Divergent axon collaterals in the regenerating goldfish optic tract: a fluorescence double-label study

DAVID L. BECKER and JEREMY E. COOK
Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK

Summary

In the normal goldfish, optic axons are distributed between the two arms (brachia) of each optic tract, in such a way that each axon enters the tectum close to its retinotopic termination site. We have shown previously that regenerating axons at first express little or no preference for their normal brachium. Later, however, a partial refinement of the brachia pathway takes place, implying that some axons must have sent out divergent collateral branches and then eliminated the least appropriate.

We have now studied the formation and subsequent loss of axon collaterals in regeneration using retrogradely transported fluorescent dyes. We labelled the axons in the medial brachium with Fast Blue and those in the lateral brachium with Diamidino Yellow in a way that avoided cross-contamination. In normal fish, yellow-labelled ganglion cells dominated the dorsal retina and blue-labelled ganglion cells the ventral, with only a narrow zone of overlap. Double-labelled cells were not found.

In fish labelled early in regeneration, however, both dyes were spread over the entire retina in single- and double-labelled ganglion cells. As regeneration progressed, each dye again came to dominate its appropriate retinal region; but much less strongly, confirming previous results. At the same time, double-labelled cells became harder to find. From 60 days after nerve cut onwards they were rare, and largely confined to the boundary zone between dorsal and ventral retina. Their distribution was consistent with our previous proposal that excess collaterals may be eliminated competitively. Since axons from the boundary zone terminate in a tectal strip equidistant from the two brachia, neither route would confer any clear competitive advantage over the other.

Key words: optic tract, axon collaterals, fluorescent double-labelling, goldfish.

Introduction

Fish and amphibia regenerate their retinal projections when the optic nerve is cut. In the goldfish, the regenerated projection to the contralateral optic tectum eventually regains most of its normal topographic precision, though only after several weeks of gradual refinement (Rankin & Cook, 1986).

An equivalent degree of precision never returns to the axonal pathway (Cook, 1983; Stuermer & Easter, 1984; Becker & Cook, 1987). In normal fish, axons from the ventral half of the retina run in the medial brachium of the optic tract to reach the medial part of the tectum, whereas those from dorsal retina run in the lateral brachium to reach lateral tectum (Attardi & Sperry, 1963; Horder, 1974; Springer & Mednick, 1986). The division is quite precise: apart from a narrow boundary zone of overlap between dorsal and ventral retina, only about one retinal ganglion cell in seventy has an axon in the brachium that is inappropriate to its tectal termination site (Stuermer & Easter, 1984; Becker & Cook, 1987). In the initial stages of regeneration, however, ganglion cells can be labelled from the inappropriate brachium just as readily as from the appropriate one (Becker & Cook, 1987). The proportion of inappropriately labelled cells then declines, showing that some refinement of the brachial pathway takes place, though months or even years later one ganglion cell in four retains an axon in the inappropriate brachium (Horder, 1974; Stuermer & Easter, 1984; Becker & Cook, 1987).

The refinement, though incomplete, is important. It cannot be ascribed to the death of cells with misrouted axons because little cell death occurs in goldfish during regeneration (Murray et al. 1982; Burmeister et al. 1983; Cook & Rankin, 1986), so we
were led to deduce (Becker & Cook, 1987) that regenerating axons must send collateral branches by divergent pathways towards their target sites. Pathway refinement could then take place by elimination of the least appropriate collaterals.

We have now labelled the axons in each brachium of the tract separately, with retrogradely transported fluorescent dyes, to confirm that regenerating axons do branch and can send collaterals into different brachia. We chose Fast Blue and Diamidino Yellow because they are transported well and can be distinguished clearly when present in the same cell. Fast Blue chiefly labels the cytoplasm while Diamidino Yellow labels the nucleus alone (Kuypers et al. 1980; Keizer et al. 1983). When proper precautions are taken to avoid cross-contamination of the dyes, double-labelling of a retinal ganglion cell indicates that it has an axon in each brachium. A preliminary account of our findings has been published (Becker & Cook, 1988).

Materials and methods

General

Common goldfish (Carassius auratus; 55–65 mm long, snout to tail base) were obtained locally. All surgery was performed under MS222 anaesthesia (ethyl 3-aminobenzoate, methanesulphonic acid salt; Aldrich), induced by immersion of the fish in a 1% solution. Right optic nerves were cut in mid orbit, fish with damage to the accompanying blood vessels being eliminated. During regeneration, all fish were kept in diurnally lit tanks at 20 ± 0.5 °C. At various times between 21 and 147 days after nerve cut, the brachial distribution of the regenerating axons was examined as described below. At least two normal fish were included as controls for possible cross-contamination of the dyes in every group taken for analysis. In all, 82 labelled retinae were studied: 42 with normal projections and 40 with regenerating ones.

Dye application

Suspensions containing 1–2 mg of Fast Blue or Diamidino Yellow dihydrochloride (Illy) in 100 μl of distilled water were spun down for 10 min in a microcentrifuge and the supernatants stored frozen in embedding capsules. To apply the dyes, a cranial flap was opened and the left lobe of the forebrain gently displaced to reveal the left optic tract. The lateral brachium was completely transected with an electrolytically sharpened tungsten needle, a small pledget of gelfoam (Allen & Hanbury) soaked in Diamidino Yellow was wedged in the cut, and the forebrain was replaced to apply the dyes. The glycerol-mounted retina viewed under epifluorescence illumination using Zeiss filter set 18 (395–425 nm excitation) and immersion objectives. Fast Blue labelling bleached rapidly, even when ‘anti-fade’ mountants were used, so photographs were taken immediately on Ektachrome 200 reversal film.

Results

The quality of the preparations varied considerably. In some cases, it was hard to see labelled cells at all. In others, especially where the photoreceptor layer was stripped, they stood out very clearly. No cause could be found for much of this variation, which occurred not only between batches but also within a single batch, and even between different regions of the same specimen. This made it impractical to attempt the kind of systematic quantification of label distribution previously used with horseradish peroxidase (HRP). Counts made at the microscope were also curtailed by photobleaching, while photographs were limited to a single focal plane. Only rough estimates of prevalence could be obtained, by cat-
egorizing and counting cells in individual small fields in regions of apparently typical distribution.

**Normal axons**

When ganglion cells in normal fish were retrogradely labelled with Diamidino Yellow (from the cut lateral brachium) or Fast Blue (through the intact medial brachium), a clear segregation of the two dyes was seen, yellow cells being largely confined to dorsal retina and blue cells to ventral retina. Occasional blue cells were present dorsally and yellow cells ventrally, but all appeared to be single-labelled (Fig. 2A,C). Rough estimates of their prevalence were consistent with previous evidence, obtained by the more quantitatively reliable HRP method, that about one normal ganglion cell in seventy has an axon in the inappropriate brachium (Becker & Cook, 1987). At the boundary between dorsal and ventral retina, single-labelled blue and yellow cells were intermingled across a narrow intermediate zone (Fig. 2B). The clear segregation of the two dyes in normal fish, and the absence of double-labelled ganglion cells, together show that our technique for labelling axons in the two brachia independently was effective in avoiding cross-contamination.

**Regenerating axons**

Early in regeneration (21–25 days after nerve cut), the distribution of the blue and yellow dyes across the retina appeared random. Single-labelled cells of both colours were present in all regions, intermixed with double-labelled cells (Fig. 2D–F). Rough estimates obtained as described above, both at the microscope and from photographs, suggested that about one ganglion cell in ten was double-labelled at this stage. Since some such cells were labelled more strongly with one dye than the other, there may have been others in which a second label was missed altogether.

As regeneration progressed, it became harder to find double-labelled cells. By 60 days after nerve cut they were rare, except in the boundary zone between dorsal and ventral retina, where they could be found even 147 days after nerve cut (Fig. 2H). At the same time, each dye gradually became more biased to its appropriate half retina, blue to ventral and yellow to dorsal, though the pattern never returned to normal (Fig. 2G, I).

**Discussion**

The presence of Fast Blue or Diamidino Yellow in a retinal ganglion cell was taken as evidence that it had an axon in the corresponding brachium of the optic tract. The absence of double-labelled cells in normal fish showed that cross-contamination of the two dyes was effectively prevented by the careful, indirect labelling strategy we employed, and that normal ganglion cells do not sustain axon collaterals in both brachia. Moreover, the distributions of single-labelled blue and yellow cells were qualitatively similar to those described previously after HRP labelling from the medial and lateral brachium, respectively (Becker & Cook, 1987).

In these circumstances, the presence of both dyes in a single retinal ganglion cell could be taken as evidence that it had at least one axon collateral in each brachium. Early in regeneration (21–25 days after nerve cut) we found many such cells, and estimated that about a tenth of the labelled cells fell into this category.

We did not expect to find both dyes in every ganglion cell with a branched axon. As noted above, there may have been cells in which a second label was present but weak. It is hard to label every cell strongly from the optic tract, even in a normal fish, without applying so much dye (or HRP) that its spread cannot be controlled. This variability presents a particular problem for double-labelling, where the fractional success rate would be expected to fall disproportionately, roughly according to the square of the fractional rate for each individual label. Other cells with branched axons might have sent all their collaterals into the same brachium, whether or not it was the appropriate one. Some collaterals may even have been withdrawn before others were extended. In view
of all these sources of conservative error, we suggest that the true proportion of ganglion cells developing axon collaterals during regeneration must be considerably higher than our estimate of the proportion of cells that can be double-labelled. This would be consistent with the large increase in axon profile number observed electron-microscopically in the regenerating goldfish optic nerve (Murray, 1982).

We have noted that occasional double-labelled cells persisted in the boundary zone between dorsal and ventral retina, implying that, in these special circumstances, collaterals can be maintained for long periods. We take this as circumstantial evidence in favour of a competitive model of collateral elimination (Becker & Cook, 1987). Axons from cells in this region of the retina terminate in a rostrocaudal strip of tectum equidistant from the two brachia. In this instance alone, collaterals selecting one brachium would have little or no competitive advantage over those selecting the other.

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References


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