



The Primary Visual System of Adult Lizards Demonstrates that Neurogenesis is not Obligatorily Linked to Central Nerve Regeneration but may be a Prerequisite for the Restoration of Maps in the Brain

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Following optic nerve crush in the adult lizard *Ctenophorus ornatus*, most retinal ganglion cells regrow their axons into visual brain centres: however, the regenerated projections lack retinotopic order and the animals are blind via the experimental eye. Here we have used 3H-thymidine autoradiography to demonstrate that cell division is no longer taking place in the retina of normal adult lizards. We conclude that the optic nerve can regenerate in lizard even though cells are no longer being added to the retina. However, continued retinal neurogenesis may be linked to the ability to restore topographic maps. © 1998 Elsevier Science Ltd. All rights reserved.

Regeneration Retinotopy Cell generation Reptilia

INTRODUCTION

Several lines of evidence have led to the hypothesis that axonal regeneration in the central nervous system occurs only if the population of parent cell bodies is still undergoing neurogenesis (Taylor, Lack & Easter, 1989). As an example, in the adult frog *Rana pipiens*, diencephalic lesions to sever axons of both retinal and tectal origin lead to regeneration of the retinal but not of the tectal axons (Lyon & Stelzner, 1987). One difference between these axonal populations, as the authors noted, is related to the germative status of their parent cell populations. In the adult frog, the retina continues to produce ganglion cells into adult life (Straznicky & Gaze, 1971; Coleman, Dunlop & Beazley, 1984), whereas tectal cells cease division at metamorphosis (Straznicky & Gaze, 1972). However, a more recent study has reported that retinal ganglion cells were no longer being generated in *Xenopus laevis* frogs of a very advanced age, yet optic axons were still capable of regeneration (Taylor *et al.*, 1989).

A refinement of the original hypothesis was put forward by Holder and Clarke (1988). They provided

several examples to support their argument that the link was not between neurogenesis and axonal regeneration *per se*. Rather, they argued that the link was between neurogenesis and the ability of regenerating axons to navigate to appropriate targets and there restore functionally useful connections. The reasoning was that regenerating axons are required to follow cues in their environment. These cues would be of embryonic origin (Harris, 1989) and would persist only as long as new axons grew along the pathway. Once neurogenesis was complete, the cues would no longer be required and would disappear.

One well known example cited by Holder and Clarke (1988) was that of optic nerve regeneration in adult fish and amphibians (reviewed by Sperry, 1951; Beazley, 1984; Jacobson, 1991). Regenerating optic axons grow along essentially normal visual pathways to re-innervate visual centres in the brain. Once regenerating axons re-enter visual centres, they search for appropriate sites in which to terminate and reform retino-topically ordered maps. The refinement of the hypothesis predicts that a continued generation of retinal ganglion cells (Fish: Meyer, 1978; Johns & Fernald, 1981; amphibians: Straznicky & Gaze, 1971; Coleman *et al.*, 1984) ensures the retention of signals to guide axons, both newly growing and regenerating ones, along the visual pathway and to elicit map formation in visual centres.

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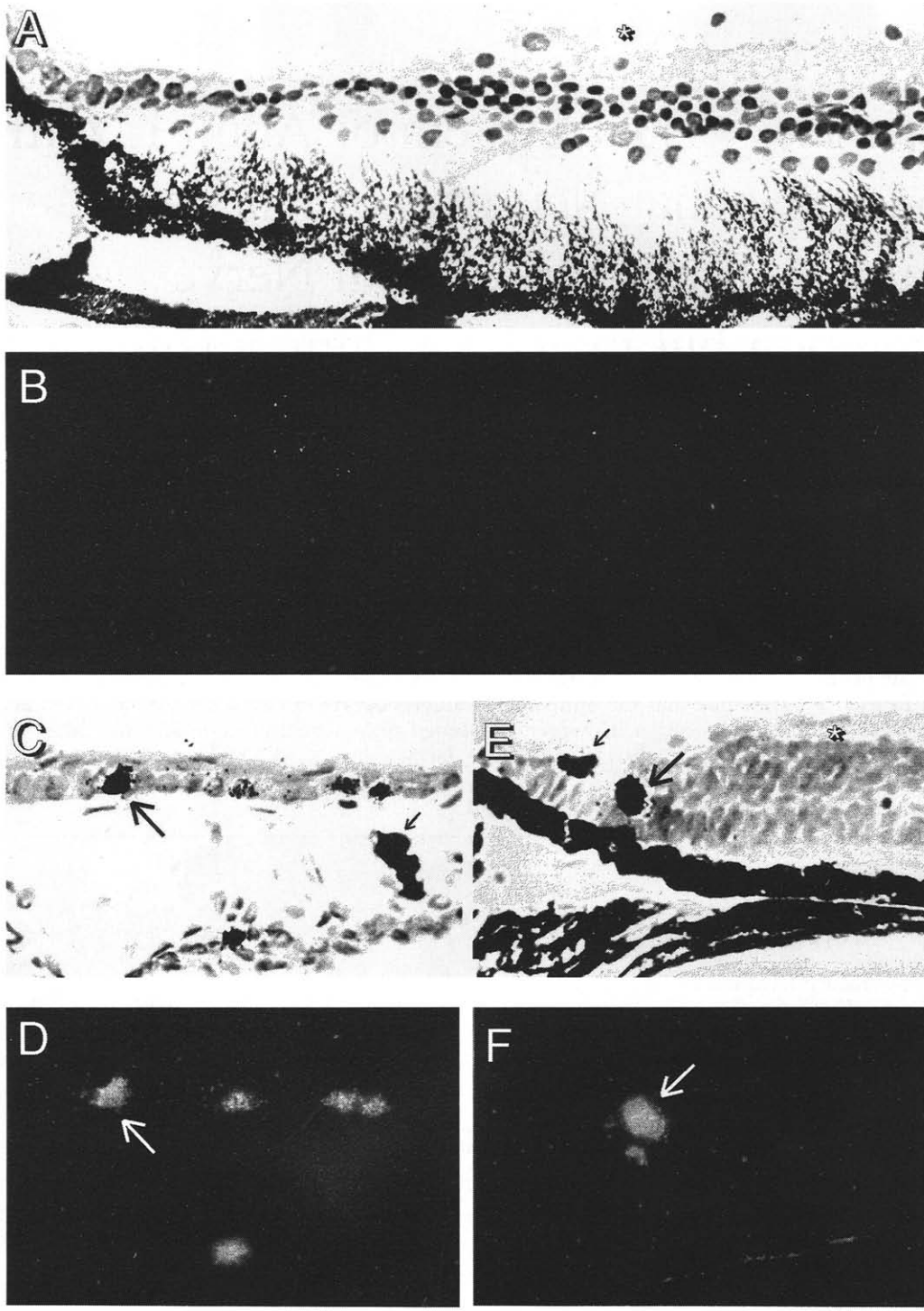


FIGURE 1. Cross-sections of the retinal ciliary margins in adult lizard (A, B) and adult frog (E, F) and of the Harderian gland (C, D) of the lizard eye shown in (A, B); (C, D) show regions adjacent to those depicted in (A) and (B). Animals were injected with ^3H -thymidine 96 and 48 hr before sacrifice. In (A, B, E, F), the ganglion cell layer (asterisk) is uppermost, the pigment epithelium towards the bottom; retinal peripheries are to the left. Differentiated cells extend to the retinal periphery in (A) whereas in (E) undifferentiated cells form a peripheral retinal band. One of the ^3H -thymidine labelled cells is indicated by large arrows in each of (C–F), being seen by both bright (C, E) and epi-polarized illumination (D, F). In (F), part of the ^3H -thymidine labelled nucleus of a pigment epithelial cell is seen immediately below the labelled cell of the neural retina, matching a previous report (Beazley *et al.*, 1996). Pigmented cells (small arrows in C, E) are not revealed by epi-polarized illumination (D, F) and thus are readily distinguishable from ^3H -thymidine labelled cells. Cresyl violet, bright field (A, C, E) and epi-polarized illumination (B, D, F), section thickness $7\ \mu\text{m}$, scale bar represents $20\ \mu\text{m}$ throughout.

However, optic nerve regeneration does not always produce such a clear-cut result in terms of axonal navigation and map restoration. We have recently reported the outcome of severing the optic nerve in the

adult ornate dragon lizard *Ctenophorus ornatus*. At one year after lesion, optic axons were found to have regenerated along the visual pathway to re-innervate visual centres in the brain. However, unlike the

regenerated projections in fish or amphibians, those in lizard lacked retino-topical order; the result has been demonstrated both by anatomical tracing (Beazley, Sheard, Tennant, Starac, & Dunlop, 1997) and by electrophysiological mapping (Dunlop, Tran, Papadimitriou, Stirling & Beazley, 1997). Furthermore, the finding was supported by an absence of behavioural responses to a range of visual tests via the experimental eye in that lizards lacked startle or optokinetic responses and failed to detect or capture live prey.

The nature of optic nerve regeneration in lizards illustrates three points. One is that their optic axons can regenerate. The second is that cues persist to ensure that axons regrow along the visual pathway and re-innervate visual centres. The third point is that no effective cues are available/recognizable in visual centres for the selection of appropriate terminal sites and hence for the restoration of a map. The dysfunctional optic nerve regeneration in lizards therefore allows us to analyse further the link between neurogenesis and central nerve regeneration.

To do so, it was necessary to determine whether retinal neurogenesis takes place in adult lizards. A finding of retinal cell generation would imply that neurogenesis may be linked to axonal regeneration *per se* and to the presence of guidance cues in the visual pathway. The result would further imply that neurogenesis is not related to cues for restoring maps in the brain. By contrast, if retinal cell generation was found to have ceased, the result would show definitively that neurogenesis is not a prerequisite for axonal regeneration *per se* or for the presence of guidance cues along the visual pathway. However, it would leave open the possibility that retinal neurogenesis is linked to the ability to restore maps in the brain.

We have examined this issue by seeking evidence of cell division in the retina of adult lizard *C. ornatus* using standard tritiated (3H-) thymidine autoradiography. As controls, we examined adjacent extra-ocular tissues such as the secretory Harderian gland (Walls, 1942) in which cell division would be anticipated. As additional controls, we carried out a parallel study of retinal cell generation in the adult frog *Litoria moorei*. We expected that the process of retinal cell division would still be taking place, as previously reported for this (Beazley, Tennant, Tomlin, Preuss, Coleman & Dunlop, 1996) and other species of frog (Straznicky & Gaze, 1971; Coleman *et al.*, 1984). Lizards and frogs were collected locally under licence (Conservation and Land Management) and were maintained and fed as previously described (Beazley *et al.*, 1997; Humphrey & Beazley, 1985). We induced brief anaesthesia for injection of 3H-thymidine by inhalation of Halothane and terminal anaesthesia by intraperitoneal injection of Saffan (1 mg/g body weight).

Lizards and frogs ($n = 3$ of each species) were injected intraperitoneally using a Hamilton syringe with 10 μ Ci 3H-thymidine (specific activity 888 Bq/mmol, Amersham). The procedure was repeated 48 hr later to provide a second opportunity for any dividing cells to be exposed

to 3H-thymidine. The animals were sacrificed after a further 48 hr. Eyes were orientated by a dorsal suture thread and the cornea and lens dissected away. The eyes with extra-ocular tissue attached were removed and fixed in buffered formalin (pH 7.4) for at least a week. Tissue was then wax-embedded and sectioned at 7 μ m, parallel to the naso-temporal axis of the eye. Tissue was processed autoradiographically using NTB emulsion (Kodak), exposed for 10–14 days at 4°C and developed in D19 before being stained with cresyl violet and mounted in Depex.

Every section of both eyes was examined at 1000 \times magnification, using both bright field and epipolarized illumination (Rapaport, Herman & LaVail, 1992), a dark field procedure particularly sensitive to autoradiographic labelling. The procedure reveals 3H-thymidine labelled cells even against an obscuring background such as the retinal pigment epithelium.

The lizard retinae conformed to our previous reports of their organization (Beazley *et al.*, 1997). A fovea was located in mid-dorsal retina and the ganglion cell layer varied from several cells thick in the horizontally aligned visual streak to a monolayer peripherally [Fig. 1(A)].

Despite exhaustive examination of both eyes of each lizard, 3H-thymidine labelled retinal cells were not found either at the ciliary margin [Fig. 1(A, B)] or within the body of the retina. Moreover, the structure of the lizard retina was compatible with mitotic activity being complete in that no undifferentiated cells or mitotic figures were seen either at the ciliary margin [Fig. 1(A)] or elsewhere. These observations therefore reinforced the 3H-thymidine analysis, indicating that cell division had already ceased amongst retinal cells of the adult lizard. The only labelled cells within the lizard retina had the appearance of macrophages, being large, irregularly shaped with a mottled appearance. These cells were rare, the maximum number seen per retina being 5; they were usually located in the inner plexiform layer, at its border with the inner nuclear layer.

The lack of 3H-thymidine labelled retinal cells in the lizard contrasted with their presence within control tissues. Labelled cells were common in extra-retinal tissue of the lizard. Examples were glial cells and cells resembling macrophages in the optic nerve, cells forming the epithelium of the Harderian gland [Fig. 1(C, D)] and connective tissue associated with the extra-ocular muscles. Moreover, in the frog retina, labelled cells were common at the ciliary margin [Fig. 1(E, F)]. A distinct zone of undifferentiated cells [Fig. 1(E)] and occasional mitotic figures were also seen at the frog ciliary margin, compatible with the autoradiographic evidence of cell division [Fig. 1(E)].

Our demonstration of an absence of cell division from the retina of adult lizard suggests that, as in birds (Kahn, 1974) and mammals (Walsh & Polley, 1985; Harman & Beazley, 1989), their retinal cell generation is limited to early stages of development. A comprehensive study in lizard would be necessary to establish whether, as in

chick (Kahn, 1974), retinal cell generation is complete before hatching.

The observed absence of retinal cell division in adult lizards reported here allows us to make some clear statements about the obligatory links between neurogenesis and axonal regeneration, the selection of pathways and of target nuclei, as well as about the restoration of maps.

Firstly, it is clear that neurogenesis is not a prerequisite for axonal regeneration *per se* since new retinal cells are no longer being generated in adult lizard, yet the optic nerve can regenerate. In this regard, our finding supports a study of aging *X. laevis* (Taylor *et al.*, 1989). Secondly, retinal neurogenesis is not linked to the presence of cues in the visual pathway. The retina is no longer undergoing cell division, yet regenerating optic axons remain within the visual pathway and re-enter visual brain centres. Guidance cues may be provided by the degenerating distal segments of severed optic axons (Matsumoto & Scalia, 1981). The argument is supported by a study suggesting that, after enucleation, such segments persist for extended periods in the reptilian visual pathway (Kruger & Maxwell, 1969). Presumably, as in frog (Humphrey, Dunlop, Shimada, & Beazley, 1992), the segments survive in lizard beyond the 1–2 months required for axons to regenerate to visual centres (Tran, Dunlop, Papadimitriou & Beazley, 1995).

The third issue is the association between retinal neurogenesis and the signals for map restoration in visual centres. We have shown that adult lizards lack both the abilities to undergo retinal neurogenesis and to re-establish organized maps in the brain; fish and frogs possess both these abilities. It is therefore tempting to conclude that the two features are obligatorily linked. Additional examples may emerge to support the relationship.

However, to prove the relationship between neurogenesis and the restoration of maps, it would be necessary to prevent the cessation of retinal cell division and determine whether regenerated projections then form organized maps. Such an approach will become feasible only when we can manipulate the factors controlling retinal cell division. As a more practical alternative, it may be possible to transplant tectal tissue excised from young lizards, at stages when the retina is still undergoing neurogenesis, into the tectum of host adult lizards. Presumably tectal cues for map formation would be present in the graft and therefore regenerating axons would restore maps therein.

If, as the results presented here suggest, there is a relationship between neurogenesis and the restoration of maps, the link has major implications for the induction of successful central nerve regeneration in mammals (Vidal-Sanz, Bray, Villegas-Perez, Thanos & Aguayo, 1987) including man. The implication is that targeted axonal regeneration will occur only in systems which are still undergoing neurogenesis. By analogy, for systems which do not undergo neurogenesis, it may be necessary

therefore to devise interventions to upregulate map-making molecules to ensure functional regeneration.

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