The early development of the frog retinotectal projection

JEREMY S. H. TAYLOR

Department of Human Anatomy, University of Oxford, South Parks Road, Oxford OX1 3QX, UK

Summary

The guidance of retinal ganglion cell axons has been investigated in embryos of the frog Xenopus. During the initial development of the brain a series of axon tracts are laid down forming a basic ‘scaffold’ or framework. Retinal axons grow through one of these tracts, the tract of the post-optic commissure (tPOC). This is the only tract that extends through the rostral part of the brain at these early stages of development. The origin and development of the tPOC has been studied using antibodies which label neurons at their earliest stages of differentiation. The first sign of the tPOC is a chain of neurons which differentiate simultaneously in the caudo-lateral part of the diencephalon. Axons from these neurons grow the short distance between adjacent cells interlinking the chain to form a descending tract. A series of other axon projections are then added to the tPOC, each of which is segregated into a particular sub-region of the tract. Retinal axons are added to the tract approximately 18 h after its formation. They grow in the sub-pial part of the tract and always occupy the rostral-most edge. Retinal axons follow the tract to the region of the developing tectum where they leave, turn dorsally, and terminate.

The reliance of retinal axons on this pre-existing pathway has been demonstrated by experimentally altering the course of the tPOC during its early development. The caudo-lateral wall of the diencephalon has been rotated through 90° at a stage just before the tPOC neurons differentiate. Confirmation of the predicted alteration in the course of the tPOC has been made using immunocytochemistry. In such manipulated brains, retinal axons maintain their strong affinity for the rostral edge of the tPOC, following its altered course through the diencephalon.

Key words: axon guidance, retinotectal, Xenopus, selective fasciculation.

Introduction

These studies concern the development of axon tracts in the central nervous system. We have concentrated upon the earliest stages of development of the retinofugal projection in the frog Xenopus. Specifically, we have investigated the guidance of growing retinal ganglion cell axons as they course through the diencephalon to their termination in the developing optic tectum. The major aim of this work has been to define, as precisely as possible, the cellular environment faced by the growing fibres. We believe that such descriptive work is essential before meaningful suggestions for mechanisms involved in guidance of axons in a particular system can be made. We have also examined the reliance of the growing retinal axons on components of their environment by performing embryonic manipulations that disrupt the normal spatial relationships of the embryonic brain.

Nature of the problem

In Xenopus the first retinal ganglion cell axons arise from the central part of the eye cup at stage 28 (Cima and Grant, 1982). They reach the diencephalon approximately 10 h later at stage 33 (Harris, 1986; Easter and Taylor, 1989). Within the diencephalon retinal axons grow as a group following a predictable course. First they run medially, meeting and passing through the fibres of the other eye to form the chiasm, then they follow the lateral wall of the diencephalon growing dorsally and forming the optic tract. At the border between the dorsal diencephalon and the midbrain they arborize and terminate over the region where the tectum will later develop (Fig. 1).

Possible mechanisms for axonal guidance in the retinotectal system

There have been many suggestions as to possible mechanisms that might be involved in axon guidance, many of which have been proposed after observations in vitro. It is the probable involvement of a multiplicity of such mechanisms that has thwarted attempts to define experimentally any general mechanism by which axons are guided. Four broad categories of mechanism
of axon guidance will be briefly outlined. These will serve as working definitions for the ensuing discussions and it will be shown how each of these mechanisms is likely to be involved in the establishment of the retinotectal projection.

(i) Local cues: the interactions between a growing axon and discrete entities within the immediate environment which may be used for axon navigation are regarded as local cues. These may be specific cells, or localized concentrations of extracellular material, preferred as a substrate by a growth cone. By definition they would be used as a series of stepping stones along which a growing axon sequentially moves (Bentley and Caudy, 1983; Hammerback and Letourneau, 1986). Specific affinities between individual neurons also have been demonstrated in the invertebrate CNS (Raper et al. 1983).

(ii) Pre-formed pathway: this can be considered as a continuous cue that predicts the course followed by an axon. This may be a tract of specifically favourable cells (Jacobs and Goodman, 1989) or an aligned concentration of favourable extracellular material (Silver and Rutishauser, 1984) or, for example, a bundle of previously grown axons (Kuwada, 1986).

(iii) Target-attraction: one of the earliest mechanisms proposed for growth cone guidance was chemotraction (Ramon y Cajal, 1890). The idea is that the target produces some diffusible molecule to which the appropriate axons respond by directed growth towards the source. Good evidence for such a mechanism has been obtained only recently (Lumsden and Davies, 1983, 1986; Tessier-Lavigne et al. 1988; Heffner et al. 1990).

(iv) Selective inhibition: as outlined above there is good evidence for differential attraction between growth cones and their substrates. Recently, evidence for selective repulsion has also been found (Patterson, 1988). Inhibition of growth cone extension by interactions with cell surface proteins has been suggested to act in confining growing axons to particular sub-regions of their environment (Walter et al. 1990).

The development of a basic framework of axon tracts in the brain

In an attempt to identify potential cues that are encountered by growing retinal axons, Steve Easter and I undertook a detailed study of the environment of the developing brain at the stages when the first retinal axons were growing towards the tectum (Easter and Taylor, 1989). This involved labelling the retinal projection with HRP in a progressive series of staged embryos and preparing serial semi-thin sections or thin EM sections through the brain. We found that the retinal axons did not grow in isolation as the optic tract, but joined a pre-existing tract, the tract of the post-optic commissure (tPOC) (Easter and Taylor, 1989). The tPOC is one of a series of tracts that form a system of axon pathways which constitute a basic framework for axon growth within the developing brain. Neurons that send out axons during these early stages of development tend to use these existing pathways rather than pioneer new routes. This mode of brain development, forming a simple framework of tracts within which multiple systems develop, each making characteristic selections from the pathways available, is remarkably similar to the situation found in the CNS of invertebrates (Goodman et al. 1984). Amongst vertebrates, this strategy is not unique to the development of the
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Within the tPOC, retinal growth cones fasciculated with the other axons but were always found restricted to the rostral edge (Fig. 2A). There was no physical segregation between the retinal and other component axons of the tract. This predilection of retinal axons for the rostral edge of the tPOC suggests a preferential affinity for, or an exclusion by, some component of the tract. Using electron microscopy, the labelled retinal axons were found in direct contact with other fibres of the tPOC and in some cases with the neuroepithelial end feet (Fig. 2B). The retinal fibres maintained their rostral position in the tract to the region of the developing tectum where they left the tPOC and immediately arborized. This suggests that there must be some cue at the position of the developing tectum telling the growing retinal axons to get off the pathway and arborize. We consider the possible nature of this cue in the light of the experimental findings detailed below.

To determine the origins of the tPOC and details of other axonal components that use this common pathway, we have now made an immunohistochemical study of the developing CNS. Using antibodies which specifically stain neurons at their earliest stages of differentiation, we have described the sequence of development of all early developing axon tracts (Taylor and Easter, unpublished observations). We have used an antiserum against goldfish GFAP (Nona et al. 1989), which in Xenopus stains developing neurons rather than glial cells, the antibody HNK-1 (Kruse et al. 1984), and an antibody against acetylated tubulin (Piperno and Fuller, 1985). Each of these have slightly differing staining patterns, but all label the majority of differentiating neurons in CNS. Their staining of the same neurons appears in the following sequence: first α-GFAP, then HNK-1, followed by α-acetylated tubulin.

At stage 24, a chain of cells was stained which extended from the rostral end of the neural tube through the caudo-lateral wall of the diencephalon to the flexure at the ventral mesencephalic–diencephalic border (Fig. 3A). These cells were the first evidence of the development of the tPOC. They initiated growth cones which grew caudally, interlinking adjacent cells and so forming a continuous pathway. By stage 25, a well defined bundle of axons was established running from the anterior end of the neural tube, dorsally to the midbrain flexure. At the midbrain flexure there was a prominent cluster of stained neurons which had descending axons. These descending axons passed from the flexure into the ventrolateral tract of the hindbrain. By stage 28 the tPOC had increased in size and had joined with its counterpart on the other side of the brain, forming a commissure at the ventral mid-line of the diencephalon (Fig. 3B). From its position we have designated this commissure, post-optic. At this time the initial tract was joined by a projection arising in the olfactory region of the forebrain which passed through the commissure into the contralateral tPOC (Dar and Taylor, 1990). Later, a substantial number of cells in the caudal diencephalon and hypothalamus also sent axons into the tract. Dorsally, a projection arising from the pineal entered the tract and turned caudally. The tPOC was finally joined by retinal axons at stage 33.

fig. 2. (A) Semi-thin section, cut transversely through the optic tract of a stage 35 brain, showing the HRP-labelled retinal axons (arrowhead), fasciculating with the unlabelled axons of the tPOC. The retinal axons are confined to the rostral-most edge of the tPOC. Scale bar, 10 μm. (B) Electronmicrograph of HRP-labelled retinal axons and their growth cones in the rostral part of the tPOC in a stage 39 brain. There is no structural barrier separating the retinal axons from other components of the tPOC. The filopodia of the growth cones are in contact with other axons of the tract and with the end-feet of neuroepithelial cells at the pial margin (P). N, nucleus of neuroepithelial cell. Scale bar, 5 μm.
that the basic components of the scaffold do not change dramatically during this time (Easter and Taylor, 1989). These first contributions to the tPOC are only the beginning; for example, in adult *Ambystoma* there are at least 15 components in the tPOC (Herrick, 1942).

**Segregation of component axons in within the tPOC**

Sumra Dar and I have used HRP to label known component systems of the early tPOC and describe their development (Taylor and Dar, 1990). The first projection we studied was that from the olfactory region of the forebrain. In semi-thin sections of labelled projections and from EM evidence, we knew that this was one of the first fibre projections to be added to the tPOC at stage 28. Axons of this projection grew diagonally across the ventral forebrain and joined the post-optic commissure just lateral to the mid-line. They then crossed in the commissure and descended through the tPOC to the hindbrain, where ultimately they terminated in the reticular formation. These first projection fibres were not clearly localised in the tPOC, but as development proceeded they tended to occupy a relatively caudal position within the tract. At stage 35, in the ventral diencephalon, olfactory axons could be seen crossing to the caudal edge of the tract traversing more recently added fibres which lie rostral in the tPOC (Fig. 4A). An ascending component of the tPOC, which arose in the rostral hindbrain, developed at stage 32. Fibres of this projection lay rostral to the olfactory axons in the mid-region of the cross section of the tract. At stage 35 comparison of the position of the these hindbrain axons with that of the olfactory and retinal fibres showed that they had a distinct central tract distribution (Fig. 4B). Retinal axons entered the diencephalon at stage 33 and, as already described, were confined to the rostral edge of the tPOC.

We have therefore identified three component systems of the tPOC which show differing spatial restrictions of their axons. This differential distribution of sub-populations of axons within a common pathway is suggestive of a mechanism of selective fasciculation. These observations are consistent with the selective affinities demonstrated by growing axons within the axon tracts of the invertebrate CNS (Bastiani et al. 1984; Bastiani and Goodman, 1986). In the tPOC, we do not know whether this partitioning is the result of exclusion from, or specific affinities for, either other axonal components of the tract or some cellular or extracellular component in the milieu of the tract. We are currently examining interactions between these component axons *in vitro*.

**Games of hide and seek with the eye and the tectum**

To test how reliant retinal axons are upon any of these potential cues one can exploit the remarkable resilience of the frog embryo CNS to surgical interference. The
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Fig. 4. Semi-thin horizontal sections through the ventral diencephalon of stage 35 brains in which the olfactopeduncular, ascending hindbrain, and retinal components of the tPOC have been labelled with HRP. (A) Olfactory axons enter the tPOC at an oblique angle and cross through the other axons of the tPOC to reach their normal position at the caudal edge of the tract. (B) Hindbrain ascending axons lie in the central region of the tract where they have a loose distribution. (C) Retinal axons occupy the rostral edge of the tract. R, rostral; tPOC, tract of the post-optic commissure; OS, optic stalk. Scale bars, 10 μm.

experiments were designed to test whether some form of target attraction plays a role in guiding growing retinal axons. One can graft an eye to an abnormal location and see whether the retinal axons can navigate to their target over abnormal territory (Harris, 1986), or, one can move the tectum to a novel position and see if the axons find it (Taylor, 1987, 1990). The outcome of these experiments is that retinal axons nearly always win (Fig. 5A). The growing axons can locate the tectum in spite of their abnormal spatial relationship. These studies support the idea of a long range signal emanating from the tectum which guides the growing axons to their correct target. Ideally, this suggested tectal attraction should be demonstrated in co-culture experiments. Unfortunately, the results of exhaustive culture experiment using retinal and tectal explants have not yielded any evidence for such a signal (Harris et al. 1985; Jack and Taylor, unpublished data).

One alternative explanation for the directed outgrowth of retinal axons towards a misplaced target is that the operation has altered the underlying axon scaffold (Taylor, 1990). However, we have now shown that in the tectal translocation experiments the consequence of the operation on the scaffold of axon tracts is minimal. The pineal, which is an early differentiating structure, forms a useful marker to show the success of the operation, but with the exception of the axons emerging from the pineal, the other axon tracts are unaffected. This lends further support to the notion that there is some form of target attraction.

Occasionally the results of hide-and-seek operations do not fit with the idea of a potent tectal lure. Axons arising from eyes very close to the tectum may follow well defined routes away from the tectum (Fig. 5B; Katz and Lasek, 1978; Harris, 1986), or in other cases they can pass by the tectum and grow the wrong way down through the optic tract (Fig. 5C). In each case the routes taken by retinal fibres that are not directed towards the tectum correlate with known component tracts of the basic scaffold. For example, retinal fibres growing away from the tectum through the hindbrain do so in a sub-region of the ventro-lateral tract, in association with the central tracts of the trigeminal nerve (Fujisawa et al. 1989).

The ultimate game of retinotectal hide-and-seek is when the tectum is removed, leaving the axons to find their way in the absence of their target. In such experiments the idea of a long-range signal being a...
Hb , major determinant of optic fibre guidance in the optic tract is seen to have been mistaken. Where the tectum was ablated before the outgrowth of retinal axons, the subsequently growing retinal axons were shown to have followed a normal pathway through the diencephalon. At the place where the tectum would normally have been developing, retinal axons either crossed over the dorsal surface of the brain and joined the optic tract on the other side, forming a closed, looping optic tract, or, disappeared down through the hindbrain and into the spinal cord (Taylor, 1987, 1990). These results suggested that the cues guiding the retinal fibres through the optic tract were local, lying within the diencephalon (Harris, 1989), and the cue to terminate was probably a short range, target derived signal (Taylor, 1990).

**Rotations of the diencephalic neuroepithelium and the effects on both tPOC and retinal axon development**

Harris (1989) investigated the suggestion that in the absence of the target there must be a local cue within the diencephalon to guide retinal axons, by performing a very difficult manipulation. A small square of lateral diencephalon was excised at stage 25, rotated clockwise or anticlockwise through 90°, and then re-implanted. At later stages of development, retinal axons entered the graft and turned through 90° corresponding to the direction of rotation. This experiment provided powerful evidence for local cues within the developing diencephalon which not only specify the permissive region for retinal axon growth but also direction.

I have extended these experiments to look at how the manipulation of the diencephalon affects the development of the tPOC and the subsequent development of the retinotectal projection. At various times after grafting, embryos were labelled using the HNK-1 antibody to show the early developing axon tracts. The grafts include members of the chain of early differentiating cells that initially form the tPOC. These cells underwent their normal sequence of development and produced descending axons which interlinked cells within the graft. Within the graft the differentiated cells formed a horizontal band of axons lying perpendicular to the rest of the tPOC (Fig. 6). At the graft borders, where the normal chain of tPOC cells was broken, axons were forced to navigate over virgin neuroepithelium. Because of variations in the healing patterns of grafts the course followed by these axons at the graft borders resulted in variable distortions of the diencephalic axon tracts. In some cases the graft was by-
Fig. 6. HNK-1 staining of the tPOC in a stage 28 brain which has had the lateral part of the diencephalon (graft indicated by arrows) rotated by 90° in an anti-clockwise direction. The stained axons and cells of the tPOC that lie within the graft can be seen to lie perpendicular to the normal course of the tPOC (arrowheads). P, pineal; dvdt, dorsolateral diencephalic tract; OS, hole left by removal of eye cup and optic stalk; MF, midbrain flexure. Scale bar, 100 μm.

passed and the axons re-formed a tPOC, which had a relatively normal course. In other cases the axons emerged from the graft and only joined with one end of the disrupted tPOC resulting in a discontinuous tract, whilst in other cases the grafted axons successfully connected with the ends of the tract, forming a sinuous tPOC. On the basis of these results it was predicted that if retinal axons are obliged to follow the tPOC then they should form a predictable series of distorted optic tracts. Unfortunately, the antibody staining protocols (Chu and Klymkowsky, 1989) are not compatible with retinal axon labelling using either Dil or HRP. We have, therefore, performed a parallel series of retinal-labelling experiments.

The graft was fluorescently labelled at the time of operation to allow for later identification and the embryos were allowed to develop to a stage when the pattern of retinal axon outgrowth could be determined. They were then fixed and Dil was applied to the retina. A day later, fluorescent micrographs were taken to show the graft and the Dil-labelled axons. The results of such grafts are variable, as one might expect on the basis of what has been shown to have happened to the tPOC. In most cases axons followed pathways which turned through 90° as they encountered the rotated graft (Fig. 7; see Harris, 1989). In some very instructive cases the labelled retinal axons deviated from the normal route of the optic tract long before they reached the graft. This means that the 'local information' for retinal axon guidance is capable of acting at a distance.

The most likely explanation for such re-routing of the optic fibres is that the graft healing resulted in a rostral discontinuity in the tPOC, which was rectified resulting in a sinuous tPOC. This preformed pathway was subsequently followed by the retinal axons. In other cases the retinal axons appeared little affected by the rotated graft (Fig. 7D–F), which would suggest that the course of the tPOC had not been affected by the operation.

To see if the prediction that the retinal axons always fasciculated with the rostral edge of the tPOC and followed its course through the diencephalon, the preparations were photo-oxidised, to yield a stable image of the Dil labelling, and serial semi-thin sections cut through the brains. This allowed us to examine the course of the retinal axons and their relationship to the fibres of the tPOC. In all cases the retinal axons occupied a position at the rostral edge of the tPOC, which they maintained through the host diencephalon and through the graft (Fig. 7F). In those cases where the optic tract was abnormally distorted, the retinal axons had followed a correspondingly distorted tPOC. Where the retinal fibres appeared to ignore the graft and showed no deviation, the tPOC appeared to be unaffected by the operation. In one case following a clockwise diencephalic rotation, retinal axons turned rostrally, rather than in the predicted caudal direction. In this case retinal axons could be followed passing from the rostral edge of the lower part of the tract through the fibres of the tPOC, to attain the dorsal edge of the tract (originally rostral) in the graft.

Conclusions

We believe that the local cue used by retinal axons within the diencephalon is the tPOC. This tract is formed by a specific population of early differentiating cells and is joined by several different axon projections which use it as a common highway. Retinal ganglion cell axons join the tPOC which guides them through the diencephalon. Within the tPOC retinal fibres have a strong affinity for the rostral edge of the tract, leading them to a position just ventral to the developing optic tectum as the tract courses around the midbrain flexure. Where the normal development of the tPOC is disrupted by experimental manipulation the distortions of the tract are faithfully followed by the retinal axons.

Two important and central problems remain unanswered: the determination of the time and position of differentiation of the chain of cells in the diencephalon, and the signal used by retinal axons to recognise the tectum. We have no clues about how a longitudinal structure such as the tPOC and its continuation into the ventrolateral tracts of the spinal cord could be specified. Suggestions for regional specification in the vertebrate CNS have mostly concentrated upon patterning which is orthogonal to that required to specify longitudinal tracts (Keynes and Lumsden, 1990; Patel et al. 1989). As to the target derived cue, we think that it must be recognisable over a short distance, since in the tectal translocation experiments the tracts in the diencephalon are unaltered, yet fibres locate the tectum.
and leave the tPOC early. We also know that the recognition of the target is a property of the growth cone, since soma-less retinal axons arborize in an abnoromal rostral optic tract well away from its normal position. At the graft the labelled fibres are seen to have turned through $90^\circ$ and continued to the tectum (T). (B) The Hoechst-stained graft (arrows) under broad band UV fluorescence, which incidently shows the rostral course of the retinal axons as a dark band. (C) The photo-oxidised Dil preparation of A. (D–F) A brain in which a clockwise rotation of the diencephalic graft has had little effect upon the course of the retinal axons. (D) The path followed by retinal axons demonstrated with photo-oxidised Dil. (E) A fluorescent image of the Hoechst-stained graft (arrows) showing its relationship to the retinal axons. (F) A 3D reconstruction of the brain, which had been sectioned as serial semi-thin sections to reveal the relationship between the retinal and tPOC axons. The retinal axons can be seen to have maintained their usual rostral position in the tPOC through the entire diencephalon including the graft, off, olfactory region; tAC, tract of the anterior commissure; sot, supraoptic tract; OT, optic tract; tPOC tract of the post-optic commissure; dVdt, dorsolateral diencephalic tract; PC, posterior commissure; T, tectum; VLT, Ventrolateral tract. Scale bars, 100 µm.

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and leave the tPOC early. We also know that the recognition of the target is a property of the growth cone, since soma-less retinal axons arborize over the tectum (Harris et al. 1987).

Whilst these studies have concentrated upon the development of the frog visual system, we know that in both mammals and chicks the supra-optic tract pre-exists and is joined by the retinofugal axons. The sequence of development of retinal and non-retinal systems in the diencephalon of both mice and chicks is currently being investigated.

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