotopic sites (Cook, Rankin, and Stevens 1983). In regeneration the search area becomes two-dimensional and the strategy, in consequence, very much less efficient. However, its efficiency would be maximized if each regenerating axon began by branching extensively. There is evidence to suggest that this may happen, both from estimates of axon number in the nerve, tract and tectum (Murray 1976; Murray 1982; Murray and Edwards 1982) and from direct observations of HRP-filled axons (Fujisawa et al. 1982; Schmidt et al. 1984; C.A.O. Stuermer, personal communication). Certainly, the wide initial scatter seen here would be consistent with such branching.

Distributions later in regeneration

Later in regeneration, dense clusters of labelled cells began to appear in approximately retinotopic locations, while scattered cells became fewer. In general these results reinforce the conclusion, first drawn tentatively from electrophysiological studies and later strengthened by autoradiography, that map refinement consists of a gradual but almost total elimination of non-retinotopic terminal structures, leaving only convoluted axons bearing retinotopic terminals.

Only two aspects of the late distributions merit specific comment. The first is the apparent lack of any further refinement beyond 70 days after nerve cut. Here we are pushing our assay to its limit: when the area index is so small even a few isolated cells can increase it significantly, and more data would be needed to exclude any further change. Nevertheless, it is clear that for practical, experimental purposes little is gained by allowing regeneration to proceed for longer than about three months.

The second is the sporadic incidence of paired retinal clusters labelled from single tectal injections. One such pair, after 88 days, was found in an earlier study (Cook and Rankin 1984). The basis of the duplication is not self-evident: in principle, terminal arbors from the two clusters might either have overlapped and interdigitated within the injected region or have been segregated into adjoining fragments of a piece-wise continuous map composed of internally ordered but misrelated part-maps (Cook, Pilgrim, and Horder 1983). We favour the second interpretation, because Meyer et al. (1985) have recently discovered, by anterograde WGA-HRP tracing, that the regenerated projection is *usually* piece-wise continuous, though often on a scale too fine to be demonstrated reliably by our injections. In most electrophysiological studies, duplicated and enlarged receptive fields persisting late in regeneration have been found only very infrequently, a notable exception being the automated mapping study of Northmore and Masino (1984). The constant radial alignment of the five cluster pairs produced by caudal injections is intriguing, in that it suggests that regenerating axons in the caudal tectum may make more errors in their relative radial positions, which are normally determined largely by their sequence of growth (Cook, Rankin, and Stevens 1983), than in their circumferential positions, which are not.

Previous quantitative studies

Weiler (1966) measured the visual acuity of the cichlid *Astronotus ocellatus* after nerve regeneration, using a conditioned response to an array of dots. The average minimum separable visual angle was only 28% greater than normal once regeneration and conditioning were complete. However, no estimate of acuity could be made early in regeneration. Every other quantitative study of map refinement seems to have used electrophysiology to estimate the sizes of presynaptic MURFs: some limitations of this approach were mentioned in the Introduction. Cronly-Dillon (1968) compared MURF sizes in normal newts with those after regeneration of an ablated retina. Five months after surgery, MURFs (but not SURFs) for recording sites in peripheral tectum were significantly enlarged (equivalent in average solid angle to a circular field subtending 31°). By nine months, MURFs in all regions were almost normal. Uncertainty about the timing of retinal regeneration complicates these findings. Horder (1971) estimated MURF sizes in goldfish kept at room temperature after optic nerve cut or crush. The mean MURF diameter, normally 10.5°, was 24.7° after 43–69 days of regeneration, falling gradually to 14.0° after nine months. Humphrey and Beazley (1982) reported a similar gradual decline in the frog *Hyla moorei*, with MURF diameters as great as 70° after 26–30 days but falling to normal by about 50 days; and Adamson et al. (1984) found MURFs in *Rana pipiens* to be about double their normal diameter at 20–40 days, returning to normal in most cases by 60 days. Northmore and Masino (1984) used an automated mapping technique to examine MURFs after optic nerve regeneration in the bluegill sunfish (*Lepomis macrochirus*), and found MURF areas (defined as areas of greater than half-peak response amplitude) to be about three times normal after 39–58 days at 21–24° C, falling significantly during the period up to 152 days from nerve crush but never reaching normal. In contrast to these studies, Schmidt and Edwards (1983) estimated that MURF diameters in goldfish kept at 20° C for 35 days after nerve crush averaged only 11.85°. Since SURF diameters averaged 11.26° they concluded that retinotopy was almost perfect by this time, the earliest at which reliable responses could be obtained.

To simplify comparison of our anatomical findings with these results, we have added a second ordinate to Fig. 10, showing the angle subtended by an equivalent circular receptive field (for equation see Methods). The most striking observation is that our equivalent fields are consistently larger, both in normal fish and throughout regeneration, than typical MURFs. Some of the difference may arise because recording electrodes pick up action potentials over an area smaller than our injection sites, which covered about 1% of the tectum. However, this should be partly offset because labelled ganglion cells are treated by us as points, while their individual SURFs, each covering some 0.5% of the retina, make a large contribution to the normal MURF. Together, these factors might contribute about 0.005 to the area index: this would account for 4.2° of the mean normal equivalent field of 18.3° and bring it appreciably closer to the normal MURF, but would only account for 0.9° of the 86.5° field that fits the regression equation at 35 days. We must therefore seek another explanation; and the most plausible is that electrophysiological MURFs, at least in fish, tend to represent only the areas of the relatively compact labelled cell clusters that begin to emerge at about 35 days, just when reliable unit recording first becomes possible. This would imply that recording electrodes are intrinsically selective for the relatively small, densely branched and retinotopic arbors that represent the end-points of regeneration. Early in regeneration, these 'bushy' arbors may be few and bear only a fraction of the total synaptic population. Studies in other contexts have found anatomical methods to be better than electrophysiology at revealing diffuse projections (see Udin and Gaze 1983).

Finally, it is useful to note some relationships between map refinement and other aspects of regeneration for which there are quantitative data. After axotomy, ganglion cells become basophilic, undergo hypertrophy and develop prominent nucleoli. These changes begin three days after optic tract section and reach a maximum by 21–28 days, when ganglion cell area is increased two-fold, nucleolar area nine-fold, and incorporation of leucine three-fold (Murray and Grafstein 1969). The rate of slow axonal transport begins to rise after six days, reaches a maximum by 17 days, and then falls, though it is still above normal at 66 days (Grafstein and Murray 1969). Morphological recovery is evident after 35 days and virtually complete after 70–90 days (Murray and Forman 1971; Burmeister and Grafstein 1985). All these changes match changes in the area index, as we should expect if this were an indicator of exploratory growth. Complementary trends are shown by indicators of synaptic maturity such as postsynaptic field potential amplitude and response latency (Schmidt et al. 1983).

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Impaired refinement of the regenerated retinotectal projection of the goldfish in stroboscopic light: a quantitative WGA-HRP study

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Summary. The retinotectal projection of the goldfish was studied after regeneration of a cut optic nerve in stroboscopic light, constant light or diurnal light, with the lens removed to blur the retinal image. Retrograde transport of wheatgerm agglutinin, conjugated to horseradish peroxidase, from a standard tectal injection site was used to measure the topographic precision of the projection. The dispersion of labelled retinal ganglion cells, which reflects this precision, was assessed by a method based on distance to nearest neighbour. In normal fish treated similarly, these cells are known to be clustered into about 1% of the retinal area. Early in regeneration, however, they are widely dispersed. The projection map then re-acquires its precision over two or three months.

In diurnal light, lens ablation had no effect on refinement of the regenerated map. Constant light increased the number of labelled cells but also had no significant effect on the map. But in stroboscopic light with a continuous pseudorandom pattern of flash intervals (average rate 4.8 Hz), much less refinement was seen. Even after 70–98 days of regeneration, labelled cells remained scattered, on average, over 20% of the retinal area. These retinae were indistinguishable by several criteria from those obtained in diurnal light after only 32–39 days. Mislocated axon terminals, which are largely eliminated during the second and third months of regeneration in diurnal light, evidently persist much longer in stroboscopic light that synchronizes ganglion cell activity across the retina. These results, like previous ones obtained by blocking the transmission of activity to the tectum, support a model of map refinement based on correlation in the firing of neighbouring neurons, which may have wide application within the nervous system.

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Key words: Retinotectal projection – Topography – Correlated activity – Goldfish

Introduction

Four decades of experimental work on the vertebrate retinotectal pathway have led inexorably to the conclusion that several different mechanisms must make specific, though overlapping, contributions to the establishment of topographic neural projections (Cook and Horder 1977; Meyer 1982). Nevertheless, the key feature of the many such projections (maps) in the nervous system is that *neighbouring* neurons in one nucleus or sheet of cells project specifically to *neighbouring* neurons in another nucleus or sheet. If neurons were themselves able to obtain, transmit and evaluate information about their proximity to each other, they would have the basis of a powerful mechanism for topographic ordering that could account for many experimental observations.

Taking the retinotectal system as their model, Willshaw and von der Malsburg (1976) showed how such a mechanism might work. They suggested that pairs of neighbouring retinal ganglion cells might show a temporal correlation in their spike activity patterns that was absent from non-neighbouring cells; and showed how this correlation might lead, in certain circumstances, to the selective reinforcement of any synapses that both made with the same tectal neuron, or with neighbouring tectal neurons. Applied to all possible ganglion cell pairs, this could lead eventually to topographic order throughout the projection. If one of the mechanisms contributing to map formation were based on neural activity in this way, relatively little information would have to be specified genetically, and expressed through other mechanisms, to control macroscopic features of the

map such as its orientation and metrics (Willshaw and von der Malsburg 1976; Whitelaw and Cowan 1981).

Neighbouring ganglion cells of the same response polarity (on-centre or off-centre) do indeed show correlated spike activity, while neighbours of opposite polarity show inverse correlation. This has been shown both for spontaneous activity in the isolated, dark adapted, goldfish retina (Arnett 1978; Ginsberg et al. 1984) and for maintained activity in the intact, mesopic, cat retina (Rodieck 1967) where it may be due to the effects of shared inputs from spiking amacrine cells (Mastronarde 1983). The strength of the correlation falls with increasing cell separation. This local correlation does not depend on the presence of a retinal image, though visual stimulation increases it slightly (Mastronarde 1983), as might be predicted from the fact that neighbouring cells have overlapping receptive fields.

To eliminate neural activity and study any consequences for map formation, Meyer (1983) and Schmidt and Edwards (1983) injected tetrodotoxin (TTX), a specific blocker of voltage-sensitive sodium channels, into the goldfish eye at regular intervals after optic nerve crush. Using retinal lesions and autoradiographic mapping, Meyer showed that maps regenerated in the presence of TTX were ordered at a gross but not at a refined level. In similar experiments, but using electrophysiological mapping, Schmidt and Edwards found organized maps with enlarged multi-unit receptive fields (MURFs). Both experiments suggest that neural activity may play a part in refining the regenerated retinotectal map. However, doses of TTX that are effective in blocking neural activity also alter the metabolism, and even the number, of regenerating optic axons (see Discussion), complicating the interpretation of these experiments and making it imperative to test less drastic ways of modifying neural activity such as changing the visual environment.

The experiments reported here involved three groups of goldfish with regenerating optic nerves. One group (S-Ab) was kept in stroboscopic (strobe) light throughout the experiment. By entraining the spike discharges of all retinal ganglion cells to intermittent flashes we aimed to limit their freedom to set up local patterns of correlation coding for cell proximity. A second group (C-Ab) was kept in constant light to act as a control for possible effects on S-Ab fish of the loss not only of diurnal rhythm but of any prolonged exposure to darkness. The third group (D-Ab) was kept in normal diurnal light. In all these fish the lens was ablated so that the eye could no longer form a focussed image, thereby decreasing the possibility that neighbouring ganglion cells might acquire local activity correlations from local correlations in light intensity within their overlapping receptive fields. A baseline for map refinement under standard conditions was provided by an additional group of fish (D-In), also kept in diurnal light but with the lens intact, which was run in parallel with these three but provided part of the data for the preceding paper (Rankin and Cook 1986). Map refinement was assessed in each case by retrograde transport of wheatgerm agglutinin conjugated to horseradish peroxidase (WGA-HRP) from an iontophoretic injection site in the tectum (Cook and Rankin 1984a; Rankin and Cook 1986). Some of the results have been presented in abstract form (Cook and Rankin 1984b).

Methods

All observations were made on goldfish (*Carassius auratus*; Cura and Sons, Hemel Hempstead, Herts) 55–65 mm long from snout to tail base. Anaesthesia was induced by immersion in a 0.1% solution of MS-222 (Sandoz) and maintained by perfusion of the gills with a 0.012% solution. Right optic nerves were cut cleanly in mid-orbit with iridectomy scissors, sparing the ophthalmic artery. In fish destined for groups S-Ab, C-Ab and D-Ab, a curved incision was made in the dorsal cornea, arching over the pupil, through which the right lens was seized with forceps and removed. Since the cornea is optically inactive underwater, no image can be formed without the lens. Lens ablation had no discernible effect on the morphology of the retina, and the cornea healed rapidly with little or no scarring. However, the pupil had contracted by an average of 28% by the end of the experiment. Regeneration of a functional lens was never seen, though small globules of lens tissue were occasionally found attached to the ciliary body. Fish that bled excessively or had any anatomical abnormality were excluded. After recovery, 64 fish were assigned in rotation to four similar tanks, made from opaque polypropylene of a light neutral colour.

The first tank (S-Ab) was lit only by a 2-Joule xenon discharge tube flashing continually 300 mm above the water surface. To avoid both the arbitrary selection of a flash frequency and the obvious (but impractical) alternative, the systematic testing of a wide range of flash rates, we used a pseudorandom sequence of flash intervals varying in 20 ms steps between 60 and 360 ms, with an equal probability of any of the 16 possible intervals between each flash and the next. The mean interval was thus 210 ms, corresponding to an average flash rate of 4.8 Hz; and the effective range of flash rates was from 2.8 to 16.7 Hz. The device triggering the stroboscope has been described elsewhere (Cook 1984). The peak illumination at the water surface, measured from calibrated oscilloscope traces of the short-circuit current through an 'eye-response' photodiode (BWP 21; RS Components; peak spectral response at 560 nm), was 1.8×10^6 lux; and the duration to 10% of peak brightness was 50 μs. The mean illumination, estimated from the trace area or from the averaged photodiode output, was approximately 140 lux. Over 4×10^7 flashes were delivered during the experiment: when the discharge tube failed, a second was recruited automatically within two seconds.

The second tank (C-Ab) was lit continuously by a 20 W 'Northlight' fluorescent tube, which matched the strobe light subjectively in colour temperature and on average appeared somewhat brighter. The measured illumination was approximately 600 lux. The third and fourth tanks (D-Ab and D-In) were lit by

similar fluorescent tubes for 12 h each day. The temperature in all tanks was maintained at 20 ± 0.3° C. No attempt was made to eliminate visual contrast in these tanks. All fish could therefore see each other, their daily food ('Tetramin' flake). and an assortment of heating and filtration equipment, through either the left eye (S-Ab, C-Ab, D-Ab) or both eyes (D-In).

At intervals between 42 and 98 days, fish were reanaesthetized for iontophoresis. Subsequent steps were exactly as described by Rankin and Cook (1986), all tectal injections being at the caudal of the two standard sites defined there. In summary, WGA-HRP was iontophoresed into the left tectum of each fish within 10–20 min of removing it from its tank. Two days later the right retina was dissected free and processed to reveal labelled cells. For fish injected from 70 days of regeneration onwards, the individual positions of all these cells were marked on a photomontage of the retina with the aid of a drawing tube. Cell distributions were then analysed as before to determine cluster size in terms of an *area index* (Rankin and Cook 1986) based on retinal area. number of labelled cells and mean distance to nearest neighbour.

For all comparisons between groups. non-parametric statistical tests were used because of their 'distribution-free' properties. Pair-by-pair comparisons, made with the Mann-Whitney U test, were validated by overall (four-sample) comparisons, made with the Kruskal-Wallis one-way analysis of variance by ranks (Siegel 1956). To obtain frequency distribution histograms of normalized distance to nearest neighbour (Fig. 5), all the individual distances for the 26 retinae analysed quantitatively in this experiment, and also for many of the early retinae of D-In type in the preceding study, were keyed into an Apple microcomputer and stored on disk. Each distance was then automatically divided by the expected mean for a random distribution with the same average density as that particular retina ($1/(2\sqrt{D})$; Clark and Evans 1954) to correct for variations in labelled cell number and retinal area. Finally, these normalized distances were pooled by experimental group, transformed logarithmically to increase resolution in the body of the distribution, and allocated to histogram bins.

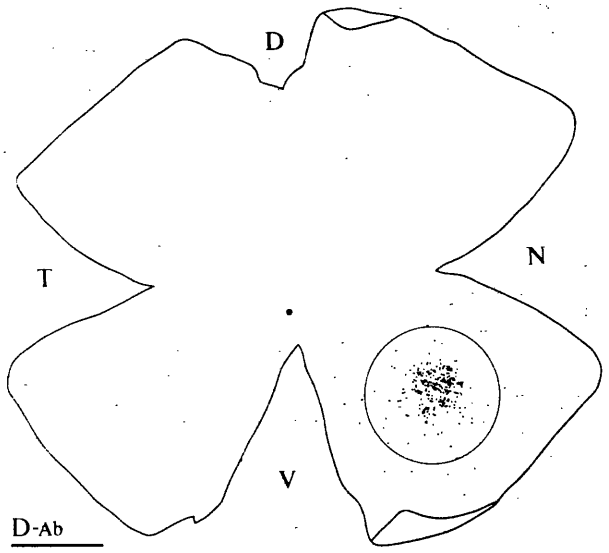

Fig. 1. Flat-mounted right retina from a goldfish kept in *diurnal light* for 70 days after optic nerve cut and lens ablation. Each dot marks the position of a single retinal ganglion cell labelled with WGA-HRP from a caudal tectal injection. The circle surrounding the cell cluster is positioned by eye but its *area*, as a fraction of the total retinal area, represents the area index (0.066) for this particular retina. (The area index is an objective measure of clustering based on retinal area, number of labelled cells and nearest neighbour distance measurements on each individual labelled cell.) This particular retina is shown because the *mean* area index for the eight D-Ab fish was also 0.066. The ventral incision (V) followed the line of the choroid fissure. D, N and T show the directions of the dorsal, rostral and temporal poles. A large, central dot marks the centre of the optic disc. The scale bar represents 1 mm

Results

After iontophoretic injection of WGA-HRP into the left tectum, labelled ganglion cells were present in the right retina in all four experimental groups, at all stages between 42 and 98 days after optic nerve cut. Most were in the expected retinal quadrant, which was always the ventronasal since all injections in this experiment were caudomedial. No consistent differences among groups could be found in the intensity of labelling, or in the morphology of the labelled cells (for representative examples, see Fig. 1D in Rankin and Cook 1986).

At 42 and 49 days, systematic inspection also failed to reveal any consistent differences in labelled cell distribution (6 fish per group). At 56 days, however, cells were becoming noticeably localized into discrete ventronasal clusters in all groups *except* that kept in strobe light with the lens ablated (S-Ab; 3 fish per group). The pattern of labelled cells seen from 70 days onwards in fish kept in diurnal light with the lens intact (D-In) was described and illustrated in the preceding paper (Fig. 7B in Rankin and Cook 1986). Very similar patterns were seen in eight fish kept for 70–98 days in diurnal light with the lens ablated (D-Ab), showing that lens ablation alone had little or no effect on map refinement: a typical cell distribution for this group is shown in Fig. 1. Only four fish survived this long in constant light with the lens ablated (C-Ab); but each of these also showed a dense ventronasal cluster, together with some scattered cells, as in Fig. 2. In contrast, seven S-Ab fish showed little or no such localized clustering, even after 98 days: in most cases, labelled cells were more or less evenly distributed over a large part of the ventronasal retina, as in Fig. 3. In fact, no consistent difference was visible between these S-Ab retinae at 70—98 days and retinae of D-In type that were prepared after only 35 days of regeneration for the preceding paper.

For each of 26 fish that were successfully injected between 70 and 98 days after optic nerve cut, the position of each labelled ganglion cell was plotted on a retinal photomontage and the cell distribution was analysed by the methods described in the preceding

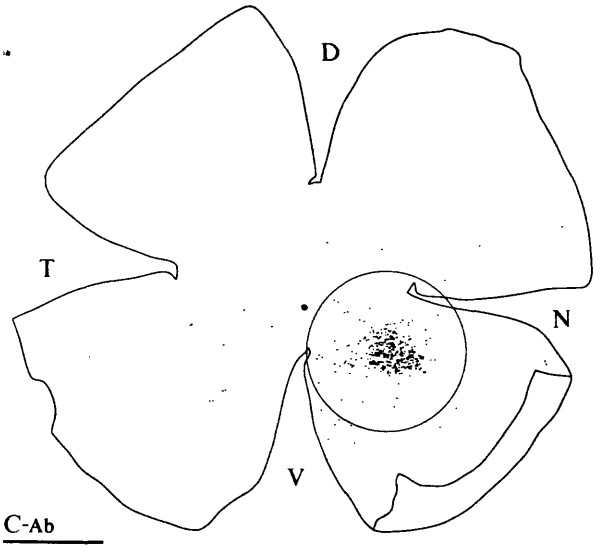

Fig. 2. Flat-mounted right retina from a goldfish kept in *constant light* for 91 days after optic nerve cut and lens ablation. For conventions see Fig. 1. The area of the circle represents the area index (0.097) of this particular retina, which is shown because its index is closest to the mean (0.102) for the four C-Ab fish

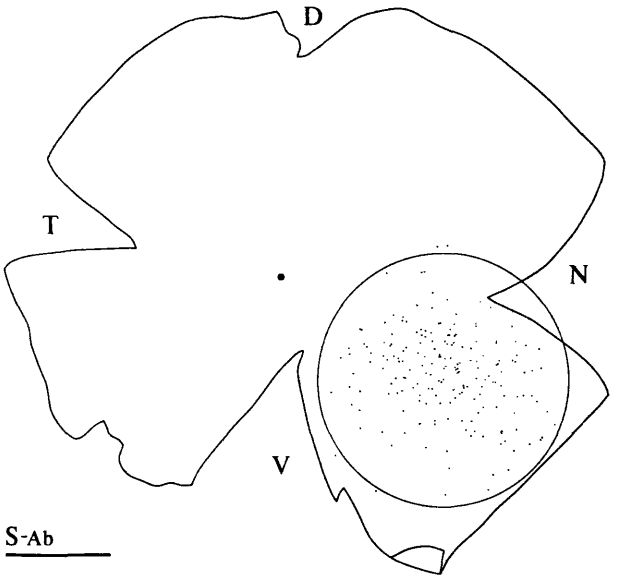

Fig. 3. Flat-mounted right retina from a goldfish kept in *stroboscopic light* for 70 days after optic nerve cut and lens ablation. For conventions see Fig. 1. The area of the circle represents the area index (0.216) of this particular retina, which is shown because its index is the median value, and also close to the mean (0.208), for the seven S-Ab fish

paper (Rankin and Cook 1986). In general, numerical analysis served to clarify and confirm the impressions gained from direct retinal inspection.

The *mean distance to nearest neighbour* was much greater in S-Ab retinae than in any other group (Fig. 4A), as would be expected if strobe light interfered with map refinement by suppressing local correlations in spike activity. Highly significant differences were found, using one-tailed Mann-Whitney U tests, between S-Ab (n = 7) and each of the other groups: C-Ab (n = 4; $P = 0.003$), D-Ab (n = 8; $P = 0.0003$) and D-In (n = 7; $P = 0.0003$). A four-sample Kruskal-Wallis test (see Methods) confirmed that the overall variation among groups was highly significant ($P < 0.002$). No significant variation existed among the three non-strobe groups, either taken in pairs or all together.

However, distance to nearest neighbour is a function not only of cell distribution but also of cell number, and the *number of labelled cells* in each retina also showed significant variation among groups (Kruskal-Wallis test; $P < 0.02$; Fig. 4B). In particular, two-tailed U tests revealed that C-Ab retinae contained significantly more labelled cells than retinae of groups S-Ab ($P = 0.006$), D-Ab ($P = 0.008$) and D-In ($P = 0.042$). The reason for this difference is not clear (though one possibility is considered in the Discussion); but it highlights an important limitation in the use of mean distance to nearest neighbour, by itself, as a measure of cell dispersion.

The *area index*, like the dispersion index (Clark and Evans 1954) of which it is the square, overcomes this limitation by including as one of its terms the average population density of labelled cells: differences between individual retinae or experimental groups in either cell number or retinal area are therefore automatically taken into account (see Rankin and Cook 1986). The area index, too, showed highly significant overall variations among the four groups (Kruskal-Wallis test; $P < 0.002$). In particular, labelled cells covered a larger fraction of the total retinal area in S-Ab retinae than in any other group (Fig. 4C). Despite the correction for cell number, a highly significant difference remained between S-Ab and its closest rival, C-Ab (one-tailed U test; $P = 0.006$); while even higher significance values were found in tests of S-Ab against D-Ab ($P = 0.0003$) and D-In ($P = 0.0003$). As in the case of mean distance to nearest neighbour, no significant variation could be demonstrated among the three non-strobe groups. These results show plainly that the retinotectal projections which existed after 70–98 days regeneration under strobe light were very different from those which regenerated under all the other lighting conditions.

On the other hand, we have already mentioned the resemblance we saw between late S-Ab retinae and much earlier retinae of D-In type. Numerical analysis also confirmed this impression. First, we

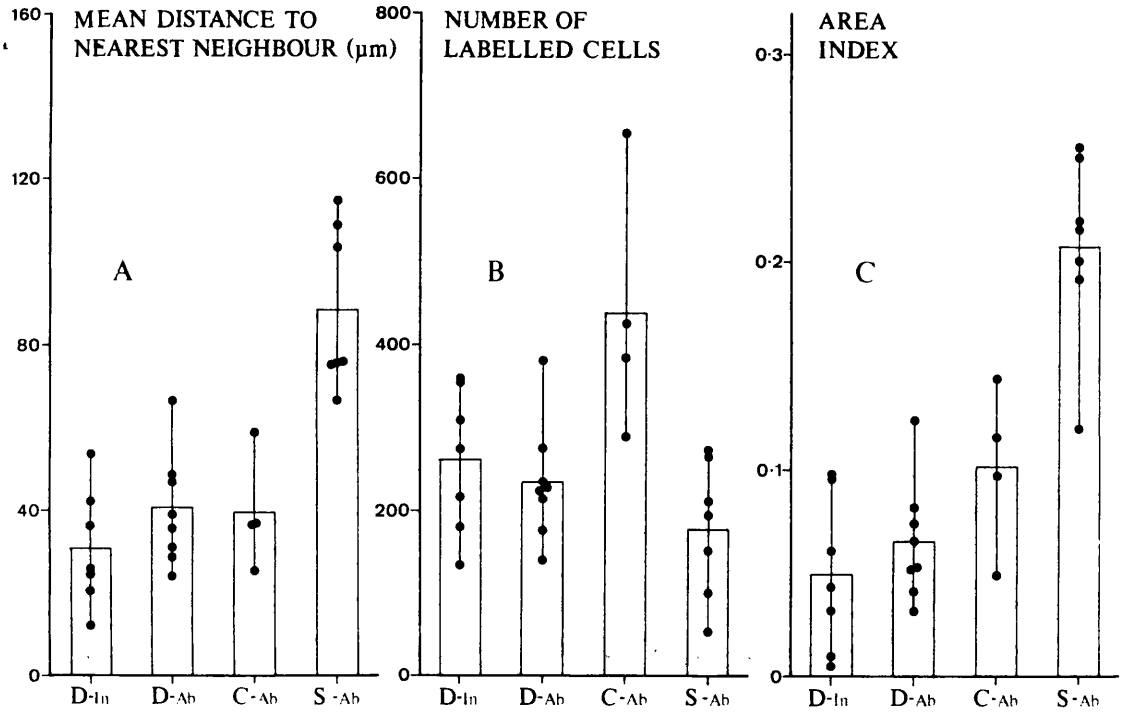

Fig. 4. **A** Values of the mean distance from each labelled cell to its nearest labelled neighbour (filled circles) for each of the 26 retinae analysed numerically. In each case an injection of WGA-HRP was made into the caudal tectum, 70–98 days after optic nerve cut. D-In and D-Ab fish were kept in diurnal light, C-Ab fish in constant light and S-Ab fish in stroboscopic light throughout regeneration. The lens was ablated in all groups except D-In. A histogram bar shows the mean of these values for each individual group. Labelled cells in the S-Ab group were significantly further apart than in any other group. **B** Numbers of labelled cells found in these retinae, with their group means. C-Ab retinae contained significantly more labelled cells than any other group. **C** Area indices for the same retinae, with their group means. Labelled cells occupied a significantly larger fraction of the retina in the S-Ab group than in any other, even though differences in labelled cell number were taken into account. The three non-strobe groups did not differ significantly among themselves

calculated the mean area index for the seven late S-Ab retinae (0.208) and inserted it into the regression equation derived previously for retinae of D-In type between 18 and 70 days (Rankin and Cook 1986). The outcome was an estimated mean regeneration time of 39 days, well under half its actual value (87 days). Then, fearing that we might have been oversimplifying the situation by judging retinae only by their area indices, we made a further series of comparisons using more of the data to show the fine structure of the cell distributions.

From each of the 26 retinae analysed numerically in this experiment, and from many of the early retinae of D-In type in the preceding study, we derived frequency distribution histograms of the distances from all the individual cells to their nearest labelled neighbours, normalized to compensate for variations in labelled cell number and retinal area (see Methods) and then pooled by experimental group. The results are shown in Fig. 5. Late D-In and D-Ab retinae (Fig. 5A and B) yielded distributions with well-defined peaks around 0.1: that is, the commonest distances found were about one-tenth of those which would be expected if the cells had been randomly distributed over the entire retina. Late C-Ab retinae (Fig. 5C) also yielded a distribution with a definite peak, though at a slightly greater distance. Late S-Ab retinae, however, (Fig. 5D) yielded a much broader distribution with a different degree of skew, and a poorly-defined peak at about three times the usual distance. The closest match we could find to this distribution, using retinae of D-In type, was from seven fish labelled, like the S-Ab group, by caudal injections but between 28 and 35 days after nerve cut (Fig. 5E). Any addition of later groups (even the 42 day group) skewed this distribution too far to the left, taking it distinctly closer to the late D-In/D-Ab pattern. The mean regeneration time for these matching D-In retinae was only 32 days. Thus taking more of the data into account only strengthened our conclusion that the late S-Ab retinae were indistinguishable from much earlier D-In ones.

Discussion

We have shown that the refinement of the goldfish retinotectal projection which follows regeneration of

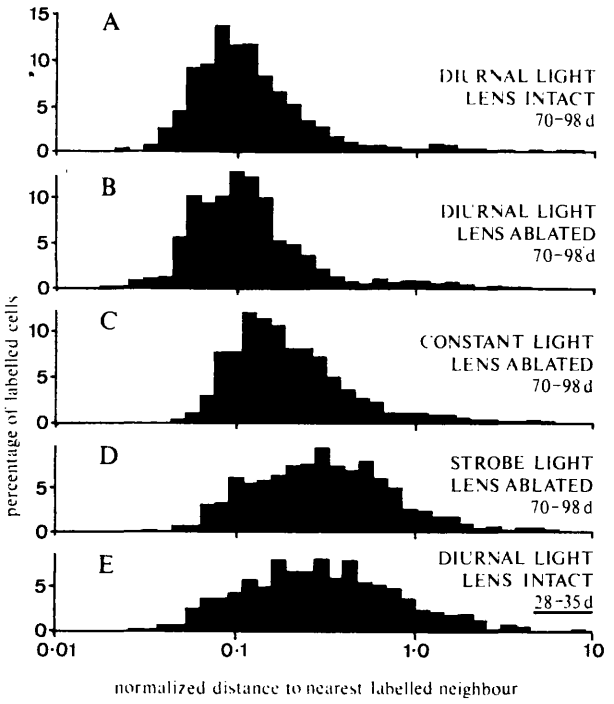

Fig. 5A–E. Frequency distributions of distance to nearest labelled neighbour for the four experimental groups (**A–D**), and for a 28–35 day comparison group (**E**) drawn from the data of Rankin and Cook (1986). To compensate for variations in labelled cell number and retinal area, distances for individual cells were normalized (see Methods) before being pooled. The distribution found after 70–98 days of regeneration in strobe light (**D**) closely matches that found after only 28–35 days in diurnal light (**E**). Both are conspicuously broadened and shifted to the right, relative to the distributions found after 70–98 days in diurnal or constant light (**A–C**)

a cut optic nerve is affected by the visual environment. Exposure to stroboscopic light throughout regeneration, with the lens of the eye ablated, led to the retention even after three months of a diffuse pattern of retrograde labelling normally seen only transiently and at a much earlier stage. Exposure to constant light or lens ablation alone, in contrast, had little or no effect. These results are just what would be expected if local correlations in neural activity were to make an important contribution to map refinement (Willshaw and von der Malsburg 1976; Whitelaw and Cowan 1981). However, other explanations must also be considered.

Interpreting the labelling pattern

In the preceding paper (Rankin and Cook 1986) we considered several explanations for the wide scatter of labelled ganglion cells in retinae of D-In type labelled early in regeneration. We showed that it was caused neither by widespread diffusion of the injected WGA-HRP nor by any special relationship between the injection site and the point of entry of regenerating axons, and must therefore have reflected the striking lack of 'goal-directedness' in axon growth seen repeatedly by other methods in previous studies of optic nerve regeneration. Although the exact nature of the terminal uptake site for WGA-HRP early in regeneration remains unknown, all the available evidence indicates that synaptic terminals in non-retinotopic positions are common at first, becoming rarer as regeneration proceeds.

Because the late S-Ab retinae described in this paper were indistinguishable from these earlier retinae of D-In type, in respect not only of scatter but also of labelled cell number, label density and cell morphology, the same arguments apply to them too: and in this case, an additional argument for a predominantly synaptic site of WGA-HRP uptake can also be proposed. The scatter in late S-Ab retinae, although greater than in late D-In retinae labelled in the same way, was always much less than in comparable retinae labelled in earlier experiments by uptake of native HRP through cut axons of passage (Cook 1983): thus after three months of regeneration, even in strobe light, WGA-HRP uptake was demonstrably confined, as in normal fish (Cook and Rankin 1984a), to the most retinotopic part of each axon. As it seems very unlikely that growth cones could have remained numerous enough after three months to account for all the diffuse labelling present, and as synaptogenesis is known to occur even when activity is blocked (Schmidt et al. 1983), we conclude that imprecisely located synapses, most of which would have been eliminated during the second and third months of regeneration in diurnal light, tended to persist in strobe light.

Action of strobe light

The evidence that strobe light achieved this effect by masking local activity correlations is persuasive, though mostly circumstantial. First, it is possible to show that masking must occur. Evidence for the existence and local nature of ganglion cell activity correlations in normal lighting conditions was reviewed in the Introduction. In strobe light, however, electrophysiological recording shows that both normal and regenerating ganglion cells fire in short bursts driven by individual flashes (Schmidt and Eisele 1985). All ganglion cells with illuminated receptive fields must therefore be broadly synchronized, regardless of retinal position: and when

the whole retina is illuminated by each flash, as it is in fish kept in tanks with light-reflecting surfaces, without shade and without a focussed retinal image, synchrony must be the dominant pattern.

Next, there is evidence from several sources to indicate that strobe light produces its effect on refinement through this masking, rather than through nonspecific effects on the condition of the fish. To begin with, gross effects on general health or axonal regeneration were not seen: the fish kept in strobe light appeared just as healthy as those kept in diurnal light, and their retinae contained just as many labelled cells. However, these are crude indicators. Better evidence comes from a follow-up study in progress, from which four experimental groups have so far been analysed (Cook 1986). These not only confirm the present finding, that map refinement is impaired by strobe light, but also show that ablation of the lens is a necessary precondition (in our experimental conditions) for its full effect: a focussed image can apparently 'rescue' the map in strobe light, even though it has no observable effect in diurnal light. This argues strongly in favour of a central role for local activity correlations. In normal light, these are known to be independent of any retinal image (see Introduction). In strobe light, however, the normal temporal patterns of activity are masked. Brightness variations, which must still occur within the static image cast during each flash by an intact lens, may then become the only sources of local correlation. Ganglion cells with overlapping receptive fields may, for example, show correlations in the number, or latency, of the spikes they generate in response to each flash.

We can also rule out any effect on the map of the loss of diurnal rhythm which occurs in strobe light, by comparing the S-Ab retinae with C-Ab retinae from fish kept in constant light under otherwise identical conditions, which showed little or no blurring of the regenerated map.

Finally, because constant light is known to cause receptor and ganglion cell loss (Marotte et al. 1979), we have begun to check for similar effects in strobe light (J.E. Cook, unpublished results). Fish with regenerating projections, and normal fish matched for size, were kept in either strobe or constant light for 69–97 days; the optic nerve was then cut and packed with HRP; after two days the retina was processed to reveal the labelled ganglion cells; and all such cells were counted in a known area close to the centre of the ventronasal quadrant, where labelled cells are found after caudal tectal injections. As in previous studies on goldfish (Murray et al. 1982; Burmeister et al. 1983; D.W. Burmeister, personal communication), only small differences in ganglion cell number were found between normal and regenerated retinae exposed to the same lighting conditions. However, eight retinae exposed to the type of strobe light used in these experiments contained significantly more surviving ganglion cells than four exposed to constant light for similar periods ($P < 0.01$, Mann-Whitney U test). Thus strobe light seems to cause less ganglion cell death than constant light, while having a much greater effect on the map.

In view of the evidence that the C-Ab retinae probably suffered the highest level of ganglion cell death, it is odd that they contained significantly more *labelled* cells than any other group after tectal injection. One possibility is that the surviving cells formed unusually large and bushy terminal arbors as a consequence of reduced competition for terminal space, and that these were more efficient at taking up WGA-HRP from the margins of the injection site.

Relation to previous studies

Two important experiments were described in the Introduction, in which the results of chronic TTX application to regenerating optic axons suggested that neural activity might play an important part in map refinement (Meyer 1983; Schmidt and Edwards 1983). Their interpretation was complicated by evidence that a similar dosage schedule of TTX has an adverse effect on the number of regenerating axons and the level of amino acid incorporation by ganglion cells (Edwards and Grafstein 1983), and on some aspects of axonal transport (Edwards and Grafstein 1984). Schmidt et al. (1983) also found a small effect of TTX on response latency after 28–31 days of regeneration, though current source-density analysis of tectal field potentials revealed no reduction of synaptic efficacy. Evidence suggesting that these additional effects might be largely independent of the effect on map refinement came from the finding that a single TTX injection at the time of nerve crush, or a schedule of repeated injections during the first 14 days of regeneration alone, retarded axonal outgrowth but affected neither the recovery of a tectally mediated behaviour pattern (Edwards and Grafstein 1983) nor the precision of the electrophysiological map (Schmidt and Edwards 1983).

It is unlikely that strobe light would mimic all these additional effects of TTX. Tectal activity levels in strobe light, in particular, appear to be very much like those in either normal light or total darkness, and much greater than those found after TTX block (Edwards and Grafstein, 1983). In consequence, the finding that strobe light impairs map refinement in much the same way as TTX greatly strengthens the

conclusion that this effect of TTX is independent of its other effects on regeneration and depends only on its ability to silence optic axons, and thereby to prevent locally correlated activity reaching the tectum. Supporting evidence that it is the pattern of activity reaching tectal cells that is important, rather than any metabolic effects of changed activity on axon regeneration, comes from the finding (Schmidt 1985a) that local application of a postsynaptic blocker (alpha-bungarotoxin) to the tectum caused terminal arbors to become destabilized and to seek new postsynaptic partners. This only occurred when the arbors were themselves active, and when unblocked alternative partners were available nearby.

Schmidt and Eisele (1985) have drawn similar conclusions from an independent study of the effects of strobe light on optic nerve regeneration. Goldfish about twice the length of ours were kept in solitary confinement after optic nerve crush, in tanks with white featureless walls designed to reduce visual contrast (but with the lens intact), and exposed to strobe light at a constant flash rate of 1 Hz. The retinotectal map was then analysed electrophysiologically. As in earlier TTX experiments, the regenerated projection was always grossly retinotopic and presynaptic single-unit receptive fields were normal in size. Multi-unit receptive fields (MURFs), in contrast, were several times larger than normal. The similarity between these results and ours is highly encouraging, especially in view of the many differences of method. Of these, perhaps the most important relates to the measurement of retinotopy: some limitations of MURF size for this purpose, as well as some current uncertainties about mapping with WGA-HRP, were noted in the preceding paper (Rankin and Cook 1986). In addition, however, it would seem that neither the flash rate nor the means by which retinal image contrast is suppressed is of critical importance.

Mention must be made of two negative results, and one positive one, obtained with strobe light in other species. Chung et al. (1973) studied the retinotectal projection electrophysiologically in two frogs (*Xenopus laevis*) after optic nerve regeneration in 0.2 Hz strobe light, and reported it to be normal. However, their emphasis was on single-unit properties, and multi-unit field measurements were not reported. Map refinement may not, in fact, have occurred; or it may have resulted either from the focussed retinal image (Cook 1986) or from correlations in spontaneous activity during the long intervals between flashes. Chalupa and Rhoades (1978) studied the visual response properties of tectal neurons in hamsters reared from birth in a diurnal cycle of darkness and 2 Hz strobe light, and found no change in the distribution of receptive field sizes. These neurons being a mixed population, their fields may not have reflected minor changes in the presynaptic map; or map refinement may have resulted either from the retinal image or from spontaneous activity during the daily dark period. However, Pearson and Murphy (1983) studied tectal neurons in rabbits reared from birth in a diurnal cycle of darkness and 4 Hz strobe light, and found significant receptive field enlargement in all laminae. Presynaptic receptive fields were not studied.

The fact that the spontaneous activity of retinal ganglion cells, as well as their driven activity, is locally correlated (Arnett 1978; Ginsburg et al. 1984; Mastronarde 1983) may be important. Schmidt and Eisele (1985) have reported that darkness is less effective than strobe light in impairing map refinement in the goldfish, even though the level of spontaneous activity is low. Higher levels might be sufficient to mediate map refinement in birds in ovo, or in mammals in utero, or after birth but before eye-opening. However, it is not yet clear just when map refinement does occur in birds and mammals. Most studies have only demonstrated retinotopy at a gross level, and even this is still developing at birth in both hamster (Schneider and Jhaveri 1984) and rat (O'Leary et al. 1984), though it exists several days before hatching in the chick (McLoon 1982). That activity-dependent map refinement might occur postnatally in some species is also suggested by the observation that intraocular injections of TTX in the kitten, starting shortly after birth and continuing for 5–8 weeks, caused significant receptive field enlargement in a proportion of lateral geniculate neurons (Archer et al. 1982). Cortical neurons, too, showed some receptive field enlargement in kittens reared in strobe light (Olson and Pettigrew 1974). Other examples of projections that appear to be refined or aligned by activity-dependent mechanisms have been reviewed by Schmidt (1985b).

Contribution of other ordering mechanisms

Strobe light seemed, in these experiments, to halt map refinement at just the stage when regenerated arbors first become electrically detectable, about 35 days after nerve cut or crush at 20° C. However, this may be coincidental. Synaptogenesis begins 10–15 days before this (Schmidt et al. 1983; Stuermer and Easter 1984), and the effectiveness of TTX applied between 14 and 34 days (Schmidt and Edwards 1983) suggests that the refinement process may be activity-dependent from the first. It is safer to conclude that

some map refinement probably occurred as the experiment proceeded, even in our S-Ab fish, by means of spatial information that filtered through the masking.

Even the gross order seen in the S-Ab group must therefore be regarded as an overestimate of the amount of retinotopy that can be generated among axon terminal structures, during regeneration, by mechanisms independent of activity. Since labelled ganglion cells in these fish occupied, on average, 20% of the retinal area (as against 1% in normal and some regenerated fish) it is plain that mechanisms independent of activity cannot begin to account for the detail of the regenerated projection, though they must be able to fix its orientation. In normal development, a further important contribution is made by the orderly pattern of ganglion cell development (Johns 1977), which leads to an orderly arrangement of axons in the optic nerve and tract (Easter et al. 1981; Bunt 1982) and within the tectum (Cook 1983). Thus there is no longer any apparent need for the sophisticated systems of differentiated cell markers that have previously been proposed to account for the high resolution of the retinotectal map: only simple markers are needed to fix its orientation. Most of the information used to generate and regenerate the detail of the retinotectal map seems to arise as a direct and natural consequence (almost a by-product) of some quite basic features of retinal neurogenesis and neurophysiology.

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