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Retinal regeneration

Peter F. Hitchcock and Pamela A. Raymond

The goal of research on neural regeneration is to restore brain function following injury. To many, this suggests regrowing damaged axons and re-establishing the interrupted pathways. A second, but little studied aspect of brain regeneration, is the replacement of lost neurons. For example, in some animals the neural retina is reconstituted by regenerative neurogenesis following its partial or total destruction. Two separate processes underlying retinal regeneration have been described: transdifferentiation of retinal pigmented epithelial cells into retinal neural progenitors (in adult urodeles, tadpoles, and embryonic chickens), and alteration in the fate of photoreceptor progenitors intrinsic to the retina (in adult fish).

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The retina is a thin slab of central nervous tissue that forms the innermost lining of the vertebrate eye. In cross section it consists of interleaved somatic and synaptic laminae (Fig. 1A), which in the tangential plane are organized into overlapping mosaics of functionally related somata and processes[1]. [This organization is disrupted upon damage of the inner layers (Fig. 1B) (see below).] At the level of the photoreceptor outer segments, the retina is tightly apposed to a monolayer of melanin-containing cuboidal epithelial cells, called the retinal pigmented epithelium (RPE), which supports many of the physiological requirements of the retina, such as nutrient exchange and phagocytosis of shed photoreceptor discs. In some animals this complex piece of central nervous tissue can regenerate. Selected studies of retinal regeneration by cellular transdifferentiation of the RPE and by proliferation of photoreceptor progenitors are briefly reviewed below.

Retinal regeneration by transdifferentiation of the RPE

Transdifferentiation (also known as metaplasia) is the cellular process whereby a differentiated cell reverts to an undifferentiated state and proliferates to give rise to cells of a new phenotype. A well-known example of this type of cellular re-programming is limb regeneration in urodele amphibians (newts and salamanders)[2]. The study of metaplastic retinal regeneration can be traced to Griffini and Marchio[3], who in 1889 reported that cutting the optic nerve in an adult urodele (*Triturus* sp.) caused degeneration of the existing retina and regeneration of a new one. Subsequent studies (reviewed in Refs 4, 5) have confirmed and extended this report, while others have demonstrated that tadpoles (*Rana catesbienna*)[6] and, under certain experimental conditions, chicken (*Gallus domesticus*) embryos[7–9] can regenerate their retinas by a similar process.

In these studies, regeneration was induced by one of two quite different manipulations: surgical removal of the retina (retinectomy) through an incision in the globe, or ischemia-induced retinal degeneration. To induce ischemia, the ophthalmic artery was cut intraorbitally (usually by enucleating and reimplanting the eye), which leaves the retina temporarily unperfused and results in its degeneration over the next several days. Despite the very different intraocular environments created by these two manipulations, in both cases the retina regenerates.

The cellular source of the new retina in these animals has been disputed and extensively debated in the literature. However, it is now widely accepted that the regenerated retina is derived from transdifferentiated cells of the RPE. The morphological changes in the RPE cells during regeneration have been thoroughly described[6,10,11], and are illustrated in Fig. 2. A few days after either retinectomy or ischemic degeneration, a subset of the RPE cells lose their cuboidal shape, detach from the underlying basement (Bruch's) membrane, disgorge their pigment granules, and proliferate to form a layer of pseudostratified cells, typical of a germinative neuroepithelium. (When the retina is destroyed by ischemia, other RPE cells appear to phagocytose the retinal debris and leave the eye[6,10].) Proliferation continues for several days within both the neuroepithelium and the RPE until they form a vitread layer of neuroepithelial cells and a sclerad monolayer of pigmented cells, similar to those layers seen in the embryonic eye. New retinal cells then differentiate from the neuroepithelium in an orderly sequence that recapitulates their normal development[12]. If, instead of complete retinectomy, a small patch of retina is removed, the RPE cells respond similarly at the site of the lesion only, and the missing piece of retina is regenerated[10].

Although retinal regeneration has been studied for over a century, only recently have studies begun to reveal the putative cellular factors involved. Using an antibody that recognizes amphibian retinal neuroepithelial cells, Reh and Nagy[6] found that the migrating RPE cells lose their pigment and assume a neuroepithelial phenotype only when in proximity to the retinal vascular layer. In a separate study[13], they showed that the basal lamina associated with the retinal vascular layer contains a large amount of laminin, and that dissociated RPE cells placed onto a laminin-coated substrate *in vitro* extrude their pigment, extend neurite-like processes, and express neuron-specific proteins.

Reh proposed that laminin could regulate the phenotypic expression of RPE cells. High levels

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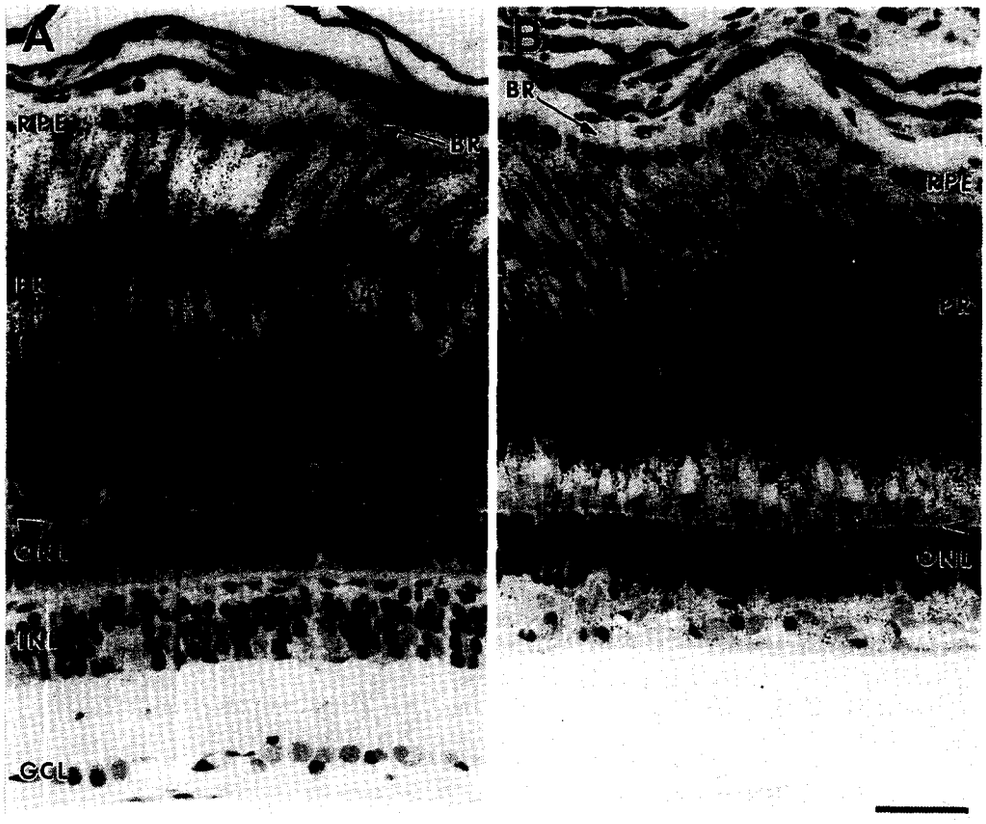

Fig. 1. *Goldfish* (Carassius auratus) *retina.* **(A)** *Photomicrograph of a normal retina. The arrowheads indicate the external limiting membrane.* **(B)** *Photomicrograph of a retina ten weeks after an intraocular injection of ouabain that destroyed the inner retina but did not stimulate regeneration. Note the absence of the inner layers, whereas the outer nuclear layer and photoreceptors appear normal. The scale bar in (B) is 25 μm and also applies to panel (A). Abbreviations: BR, Bruch's membrane; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors; RPE, retinal pigmented epithelium.*

favored a neural phenotype, whereas low levels (as might be found on Bruch's membrane) favored the RPE phenotype. However, in the experiments carried out by Reh[6] *in vivo*, the original retina was destroyed by devascularization (see above), which left the retinal vasculature intact. It should be noted that retinectomy, which removes both the retina and the overlying vasculature, induces apparently identical changes in the RPE compared to those caused by devascularization, and is followed by regeneration of the retina in adult salamanders (*Triturus viridescens*)[10]. This indicates that the vascular basement membrane may not be the sole source of inductive signals that stimulate RPE transdifferentiation. Alternatively, following retinectomy, the retinal vasculature may regenerate to quickly cover the denuded RPE. If rapid neovascularization does take place, the inductive stimulus for this process could be identical regardless of whether the original lesion was retinectomy or ischemia.

Soluble growth factors also may be involved in retinal regeneration. In a seminal paper, Coulombre and Coulombre[7] showed that following retinectomy the RPE in the four-day-old embryonic chick would not, as in amphibians, spontaneously transdifferentiate into retina. However, the RPE could be induced to regenerate retina if a piece of the embryonic retina was placed back into the globe at the time of the surgery. The implant did not contribute cells to the regenerating tissue, but was the source of inductive signals. Further, this effect was neither tissue nor species specific: chicken otocyst or mouse retina placed into the eye had similar effects[8].

Curiously, and in contrast to that seen in amphibians, the retina regenerated in the chick with a reversed polarity: ganglion cells were apposed to the RPE and photoreceptors projected into the vitreal chamber. Coulombre and Coulombre interpreted this as being a consequence of the fact that the polarity of the RPE, which is inverted with respect to the normal retina (i.e. the basal surface of the RPE is Bruch's membrane, whereas the basal surface of the retina is the inner limiting membrane), was then preserved within the RPE-derived neuroepithelium (see Fig. 2). This interpretation assumes that transdifferentiating RPE cells retain and pass on to their progeny the 'memory' of their original polarity. An alternative interpretation of these results is to assume that retinal polarity is established by a transretinal gradient of a putative soluble factor. During normal retinal development, polarity might be established by a factor that is at a high concentration near the RPE and is low near the vitreal chamber. Retinal polarity would be reversed if this gradient were reversed, for example, by release of the putative factor from an intravitreal implant of embryonic tissue (see below).

Stimulated by the results of Coulombre and Coulombre, and by recent data demonstrating the presence of growth factors within the eye and retina[14], Park and Hollenberg[9] combined retinectomy in chicken embryos with implantation of a polymer bead that slowly released basic fibroblast growth factor (bFGF). They found that bFGF stimulated retinal regeneration in a dose-dependent fashion. At low doses the regeneration was incomplete; isolated patches of retina were surrounded by RPE that appeared to be normal. However, at high doses the entire fundus was lined with new retina. Here too, the retina had a reversed polarity and, in the eyes that received the high doses of bFGF, the RPE was missing, indicating that all of the RPE cells had been recruited to transdifferentiate.

Retinal regeneration from intrinsic neuronal precursors

Similar to amphibians, fish can also spontaneously regenerate their retinas. However, the source of the regenerated tissue is not the RPE, but a population of neural progenitors that are intrinsic to the retina (Fig. 3). The first evidence for this was provided by Lombardo[15,16], who surgically removed retinal quadrants from adult goldfish (*Carassius auratus*) and reported that during regeneration mitotic figures

were present at two locations: the marginal germinal zone adjacent to the lesion, and the outer nuclear layer along the cut edges of the extant retina. He suggested that the retina was regenerated from the proliferative margin (which continually produces new retina as part of a normal growth process, see Ref. 17), and from dividing cells at the cut edges of the retina. These cells at the cut edges of the retina were thought to be derived from differentiated neurons that retained some neuroepithelial-like properties. In striking contrast to what is seen in amphibians, cells of the RPE denuded by the surgery remained attached to Bruch's membrane and *did not* undergo proliferation in fish.

Several years later, Wolburg and his collaborators[18,19] described the cellular proliferation in goldfish and trout (*Salmo gairdneri*) retina following a cytotoxic lesion using the metabolic poison ouabain. During regeneration, mitotic figures and cells labeled with [^{3}H]thymidine were seen at the marginal germinal zone, which was undamaged by the toxin, and in the former outer nuclear layer in the central retina. Wolburg initially claimed that the damaged retina was replaced only by retina added at the marginal germinal zone that migrated in concentric circles towards the center of the retina. The dividing cells in the central retina were thought to be neuroepithelial cells that had migrated from the margin. Later, Wolburg modified this to claim that the dividing cells in the central retina were not migrating neuroepithelial cells, but were photoreceptors that underwent dedifferentiation and proliferation *in situ*.

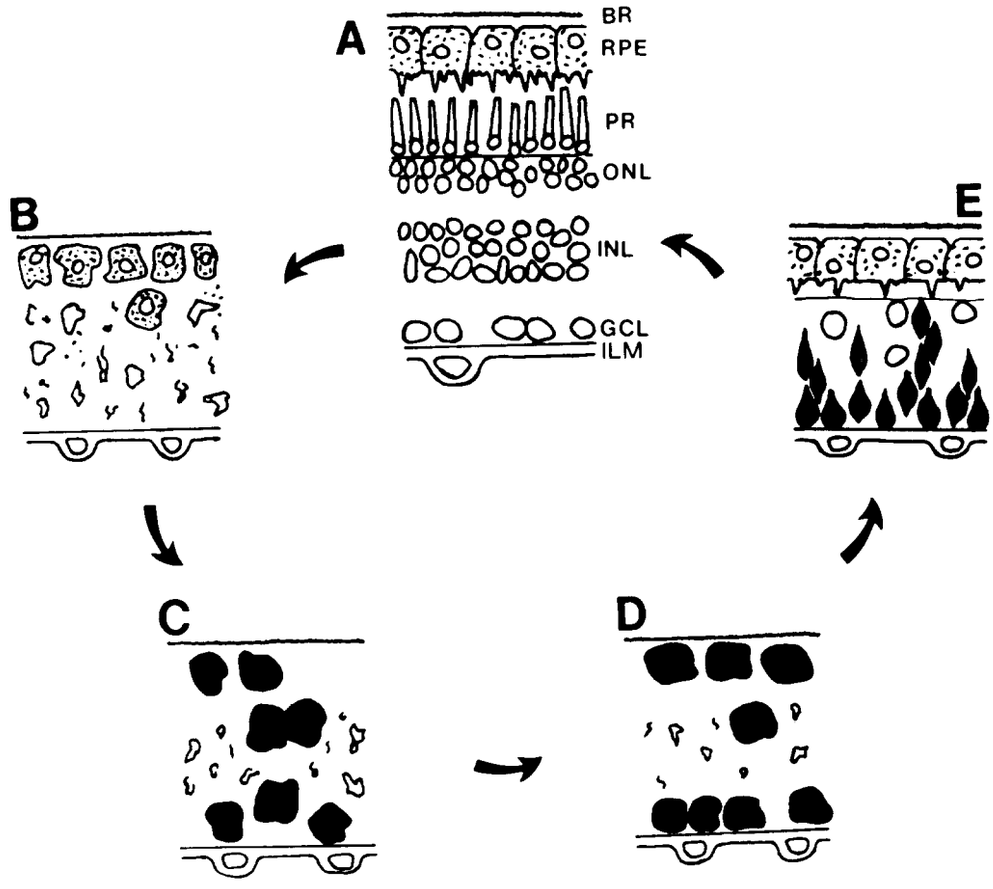

Fig. 2. *Retinal regeneration in urodeles and anurans following ocular devascularization.* **(A)** *Normal retina. As the retina degenerates* **(B)**, *cells of the RPE that detach from Bruch's membrane phagocytose the cellular debris or begin to proliferate* **(C, D)** *to reform the RPE layer and a germinative neuroepithelium* **(E)**. *From this neuroepithelium, a new retina (A) differentiates. The shading designates proliferating cells. Abbreviations: BR, Bruch's membrane; GCL, ganglion cell layer; ILM, inner limiting membrane and vascular layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors; RPE, retinal pigmented epithelium.* (Modified from Ref. 6.)

Neither Lombardo nor Wolburg could identify the source of the proliferating, non-marginal cells seen in the central retina during regeneration. Retinal anatomists had known for some time that new neurons are continually added to the retina of the fish from the marginal germinal zone[20], but only relatively recently was it discovered that neural progenitors are also scattered throughout the differentiated retina. These specialized neuroepithelial cells, called rod precursors, lie within the outer nuclear layer and divide to produce new rods that intercalate into the lawn of existing photoreceptors[21–23] (Fig. 3). Three recent studies, using techniques similar to those of Lombardo and Wolburg, have confirmed that intrinsic cells give rise to the regenerated retina[24–26], and the weight of evidence is that these proliferating cells are derived from the rod precursors. The results of these studies are summarized in Fig. 4.

Using a trans-scleral surgical approach, Hitchcock and colleagues[25] excised small patches of retina, and at various survival times made intraocular injections of the thymidine analog, bromodeoxyuridine (BUdR) to mark proliferating cells. One week after lesioning (Fig. 5A), the wound was filled with dividing (presumably phagocytic) cells, whereas within the retina, dividing cells were clustered on the edge of the wound and within the surrounding outer nuclear layer, confirming the results of Lombardo[15,16]. The dividing cells at the wound margin formed a continuous band, which was called the 'blastema'. Over the subsequent

Fig. 3. *High-magnification photomicrograph of a goldfish* (Carassius auratus) *retina labeled with [^{3}H]thymidine and processed for autoradiography. Rod precursors are identified by the silver grains in the overlying emulsion. The arrowheads indicate the outer limiting membrane. The scale bar is 10 μm. Abbreviation: ONL, outer nuclear layer.*

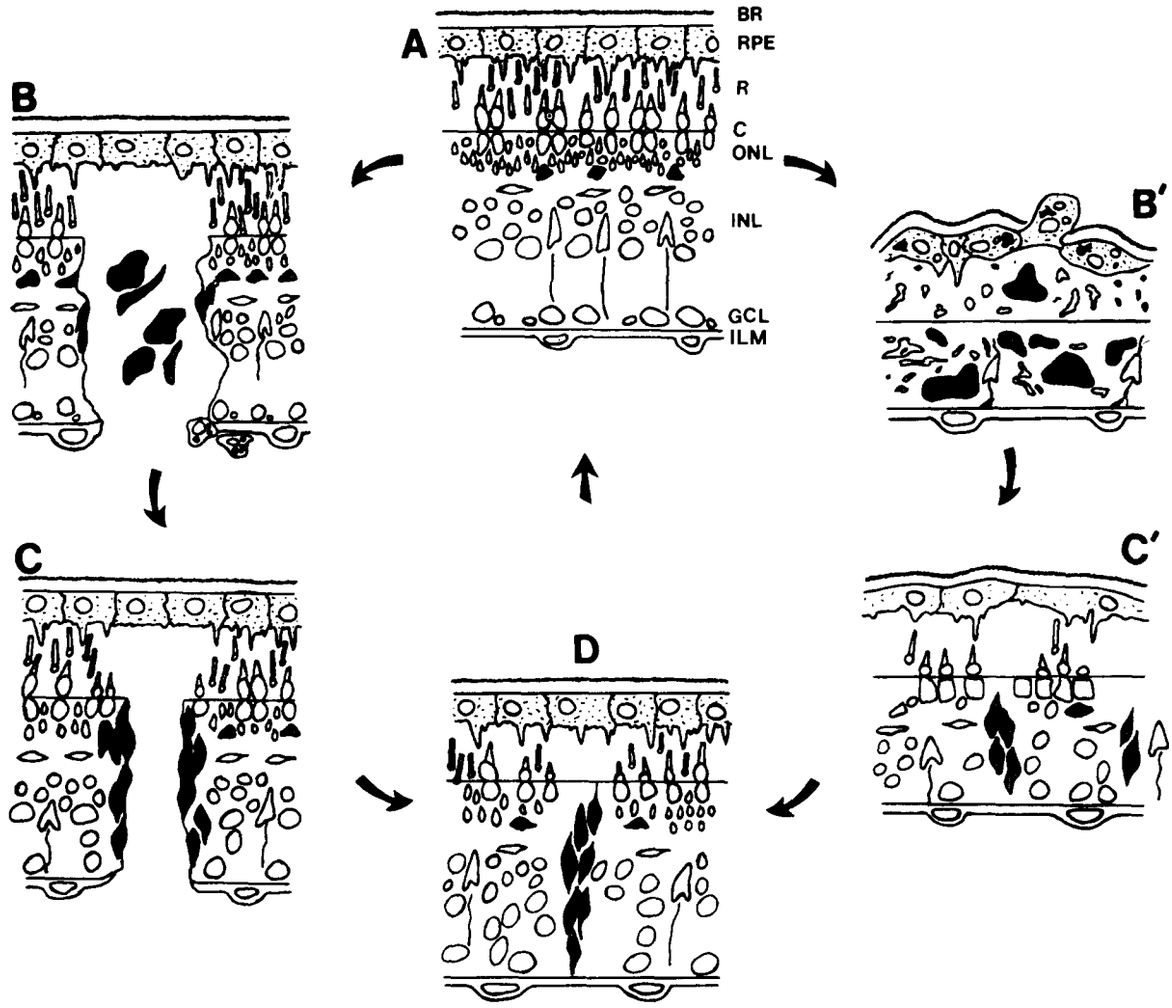

Fig. 4. *Retinal regeneration in the goldfish* (Carassius auratus). **(A)** *Normal retina.* **(B, C)** *Regeneration following a surgical lesion.* **(B',C')** *Regeneration following a cytotoxic (ouabain) lesion.* **(D)** *The common endpoint of the regenerative neurogenesis. Proliferating cells (both macrophages and neuroepithelial cells) are shaded. Note that the neuroepithelial cells form clusters at the margins of the wound (B, C) or are scattered across the degenerating retina (B', C'). As regeneration progresses, these foci of proliferation are flanked by new postmitotic neurons (C, C', D). Cones (shorter and wider) regenerate before rods (smaller and slimmer) in both situations [note the relative paucity of rods in (D) compared to (A)], which is similar to what happens in normal development. Retinal lamination is somewhat disrupted in the early regenerate, as shown by fusions between the inner and outer nuclear layers (C, C', D; see also Fig. 5B). Abbreviations: BR, Bruch's membrane; C, cone photoreceptors; GCL, ganglion cell layer; ILM, inner limiting membrane and vascular layer; INL, inner nuclear layer; ONL, outer nuclear layer; R, rod photoreceptors; RPE, retinal pigmented epithelium.*

weeks, the margin of the retinal wound, always capped by the blastema, grew together. By eight weeks, the original lesion was filled with regenerated retina. These data suggested that the retinal lesion was repaired by the migration of the blastema into the center of the wound, and the appositional addition of new retina to the old. A separate set of experiments using a cumulative labeling schedule confirmed this. By five months (Fig. 5B) the patch of regenerated retina was qualitatively and quantitatively similar to the surrounding intact one.

Raymond and her collaborators[17,24,26] undertook experiments similar to those of Wolburg, and made several new observations. (1) After ouabain administration and retinal degeneration, cells at the marginal germinal zone did not migrate into central retina. (2) The rod precursors, like cells in the marginal germinal zone, were spared by the ouabain. (3) During the entire period of regeneration, clusters of dividing cells, called 'neurogenic foci', were seen scattered across the central retina with a distribution roughly similar to that of rod precursors in the intact retina. As regeneration proceeded, the neurogenic foci became flanked by patches of regenerated retina. (4) At lower doses the ouabain did not damage the outer nuclear layer, and in those cases there was no regeneration (Fig. 1B). The last observation was paradoxical; severe damage stimulated regeneration, whereas a modest amount did not. Raymond resolved this paradox by hypothesizing that the local microenvironment around the rod precursors had to be disrupted in order to stimulate their proliferation. Consistent with this, the amount of cell proliferation in cytotoxin-treated retinas was proportional to the extent of loss of the photoreceptor cells[24].

Results from experiments in which selective neurotoxins were used to destroy specific types of retinal neurons provided further support for the hypothesis that damage to the outer nuclear layer is necessary to trigger a regenerative response in the rod precursors.

Hitchcock[27] used 'suicide transport' of propidium iodide to selectively ablate goldfish retinal ganglion cells, which failed to regenerate. Negishi and colleagues[28] demonstrated that at low doses intraocular injection of 6-hydroxydopamine destroys dopaminergic interplexiform cells in the goldfish retina, and these cells also fail to regenerate. However, at suprathreshold doses of this toxin, the dopaminergic neurons reappear after about two months. Braisted and Raymond[26] showed that this replacement of dopaminergic neurons was associated with nonspecific damage to the inner and outer nuclear layers, including a loss of over 30% of the cells in both layers. Regeneration of dopaminergic neurons took place as part of a global regenerative response that involved generation of multiple cell types by a process that recapitulated many of the normal developmental events. Together, the results of these experiments suggest that regeneration of a single class of neuron following its selective ablation does not occur, with the possible exception of rods[29] (Braisted, J. E. and Raymond, P. A., unpublished observations).

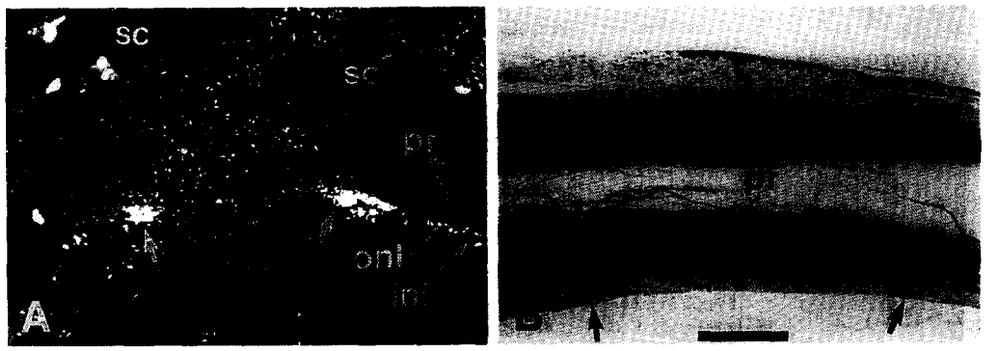

Fig. 5. *Retinal regeneration in the goldfish* (Carassius auratus) *following a small surgical lesion.* **(A)** *Photomicrograph of a regenerating retina seven days after lesioning. Bromodeoxyuridine (BUdR) was injected into the eye 24 hours prior to sacrificing the animal. The BUdR-labeled cells were visualized using indirect immunofluorescence, and are seen here as bright spots scattered within the lesion and clustered on the cut edges of the retina (arrows).* **(B)** *Photomicrograph of a regenerated patch five months after lesioning. The laminar fusions within the retina indicated by the arrows mark the original boundaries of the lesion. Scale bar is 200 μm. Abbreviations: INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptor layer; SC, scleral cartilage.*

Although the studies cited above demonstrated conclusively that in fish the RPE is not the source of the regenerated retina under these experimental conditions, they did not rule out an indirect role of the RPE in regeneration in these animals. Indeed, there is some circumstantial evidence that the RPE is important. When large areas of retina – together with the adjoining RPE – are surgically removed from the eye of the goldfish, the retina fails to regenerate[30]. This suggests that the integrity of the regenerated tissue may depend upon the presence of the RPE, which perhaps functions as a source of growth factors[31].

If rod precursors are the source of the regenerated retina, then, under conditions that induce regeneration, their fate must change from producing only rods to producing all kinds of retinal cells. This could happen in one of two ways. (1) Rod precursors are normally restricted to the rod lineage, and retinal damage induces them to become pluripotent. (2) Rod precursors, like the primitive neuroepithelial cells that give rise to retina during normal development[32–34], are pluripotent, but the fate of their progeny is restricted to the rod phenotype, perhaps because of local positional cues or cell–cell interactions. Disrupting the local microenvironment around the mitotic rod precursors or their postmitotic progeny may release them from the fate of producing only rod cells, and allow them to differentiate into multiple cell types[17,35]. Although there is no direct evidence to indicate which of the two alternatives is correct, rod precursors do not express opsins[36], suggesting that they are not partially differentiated photoreceptors.

Unanswered questions

Retinal regeneration is an important and fascinating phenomenon, and several questions, also common to development, demand further study. For example, which molecules stimulate transdifferentiation of the RPE or the alteration in the differentiated fate of rod precursors? What are the cellular sources of these putative factors? What regulates neurogenesis as new retina is reconstituted? What specifies the phenotypes of the regenerated neurons, and is each class of neuron replaced in the appropriate proportions? Does the surrounding intact neural tissue influence local regeneration?

Finally, a hallmark of the mature mammalian brain is that damage results in a permanent loss of function: lesions stimulate, in part, gliosis and scarring[37]. This is in striking contrast to amphibians and fish in which regeneration of several brain regions has been described, including the optic tectum[38,39] and telencephalon[40]. What are the essential differences between our brains and theirs? Is it simply that we lack cells capable of generating new neurons? In amphibians and fish, regeneration of brain structures is associated with stimulation of mitotic activity in neuroepithelial germinal zones located in discrete regions of the ventricular epithelium, and extirpation of these germinal zones prevents regeneration[38,40]. It is now known that damaged axons in the mature mammalian brain can regenerate if their growth cones are presented with an appropriate non-neuronal substrate[41]. By analogy, could experimental conditions be created whereby neurogenesis and tissue restitution is supported in the mature mammalian brain, and might this be a therapeutically useful strategy for repairing damage to the CNS? A complete understanding of retinal regeneration should provide important insights for answering these challenging questions.

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Neurons that say NO

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Thirty years ago, Thomas and Pearse discovered what they termed 'solitary active cells' – neurons containing an unusually high nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-diaphorase) activity that could be detected histochemically. Although these neurons were considered as something special, an appropriate mechanism to account for their outstanding metabolism was not provided until the recent identification of neuronal NADPH-diaphorase as nitric oxide synthase. This simple histochemical method now allows the precise anatomical localization of the neurons generating the exotic messenger molecule nitric oxide. This article reviews the functional implications that arise from our new knowledge of the anatomy of the nitric oxide signal transduction pathway in the nervous system. The widespread distribution of this system indicates that for those interested in cellular communication nitric oxide is a gas to study.

Only a few years ago, the idea that a highly toxic gas might be a physiological intercellular messenger would have been greeted with more than a little skepticism. However, it now appears that nitric oxide (NO) is indeed such a messenger, and may also represent a new type of neurotransmitter. An enzyme responsible for synthesizing NO has now been purified, cloned and expressed[1]. This enzyme, NO synthase, catalyses the synthesis of NO from L-arginine via a Ca^{2+}/calmodulin-dependent mechanism. Thus, increases in intracellular Ca^{2+} resulting from activation of voltage-gated Ca^{2+} channels, ligand-gated Ca^{2+} channels or the mobilization of intracellular Ca^{2+} stores could activate the enzyme. The target for NO action – soluble guanylyl cyclase – has also been characterized at the molecular level (see Box 1). By activating this enzyme, NO appears to be responsible for agonist-induced increases in cGMP levels throughout the nervous system. The cGMP formed may then regulate protein kinases, phosphodiesterases and ion channels.

The identification of NO synthase as the enzyme responsible for the neuronal nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-diaphorase)[2] histochemical reaction, together with the development of antibodies to the purified NO synthase[3], have now made possible the detailed anatomical localization of the sites of synthesis of this novel messenger in the nervous system. This review highlights some of the cell groups (indicated by these techniques) that might serve as useful model systems for functional studies aimed at elucidating the role of NO in the nervous system (see Fig. 1).

The cerebellum

The NO signal transduction system has been most intensely studied in the cerebellum, which has the highest levels of NO synthase activity[4] and cGMP[5] in the brain. NO-dependent cGMP production in the cerebellum has been recently reviewed[6], and will therefore only be summarized briefly here. In the cerebellar cortex, NADPH-diaphorase histochemistry[7] and immunohistochemistry for NO synthase[3] indicate that the enzyme is present in basket and granule cells, but not in Purkinje cells. In contrast, immunohistochemical studies with monoclonal antibodies to soluble guanylyl cyclase have found that Purkinje cells are intensely stained, while other cells in the molecular and granule cell layer were either faintly stained or unstained[8,9]. This localization of soluble guanylyl cyclase in the Purkinje cells is consistent with the presence of high levels of cGMP-dependent protein kinase and its target G substrate in these neurons[10,11].

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