Non-neuronal cell response to axonal damage in the visual paths of goldfish

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Chapter II

Abstract

Patterns of proliferation and changes in total cell number in the optic nerve, tract and tectum of goldfish have been examined following optic nerve crush or optic enucleation, using bromodeoxyuridine to label the proliferating cells. In general, an increase in proliferation and total cell number in all parts of the visual system was observed peaking between 7 and 14 days and resolving itself to normal or near normal levels by 32 days postoperative. Enucleation resulted in elevated proliferation values as compared to animals with an optic nerve crush, at at least one early timepoint in each part of the visual system, but overall, there is little to suggest that axons are exerting a major effect on the cellular response. Finally, a seasonal effect on the proliferative response of non-neuronal cells and axonal regrowth has been demonstrated. Fish acclimatized under autumn-like conditions showed a faster initiation of the non-neuronal cell response and an enhanced rate of axonal regrowth when compared to fish acclimatized under spring-like conditions. It is believed that photoperiod plays a major role in the seasonal effects observed with temperature playing only a minor one.

Résumé

Les séquences de prolifération et les variations dans le nombre total de cellules de nerf, tract, et tectum optique de poissons rouges furent examineés, suviant l'écrasement de nerf optique ou aprés l'énucléation optique en bromodeoxyuridine pour marquer les cellules en croissance. En général, une augmentation dans la prolifération et du nombre total de cellules dans toutes les compossantes du système visuel fut observée avec un plateau entre 7 et 14 jours pour ensuite retomber à des niveaux normaux ou quasi normaux par 32 jours après l'opération. L'énucléation optique a donné des valeurs de prolifération élevés comparé à des animaux ayant subit l'écrasement du nerf optique, à au moins un point temporel tôt, dans chaque composante du systéme visuel mais en ensemble, il y a peu pour suggérer que le axons exercent un effet majeur sur la réponse cellulaire. Finalement, un effet saisonnier sur la réponse proliférative de cellules non-neuronales et sur la croissance des axons fut démontré. Des poissons acclimatés à des conditions autumniéres démontrent une initiation de la réponse non-neuronale plus rapide et un amplification de taux de recroissannce des axons lorsque comparé à des poissons acclimatés à des conditions printanières. La croyance est que la photopériode joue un rôle majeur dans les effets saisonnieres observés et que la température n'a qu'un rôle secondaire.

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Preface

"If the manuscipt-based structure for the thesis is chosen, the text of the following paragraphs must be reproduced in full in the preface of the thesis"

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, of the clearly duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidates and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the author and co-authored papers.

The work contained within Chapter I of this thesis will be submitted for publication with Dr. Levine as co-author. Dr. Levine collaborated through-out the evolution of the project and edited the manuscript while I was responsible for performing all the surgeries, processing for BrdU immunohistochemistry, cell counts, analysis of data and take responsibility for the validity of the work.

Literature Review

Introduction

Researchers have been trying to unravel the mystery behind axonal regeneration since early this century (Ramón y Cajal, 1928). A particularly intriguing problem researchers have been investigating is the mechanism that allows the peripheral nervous system (PNS) of mammals and the central nervous system (CNS) of lower vertebrates to undergo regeneration following damage (Attardi and Sperry, 1963; Levine and Cronly-Dillon, 1974; Perry and Brown, 1992). This is in contrast to the CNS of mammals where not only does regeneration fail, but eventually, death of axotomized neurons occurs (Villegas-Pérez et al, 1992). The differences in regenerative capabilities between these systems can, in part, be attributed to the non-neuronal environment the axons are in and not an intrinsic deficiency in the axon itself. It has repeatedly been shown that the mammalian CNS environment is nonconducive to axonal regeneration (Caroni and Schwab, 1988a,b; Milligan et al, 1992; Blaugrund et al, 1993) whereas the mammalian PNS can support growth of CNS axons (David and Aguayo, 1981; Benfey and Aguayo, 1982). In addition, rapid proliferation of mononuclear cells following axon damage is seen in the mammalian PNS (Stoll et al, 1989; Perry et al, 1993) and lower vertebrate CNS (Battisti et al, 1995) while at best, a much slower response is observed in the mammalian CNS (Lawson et al, 1994) where regeneration fails. The following chapter will explore what is currently known about the non-neuronal cell response during axonal regeneration in the lower vertebrate CNS and mammalian PNS highlighting similarities between the two systems and contrasting them to what occurs in the mammalian CNS.

The Lower Vertebrate CNS Response to Axonal Damage

The role of oligodendrocytes during CNS axonal regeneration

Oligodendrocytes are glial cells whose major function is to produce CNS myelin (Bastmeyer et al. 1993; Nona et al. 1994). In the mammalian CNS, oligodendrocytes appear to be a major impediment to axonal regeneration through their expression of inhibitory cell surface proteins (Caroni and Schwab 1988a,b) whereas in fish they appear to aid in regeneration (Stuermer et al. 1992). One may ask whether this difference is due to differences in the expression and/or secretion of proteins between the mammalian and fish oligodendrocytes or is it due to interactions between these cells and other glial cells following axonal damage? Work done by Stuermer and co-workers (1992) has shown that when retinal explants from fish are placed on cultured rat oligodendrocytes, the regenerating axons will either collapse and pull back when they come into contact with these cells or they will avoid them altogether. The growing axons never crossed the rat oligodendrocytes. In the presence of IN-1, an antibody known to inhibit rat myelin growth inhibitors (Caroni and Schwab, 1988b), growing fish axons crossed the cells suggesting that teleost axons are sensitive to a mammalian oligodendrocyte-associated inhibitory protein. In contrast, Stuermer et al (1993) and Bastmeyer et al (1993) have shown that when fish retinal explants are placed on cultured fish oligodendrocytes, axons extended over these cells vigorously, reaching a density not seen on any other substrate (Bastmeyer et al, 1993). Growth cones were often seen in association with the processes of oligodendrocytes and in fact, in cultures which had astrocyte rich clusters in them, regenerating axons would turn away from these clusters and continue to grow on the

oligodendrocytes. Experiments performed by Stuermer et al (1993) also showed that when retinal explants were placed on untreated coverslips, axons failed to regenerate but when fish oligodendrocytes were added, growth occurred. Regenerating axons seemed to preferentially grow in close association with oligodendrocytes suggesting the presence of a cell-surface molecule which acts as a favourable substrate for growth. It appears from these observations that fish oligodendrocytes may lack the growth inhibitory protein which mammalian cells express. Taken together, these observations indicate that goldfish axons are sensitive to the myelin inhibitory proteins but that their oligodendrocytes do not express it

In contrast to the work just described, Sivron et al (1994) and Lang et al (1995) showed that teleost myelin may actually inhibit axonal growth. Sivron et al (1994) showed that neurite outgrowth did not occur when goldfish retinal explants were placed on uninjured fish optic nerves or a myelin extract from uninjured fish optic nerves. Substantial neurite outgrowth did occur when explants were placed on injured optic nerves (21 days postcrush). Lang and colleagues (1995) demonstrated that myelin from *Xenopus* spinal cord is not a good growth substrate because neurite outgrowth from retinal explants was much lower than that seen on other substrates such as laminin or cell surface membrane fractions free of myelin (Bastmeyer et al, 1991). In both of the studies described above (Sivron et al, 1994; Lang et al, 1995), the monoclonal antibody IN-1 which prevents the inhibitory effects of mammalian oligodendrocytes was used to completely neutralize the growth inhibition caused by myelin. This suggests that teleost myelin does indeed contain a similar growth inhibitor as mammals contrary to the evidence shown by Stuermer et al (1993) and Bastmeyer et al (1993).

A possible explanation for the conflicting observations regarding the

growth permissiveness of anamniote oligodendrocytes and myelin may lie in the source of the oligodendrocytes and myelin used in the experiments. (Stuermer et al. 1993; Bastmeyer et al. 1993; Sivron et al. 1994; Lang et al. 1995). There appears to be increased amounts of the growth inhibitor present in nonregenerating nerves (Sivron et al. 1994) compared to its expression in cultured oligodendrocytes (Stuermer et al. 1993; Bastmeyer et al. 1993) and in regenerating nerves (Sivron et al. 1994). This difference in expression may explain why neurtite outgrowth is possible on regenerating nerves compared to nonregenerating nerves (Sivron et al. 1994) and therefore the ability of the post injury response to remove this inhibitor. allowing growth to occur. becomes critical.

Schwann cell involvement in remyelination

Although oligodendrocytes are the main glial cell involved in remyelination of damaged CNS axons, it has recently been shown that Schwann cells, usually associated with myelination in the PNS, may also be involved in remyelination in the CNS following injury. Nona and co-workers (1994), using the antibody S-100 which is relatively specific for Schwann cells, demonstrated that these cells infiltrate the lesion site in a crushed goldfish optic nerve within 50 days following a crush. When sections of the nerve were double labelled with anti-S-100 and 6D2, a monoclonal antibody against myelin glycoproteins IP1 and IP2, at 180 days post crush, immunoreactivity was confined mainly to the crush site suggesting that the infiltrating Schwann cells were involved in remyelination. Following X-irradiation, there was a deficit in distal remyelination of new axons by oligodendrocytes, however, Schwann cell invasion at the lesion was only delayed--not arrested. While S-100 positive Schwann cells are usually restricted to the lesion site, following

irradiation, they were found in the distal segment of the nerve which was myelin deficient. It is important to note, however, that Schwann cells were restricted to the optic nerve and did not cross into the tract (Nona et al, 1994). Possible reasons for this may lie in the fact that there is no source for these cells nearby the tract as there is in the nerve (Nona, 1995). The more likely explanation, however, may be the presence of an astrocyte-related phenomenon preventing their access (Nona et al, 1994; Nona, 1995).

It is believed that the reason why Schwann cells become involved in remyelination of the CNS is because there is a disruption in the astrocytic glia limitans following optic nerve crush (Nona, 1995). Normally, this boundary prevents Schwann cells from entering the CNS but when damage occurs, such as an optic nerve crush, this boundary is breached and access is permitted (Nona, 1995). In fact, it has been established that the wider the optic nerve lesion, the greater the infiltration of Schwann cells (Nona et al, 1992)

Astrocyte involvement during CNS axonal regeneration

There are different subpopulations of astrocytes in different parts of the teleost visual system. Using antiserum against cytokeratin (anti-48), Levine (1989) showed that astrocytes expressing this protein are confined to the optic nerve and are free, stellate cells that lack glial fibrillary acidic protein (GFAP). In contrast, astrocytes in the optic tract were shown to express GFAP, as demonstrated with specific antisera, and are radial cells. Their cell bodies are deployed along the ventrical and their processes run laterally to the pial surface where they form a glial limitans. There are two distinct arrangements of astrocytes in the optic tectum. Below the *stratum periventriculare* they form an expanded ependymal layer that consists of tangled bundles of processes. The glial processes between the *stratum periventriculare* and the pial surface

of the tectum, on the other hand, are arranged in a strict radial fashion (Levine, 1989).

Astrocytes have been shown to undergo morphological changes which suggest cellular activation within 1 hour following crush injury to the optic nerve (Battisti et al, 1995). Battisti and colleagues (1995), using electron microscopy, also showed that within 3 days postcrush, astrocytes developed hypertrophic finger-like processes and occasionally contained debris suggesting they may be phagocytic. Following a nerve crush, the lesion has been shown to be void of astrocytes using antibodies against cytokeratin (Levine, 1991), vimentin (Blaugrund et al 1993; Cohen et al, 1994), and GFAP (Nona et al, 1994). Using electron microscopy, Cohen et al (1994) confirmed that the loss of immunoreactivity corresponded to an absence of these glial cells from the lesion site. The time course for repopulation of the lesion by astrocytes is still unresolved. Cohen and co-workers (1994) showed reentry of astrocytes one week after nerve crush, while Blaugrund and co-workers (1993) demonstrated repopulation two weeks postcrush, and Levine (1991) found reentry only after 4 weeks postcrush. It is important to realize, however, that Cohen et al (1994) and Blaugrund et al (1993) were using different antibodies to label astrocytes than Levine (1991) which may account for the discrepancy.

Using an antibody directed against GAP-43, a growth-associated protein specific to neurons that is expressed in regenerating axons, Blaugrund et al (1993) showed that one week following an optic nerve crush, axons have entered the proximal part of the lesion. By three weeks postcrush, GAP-43 positive axons had traversed the lesion and were now in the distal part of the nerve. On the other hand, Lowenger and Levine (1988), using ^{3}H-proline autoradiography to label regenerating axons, demonstrated that by 4 days post

crush, axons had extended slightly beyond the crush and by 8 days they reached the optic tract. This was confirmed in a later paper (Levine, 1991) where immunohisochemistry with an antiserum against a neurofilament subunit protein showed that regenerating axons traversed the entire intraorbital nerve by 2 weeks post crush. Once again the discrepancy in the data between the two laboratories (Blaugrund et al, 1993; Lowenger and Levine, 1988 and Levine, 1991) may be accounted for by the different methods used to label axons--autoradiography versus immunohistochemistry, with autoradiography, in general, being the more sensitive method of labelling as it is directly incorporated into the regrowing axon. It should also be kept in mind that the laboratory conditions, the fish stock and the season in which the experiments were performed in can also contribute to the observed differences. In any event, there is a lack of agreement between the rates of regeneration and repopulation of the lesion by astrocytes and depending on which work is considered, axonal regeneration either occurs concomitantly with repopulation of astrocytes in the lesion (Cohen et al, 1994) or precedes repopulation by 1-3 weeks (Levine, 1991; Blaugrund et al, 1993). One may then ask whether the initial event of axonal regrowth is a signal to recruit glial cells needed in subsequent axonal elongation or is it the initial migration of glial cells necessary to pave the way for axonal regeneration? Levine (1993) demonstrated that in the absence of regenerating axons, astrocytic repopulation of the lesion was absent suggesting that it is axonal regrowth that signals the glial cells.

Another subject of debate is the presence or absence of an astrocytic scar at the site of the lesion. Blaugrund et al (1993) defined an astrocytic scar in the fish optic nerve as a dense meshwork of astrocytic processes formed as the result of rapid proliferation without migration of astrocytes at the site of the

damage. By this definition, Blaugrund et al (1993) felt that scarring was absent following optic nerve crush because all of the astrocytes which eventually occupied the lesion arrived there through migration from areas outside of the lesion. By contrast, Levine (1991) identified a glial scar in the lesion based on the chaotic nature of the reconstituted glial stroma in that location. However, since glial migration into the lesion was also demonstrated, this would not constitute a scar under the Blaugrund et al (1993) definition. It thus appears that the nature of this debate may lie in the definition of how a scar is formed rather than its actual presence or absence. In mammals, the glial scar is thought to be a major impediment for axonal regeneration in the CNS (Reier, 1982; Blaugrund et al, 1993) but in fish, if it is present at all (Levine, 1991; Blaugrund et al, 1993; Cohen et al, 1994), clearly does not interfere with regeneration.

A final interesting observation concerns the interactions between astrocytes and Schwann cells following nerve crush. As mentioned previously, Schwann cells will enter the crush site and begin myelinating regenerating axons. Following X-irradiation, however, Schwann cell invasion is slowed and the crush site is populated with astrocytes 50 days post crush (Nona et al, 1994). From these experiments, it appears that Schwann cells and astrocytes are in competition and when Schwann cells are prevented from invading, astrocytes will enter the lesion.

Microglia/macrophage interactions with regenerating axons in the CNS

Microglia, the resident phagocytic cell in the CNS, and macrophages, phagocytic cells derived from blood monocytes and found throughout the body have been linked to the removal of axonal and myelin debris following CNS damage. These cells form a heterogeneous population and adopt a wide range of morphologies depending on their location and functional status.

Macrophages have a characteristic ameoboid appearance while microglia are more ramified and bear several processes (Thomas, 1992). Lower vertebrate microglia, however, are less ramified than their mammalian counterparts (Dowding et al, 1991; Dowding and Scholes, 1993)

Dowding and colleagues (1991; Dowding and Scholes, 1993), have used the monoclonal antibody FL.1, which specifically labels CD54 in fish leukocytes, to show that microglia constitute approximately 30% of all cells in the optic nerve and spinal cord in *Oreochromis spilurus* compared to 1-5% in the mammalian CNS. Microglia also outnumber oligodendrocytes by about 3-fold in the spinal cord. Battisti et al. (1995) report that OX-42, which recognizes the complement component receptor C3bi present on cells of mononuclear lineage (macrophages and microglia) in the CNS, labelled cells constitute 15% of the non-neuronal cell population in the optic nerve of goldfish.

Following optic nerve crush, Dowding and colleagues (1991) showed a dramatic increase in the number of FL.1 positive cells in the entire visual system indicating recruitment and/or proliferation of these cells. Three days postcrush, significant numbers of microglia were found near the ganglion cells in the retina as well as in the *stratum opticum* and *stratum fibrosum et griseum superficiale* of the optic tectum, which contained the degenerating terminals of the retinal ganglion cells. In the optic nerve, between the crush zone and the tract, a 2-3 fold increase in the number of labelled cells was seen. Many of these cells were elongated and more ramified than quiescent microglia. In the crush zone itself, it was also observed that there was a significant increase of large ameboid cells which may be phagocytically active. Using electron microscopy, Dowding and Scholes (1993) also demonstrated that about 5% fish macrophage profiles in uninjured fish contained myelin phagosomes also

suggesting a role in myelin turnover and remodelling. OX-42 labelled cells have been shown to increase in number in the optic nerve of goldfish following crush injury until they reach a peak at 4 weeks (Battisti et al. 1995). These cells have also been shown to fill the crush zone 1 week postcrush suggesting invasion of macrophages from the periphery.

The Role of Macrophages in Axonal Regeneration in the Mammalian PNS

Macrophages comprise 1% of the total endoneurial population under normal conditions in the PNS (Monaco et al. 1992) and their response to axonal damage in the mammalian PNS is very similar to that described in the lower vertebrate CNS. This is in contrast to the mammalian CNS whose mononuclear cell response to axonal damage is mediated through indigenous microglia at a slower rate (Milligan et al. 1991; Lawson et al 1994). Immunologically, resident macrophages in the PNS express several monocyte/macrophage markers including the complement 3 receptor, ED1 and ED2. They also label with MUC 101 and 102 antibodies which recognize rat CNS microglia and other mononuclear cells in the peripheral organs and express high levels of the MHC class II product Ia (Monaco et al. 1992). Following γ-irradiation, MHC class II-positive macrophages were almost completely eliminated from the PNS while the number of ED-1$^+$ cells remained unchanged suggesting a heterogeneity of PNS macrophages in terms of lineage and functional properties (Monaco et al. 1992).

Following nerve transection, macrophages invade the site of trauma as well as the distal part of the nerve which undergoes Wallerian degeneration

(Monaco et al. 1992; Frisen et al. 1993; Stoll et al. 1989). It has been shown that following transection of rat sciatic nerve, there is a 4 day delay in macrophage recruitment at the lesion following which they increase in number until day 14. At this point, macrophages are found in both close association with the degenerating fibres and scattered throughout the endoneurial space (Stoll et al. 1989).

Monaco and colleagues (1992) have suggested that the macrophage response to sciatic nerve injury may be regulated differently at the site of injury compared to the distal segments of the nerve. There are MUC 101/102, OX-42, and ED1-3 positive macrophages at the site of injury as early as 1 day post injury. Based on their immunophenotype and morphology as well as the fact that the blood-nerve barrier is disrupted, these cells probably represent invading heamatogenous monocytes. In the distal segment, however, ramified MUC-101/102, OX-42, and ED1-3 positive macrophages assume a more rounded appearance similar to that of foamy macrophages (Stoll et al. 1989; Monaco et al. 1992) . At later stages of Wallerian degeneration these cells formed strings of round cells and maintained a high level of Ia expression (Monaco et al. 1992).

Advances in understanding the larger role of macrophages in response to nerve damage was greatly helped after the discovery of a mutant strain of C57BL mice, the C57BL/Ola strain which undergoes very slow Wallerian degeneration in the distal portion of an axon (Perry et al. 1993; Perry and Brown, 1992). In these mice, the distal axon will remain intact for weeks after transection and can conduct action potentials for up to 2 weeks following injury. Macrophage recruitment is also very poor in these mice (Perry and Brown, 1992) which leads to the question of which event has influence over the other: does the nerve fail to degenerate because macrophages are not

present or are macrophages not present because the nerve is not degenerating and releasing a chemotactic signal? Recent work suggests that the latter is probably true (Perry et al. 1993; Perry and Brown, 1992). Using irradiated C57BL/Ola mice injected with C57BL/6J bone marrow (as the source of normal (wild type) monocytes), these workers showed that slow Wallerian degeneration still occurred. On the other hand, when irradiated C57BL/6J mice were injected with C57BL/Ola bone marrow, they under went normal Wallerian degeneration indicating that C57BL/Ola mice macrophages are normal and the Wallerian defect in the C57BL/Ola mice lies in the axon.

Using this mutant strain of mice, these researchers have been able to study regeneration of motor and sensory neurons and have shown that sensory neurons regenerate very poorly in an environment where there is no macrophage recruitment or degeneration of distal fibres. One reason for this may lie in the absence of nerve growth factor (NGF) (Perry et al. 1993; Perry and Brown, 1992): a trophic factor known to stimulate sensory neuron survival and neurite outgrowth (Levi Montalcini, 1982). Levels of NGF mRNA are up-regulated in Schwann cells in damaged nerves as a result of interleukin-1 secretion by invading macrophages (Lindholm et al, 1987). This upregulation of NGF mRNA is parallel to the timecourse of invasion of macrophages in normal mice (Heumann et al, 1990). In the C57BL/Ola mice, there is a lack of macrophage recruitment resulting in low levels of Il-1 and therefore corresponding low levels of NGF mRNA (Brown et al, 1991).

Although macrophages thus seem necessary for effective regeneration in the PNS, these cells may also have negative effects on regenerating axons and other non-neuronal cells through the secretion of neurotoxic factors. All of this work was performed in the CNS of mammals (Giulian et al, 1993a-d), but parallels to PNS macrophages can be drawn because rat peritoneal

macrophages have also been shown to release the same neurotoxic chemicals (Giulian et al. 1993c,d). Using chick ciliary ganglia neurons grown on coverslips in culture dishes that contained enriched ameboid microglia populations in filter chambers, Giulian et al (1993b,c) showed that the secreted products of the microglia killed the neurons within 48 hours. Using scanning electron microscopy, they showed that there was a 60% loss of intact neurons with extensive cellular debris scattered throughout (Giulian, 1993a; Giulian and Corpuz, 1993b). It was also determined that the neuron killing factors released by microglia and macrophages were small, heat-stable, protease resistant molecules whose activity could be blocked by N-methyl-D-asparate (NMDA) antagonists (Giulian, 1993a; Giulian and Corpuz, 1993b; Giulian et al, 1993c). Some possible categories of agents which fit the profile for the neurotoxin are excitatory amino acids and superoxide anions (Giulian et al, 1993d).

Suppression of retinal microglia, and thus the release of neurotoxic substances, has been achieved using the tripeptide MIF--Macrophage Inhibitory Factor (Thr-Lys-Pro) (Thanos et al, 1993). Following optic nerve transection in adult mice, single or multiple intraoccular injections of MIF were administered over a period of 2 weeks which resulted in a decrease in the number of responding microglia and a corresponding threefold increase in the number of surviving retinal ganglion at 2 weeks postoperative(Thanos et al, 1993). Morphologically, microglia become less ramified and more oval-shaped and a decrease in cell number was observed (Thanos et al,1993). In contrast, when the macrophage stimulatory tetrapeptide tuftsin (Thr-Lys-Pro-Arg) was injected, its effects were opposite: there was a more rapid ganglion cell degeneration and evidence for increased microglial phagocytic activity.

Microglia are also known to release IL-1(Giulian et al 1986), a cytokine

which can stimulate astrocyte proliferation (Giulian and Lachman, 1985) and astrocytes are also known to release growth factors (Giulian, 1993a,c). So it appears that microglia are involved in a somewhat contradictory role following axonal damage: on one hand, their stimulation has detrimental effects on regeneration, while on the other, they release cytokines which stimulate other cells that can aid in the regenerative process.

Astrocyte Response in the Mammalian CNS to Peripheral Nerve Damage

Many neurons of the PNS have their cell bodies located in the CNS, e.g., the facial nerve and its central nucleus. Consequently, a peripheral nerve lesion in the mammalian PNS not only causes changes in the directly damaged neuron but will also elicit an astrocytic response in the central nucleus of that nerve somewhat analogous to that observed following transection of CNS axons in lower vertebrates--they increase in GFAP content and undergo hypertrophy (Svensson et al, 1993). As mentioned previously, reactive gliosis by astrocytes in the mammalian CNS following injury causes massive glial scarring and may impede regeneration of damaged axons by forming a physical barrier (Reier et al, 1982; Guenard et al, 1994). This idea remains controversial because other extracellular matrix molecules have been implicated in the failure of CNS regeneration and it has been shown that astrocytes actually secrete growth promoting factors which would presumably aid in regeneration (Giulian, 1993; Guenard et al,1994). In any event, this problem of poor regeneration and glial scarring is not seen following damage in the PNS of mammals since their axons are not located in the CNS

Astrocytes have been shown to be the fastest reacting glial cell to PNS axotomy. Rohlman and co-workers, (1994) showed that an increase in immunoreactivity for connexin-43 (cx-43), a prominent gap junction protein in astrocytes, occurred 0.75-1.5 hours following axotomy in the facial nucleus of rats. They found long lasting increases (4 days following transection) in the ipsilateral facial nucleus with no change on the contralateral side. These data indicates that the intercellular interactions via gap junctions between astrocytes surrounding the axotomized neurons may be the most rapid response to axotomy.

Mechanisms by which astrocytes would be activated either involve an increase in a growth promoting factor or suppression of a continuously expressed inhibitory molecule. Interleukin-1 (IL-1) has been shown to promote astroglial growth and/or proliferation and may represent one growth-promoting factor, possibly released by microglia (Giulian and Lachman, 1985; Giulian et al, 1988, 1994). Epidermal growth factor (EGF) is another astrocytic growth factor, whose activity is regulated by an inhibitor molecule which probably decreases following axonal damage (Svensson et al, 1993).

Like all cell interactions in an organism, the activity of one cell type is influenced by the activity of another. Svensson et al, (1993) has shown that following transection of the hypoglossal nerve in rats there was an increase in the amount of microglia present in the ipsilateral hypoglossal nuclei as well as an increase in the number of GFAP$^+$ astrocytes. When the rats were treated with ARA-C, a mitotic inhibitor, there was no increase in the number of microglia or astrocytes following damage. Measuring GFAP mRNA levels following damage also showed no increase in GFAP mRNA levels in rats treated with ARA-C while there was a dramatic increase in expression in the ipsilateral nucleus of control rats. Since astrocytes do not proliferate following

PNS damage, the anti-mitotic affects of ARA-C could not explain the lack of astrocytes (Svensson et al.1993). The researchers thus concluded that the axotomy-induced increase in astrocytes is mediated by microglia. The pathway of activation signals would then be from damaged axon to microglia to astrocyte. This pathway of activation has also been implicated *in vitro* from Giulian's laboratory (1993a,c, 1994). Changing levels of GFAP is probably only one way in which astrocytes alter their metabolism following peripheral axotomy but this is most likely one of the more important responses because GFAP is a major molecular component within the astrocyte and increased expression allows the cell to undergo hypertrophy (M. Svensson et al.1993).

The benefit of astrocytes on the regenerative process has already been discussed but just as with microglia, it appears that astocytes may also play a dual role in regeneration by both aiding and deterring it. Guenard and colleagues (1994) demonstrated that mature and immature astrocytes can inhibit axonal regeneration. Mature astrocytes, from E21-P1 Lewis rats, are cells which have been kept in culture for more than 14 days while immature cells are ones which have been in culture for 9 days. Three weeks after semi-permeable guidance channels were seeded with either mature or immature astrocytes and placed at the transected end of a sciatic nerve in rats, no regeneration of axons was observed. One possible explanation for this inhibition is that astrocytes in culture form a sheet-like structure which may present a physical barrier preventing elongating axons from crossing. Alternatively, the astrocytes might secrete molecules like the extracellular-matrix molecules chondroitin-6-sulfate proteoglycan and/or cytotactin/tenascin which are known to inhibit axon elongation (McKeon et al, 1991). Guenard and co-workers (1994) were able to show successful regeneration when purified Schwann cells were added to the astrocyte channels. The stimulatory

effect of the Schwann cells depended on the astrocyte/Schwann cell ratio and regeneration increased as the ratio decreased. One possible explanation for this observation could be that Schwann cells are able to suppress the inhibitory molecules expressed by astrocytes.

One further interesting observation from the work of Guenard et al (1994) was that astrocytes, from both the pure channels and the astrocyte/Schwann cell channels, showed a clear proximal migration suggesting the presence of chemotactic signals being released from the axon.

Conclusion

A great deal of uncertainty and controversy still surrounds many aspects of axonal regeneration in the CNS of lower vertebrates and the PNS of mammals. The role astrocytes play in remodelling the cellular environment following damage in the lower vertebrate CNS as well as the contrasting effects of macrophages/microglia in the PNS and CNS of mammals will remain just a few topics of intense research in the years to come in order to understand the phenomenon of axonal regeneration.

It is clear that the responses of non-neuronal cells following axonal damage are dependant upon one another. Following axon damage in both the lower vertebrate CNS and mammalian PNS, it seems that microglia and/or macrophages are activated or recruited and then release factors that stimulate astrocytes, oligodendrocytes, and Schwann cells to proliferate and/or become active. It is undoubtedly the balance of many factors in the environment surrounding damaged axons which allows axonal regeneration to succeed.

Research aims

As a basis for understanding CNS regeneration in a system where it occurs and understanding the importance non-neuronal cells have during regeneration, it was my aim to elucidate the changes in proliferation and total cell number in the visual paths of goldfish following axonal damage. Since it was also of interest to see if these changes were influenced by regenerating axons, the experiments were performed in the presence and absence of regenerating axons. The importance of this work is that it will form the basis of future experiments in that we will be able to target non-neuronal cells, in various ways, at different stages during their response to axonal damage. Future experiments may include administering anti-mitotic drugs or irradiation at times of peak proliferation to prevent their division and further assess their role. In general, it was observed that proliferation peaked between 7 and 14 days in all parts of the visual paths studied and returned to normal or near normal levels by 32 days post injury. Changes in total cell number on the other hand, were much more complex and consisted of several peaks of cellularity with cell numbers never returning to normal levels. There was also very little evidence to suggest that regenerating axons had an effect on the non-neuronal cell response. Finally, as this work was progressing, it was noticed that the proliferative response of non-neuronal cells varied depending on the time of the year. Consequently, experiments were performed to examine the effects of environmental conditions upon the non-neuronal cell response and axonal regrowth. It was found that fish acclimatized to autumn-like conditions had a faster non-neuronal cell response and enhanced axonal regeneration as compared to those acclimatized to spring-like conditions.

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Chapter I

Introduction

It is well established that sectioned optic axons in the central nervous system (CNS) of lower vertebrates successfully regenerate and re-establish a functional retinal projection in the optic tectum (Attardi and Sperry, 1963; Levine and Cronly-Dillon, 1974; Jacobson and Levine, 1975). This contrasts with the result of optic axotomy in mammals where there is a failure of regeneration associated with the eventual death of the majority of affected retinal ganglion cells (Villegas-Pérez et al, 1992). These differences between anamniotes and mammals reflect, in part, differences in the nature of the cellular environment through which the regenerating axons must grow. Indeed, it has been repeatedly shown that the CNS environment in mammals is nonconducive to axon growth (Caroni and Schwab, 1988a,b; Reier et al, 1989; Milligan et al, 1992; Blaugrund et al, 1993). Thus, if we are to understand regeneration in lower vertebrates, we must gain a greater understanding of the non-neuronal environment in the CNS of these animals.

The cells which comprise the non-neuronal environment in the CNS include oligodendrocytes, astrocytes and cells of the mononuclear lineage, i.e., microglia and macrophages. In the CNS of mammals, mature oligodendrocytes express neurite outgrowth inhibitory proteins which are believed to be one reason for unsuccessful regeneration (Caroni and Schwab, 1988a,b). In contrast, during regeneration in the CNS of lower vertebrates, oligodendrocytes not only lack a growth inhibitory protein (Bastmeyer et al, 1993; Sivron et al, 1994; Lang et al, 1995) but have been shown to provide a favourable substrate for outgrowth of mammalian CNS axons(Stuermer et al, 1992; Bastmeyer et al, 1993). Oligodendrocytes do not undergo hyperplasia after CNS damage and may, in fact, be subject to cell death in certain

situations(Sivron et al. 1991).

Astrocytes rapidly undergo morphological changes within hours of injury (Stuermer et al. 1992; Battisti et al. 1995) as well as increases in GFAP content at later postoperative times (Kreutzberg et al. 1989; Levine 1991). In addition, proliferation of astrocytes has also been reported following damage to the CNS (Reier et al. 1983; Miller et al. 1986; Miyake et al. 1988; Carbonell et al. 1991). The final outcome of CNS injury is often the formation of a glial scar which is due both to proliferation and hypertrophy of astrocytes (Giulian et al. 1989; Blaugrund et al. 1993; Cohen et al. 1994). In mammals, the glial scar is thought to be a major impediment to axonal regeneration in the CNS (Reier et al. 1983; Blaugrund et al. 1993), but this is probably not the case in the CNS of lower vertebrates (Reier, 1979; Reier et al. 1983; Levine, 1991).

Macrophages play a complex and multifunctional role in the process of Wallerian degeneration and axonal regeneration. First, they are phagocytes which remove axonal and myelin debris during Wallerian degeneration in both the lower vertebrate CNS and mammalian PNS (Giulian et al. 1989; Springer and Wilson, 1989; Stoll et al, 1989a, b). In addition, macrophages secrete interleukin-1 (Giulian et al. 1986) which drives secretion of nerve growth factor by non-neuronal cells during Wallerian degeneration in the PNS (Heumann et al. 1987; Lindholm et al. 1987) . Nerve growth factor is, in turn, a neurotrophic factor for sensory neurons which require it if they are to successfully regenerate. In fact, sensory axon regeneration in the PNS is inhibited in the C57BL/Ola strain of mice, where macrophage invasion is delayed (Brown et al. 1991; Perry et al. 1991; Perry and Brown, 1992). Finally, differences in the regenerative capabilities between the neonatal CNS and adult mammalian CNS have been attributed to the brisk response of invading macrophages in response to Wallerian degeneration in the developing CNS and the weak

response of indigenous microglia to similar lesions in the adult (Milligan et al. 1991).

By contrast with the situation in the PNS and neonatal CNS of mammals, macrophages and activated microglia in the adult mammalian CNS frequently have deleterious effects on the surrounding environment. For instance, IL-1 secreted by mononuclear cells (Giulian et al. 1986) is involved in the formation of astroglial scars which have been considered an impediment to successful regeneration in the CNS (Reier et al. 1983). In addition, activated microglia and macrophages secrete a variety of agents which have neurotoxic and gliotoxic effects (Giulian and Corpuz, 1993; Giulian et al. 1993a,b, Thanos et al. 1993).

In general, it has been held that the mononuclear response to Wallerian or retrograde degeneration is rapid and substantial in situations where axon growth or regeneration are possible, i.e., in the PNS and the neonatal mammalian CNS (Fawcett and Keynes, 1990; Milligan et al. 1991). On the other hand, in situations where regeneration of severed axons does not occur, i.e., in the adult mammalian CNS, the cellular response has usually been described as sluggish and weak (Perry et al. 1987; Stoll et al. 1989b; Barron et al. 1990; Milligan et al. 1991; George and Griffin, 1994). The anamniote CNS, however, does not fit this scheme since CNS axons in these animals regenerate readily (Attardi and Sperry, 1964). Consequently, it is of great interest to characterize the cellular response to central Wallerian degeneration is like in the anamniotes. Is it similar to the response in the PNS where axonal regeneration also occurs? Or, is there a universal CNS-specific response, i.e., sluggish and ineffectual, which would be seen in both mammals and anamniotes?

Previous workers have examined the cellular response to Wallerian

degeneration in the goldfish using a variety of model systems and methods of analysis (Stevenson and Yoon, 1978; Levine, 1982; Springer and Wilson, 1989; Battisti et al, 1995). However, none have attempted to systematically quantify the cellular changes throughout an entire central pathway. In the present paper, we do this by looking at patterns of proliferation and overall changes in cell number which occur following an optic nerve crush in the goldfish. In addition, because previous work (Johns et al, 1977; Stevenson and Yoon, 1978; Levine, 1993) has demonstrated effects of regenerating axons on tectal cell proliferation and morphology, we wanted to determine if we could detect similar effects in other parts of the visual system. To do this, we examined animals which had undergone optic enucleation. In general, we found early increases in proliferation and total cellularity which were resolved to normal or near normal levels by the end of the study. We invariably found elevated levels of proliferation in enucleated animals compared to animals receiving an optic nerve crush at at least one early timepoint, but overall, we found little to suggest that axons were exerting a major effect on the cellular response. Interestingly, we also found evidence for the recruitment of peripheral cells to the optic tectum and nerve during the first several days after surgery.

Materials and methods

Goldfish (*Carassius auratus*) 6-7 cm in body length from snout to base of tail were anaethestized in MS222 (0.1% tricaine methane sulphonate; Sigma) and the right optic nerve was crushed behind the eye using

watchmakers forceps with a 45° angle tip. The nerves were crushed for a count of 10 which produced an obvious separation of the nerve. For enucleated animals, the right optic nerve was crushed as above and the right eye was removed by creating a small scleral incision around the exit point of the nerve.

The operated fish were kept in 9 litre tanks (4 fish/ tank) at 20-22° C and allowed to survive for 1.2, 3, 5 and 7 days, and 2.3, 4, 8 and 12 weeks following surgery (n=3-4 in each group). Six hours before sacrifice, the fish were injected intraperitoneally with 0.1 mL of 5mg/ml bromodeoxyuridine (BrdU) (Boswald et al, 1990). Unoperated control animals (n=4) were also injected with BrdU and sacrificed 6 hours later.

BrdU Immunohistochemistry

The whole brain and nerve were removed and fixed in Carnoy's fluid (10% acetic acid, 60% absolute ethanol, 30% chloroform) for approximately 18 hours. The tissues were then processed for paraffin embedding. Ten µm sections were cut and mounted on 3-aminopropyltriethoxysilane coated slides (Rentrop et al, 1986) and allowed to dry at 37° C overnight. Sections were treated with 2N HCL for 20 min at 37° C to fragment the DNA and then exposed to a monoclonal antibody against BrdU (Becton Dickinson), at a dilution of 1:50. Labelled cells were visualized using an avidin-biotin peroxidase kit following the directions of the manufacturers (Vector Laboratories, USA),and counterstained with cresyl violet acetate.

BrdU positive cell counts

All BrdU positive cell counts were done using a dichroic blue filter,

which substantially enhanced the contrast of the DAB labelled cells. Labelled cells in the optic tract were counted in 4 bins, 165 µm on each side (27,225 μm^2), using a 40x objective (Fig 1a). Labelled cells in the optic nerve were counted in a 300 µm segment immediately cranial to the lesion under a 25x objective (Fig 1b). All labelled cells in the *stratum fibrosum et griseum superficiale* (*sfgs*) in a cross section of the rostral half of the optic tectum were counted under the 40x objective.

Total cell counts

Total cell counts were performed under 100x oil immersion. Counts in the optic tract were performed in the same manner as the labelled cell counts except that the counting bins were 40 µm on each side (1,600μm^2; Fig 1a). Cells were counted in the optic nerve in 6 randomly distributed counting bins (50 µm on a side, 2,500 μm^2) in the astrocytic domains cranial to the lesion (Fig 1b). Counts in sections of the optic tectum were made using 3 equally spaced counting bins, 40 µm on one side and spanning the thickness of the *sfgs* on the other (Fig1c).

Correction of long term cell counts

Both the optic tract and nerve undergo a substantial atrophy following enucleation, which only becomes apparent after 14 days postinjury (Levine, 1993). Because of this shrinkage, if an optic nerve crush (ONC) animal and an enucleated (ER) animal's optic tract contained the same number of cells, the density of cells would be greater in the shrunken ER tract. A natural concomitant of this is that since the counting bins are the same size for both tracts, the number of cells counted in the ER tract would be increased as the

ratio of the areas of the two tracts. In order to compensate for this problem, a correction factor was applied to the cell number values for ER animals at 21, 32, 56, and 91 days. First, the mean area of the optic tract was determined at each timepoint for the ONC and ER animals using the Sigma Plot program. The ER/ONC ratio was then calculated at each timepoint and the mean ER cell count values were then multiplied by this ratio. In the nerve, a similar method was used to adjust the total cell counts except that the thickness of the nerve, cranial to the lesion, was used to calculate the ratio.

Results

Proliferation Patterns

Both the optic tract and tectum showed similar patterns of proliferation following ONC (Figs 2 and 3). Between 5 and 7 days postsurgery there was a rapid rise in the number of labelled cells which was followed by a similarly rapid drop in labelled cell numbers to 14 days postsurgery. A major difference between the two groups of animals was that in the tract the peak was transient while in the tectum it persisted for several days. After this time, labelled cells increased in number again at 21 days followed by a sharp fall to control values by 32 days. Proliferation in the nerve followed a slower timecourse, reaching peak values at 14 days after surgery then dropping to low levels by 32 days (Fig 4). However, unlike the tract and tectum, the levels of proliferation in the nerve never fell back to control levels. Instead they remained elevated at about 20% of maximal levels for at least 3 months after the surgery. It is also noteworthy that the rate of proliferation in the nerve appeared to oscillate during the first two weeks after surgery rising to peak values at 3, 7 and 14 days and falling off during the intervening periods.

In all instances, enucleation enhanced some aspect of the proliferative response as compared to animals with a simple optic nerve crush. In the tract, the number of cells began to rise sooner but did not rise to higher levels than in the ONC animals (Fig 2). The values also fell more slowly in the tract of enucleated animals. In the optic tectum, the number of labelled cells began to rise sooner, and rose to greater heights in the enucleated animals (Fig 3). In both of these cases, however, it is of interest to note that the time to peak and to the disappearance of the proliferative response was the same. In the optic nerve the rising phase of the labelled cell curve was essentially the same both with and without the eye although the subsidiary peaks on the rising phase of the labelled cell curve of the ONC animals was not seen in the enucleated animals (Fig 4). However, the falling phase of the curve was affected by the removal of the eye and the levels of labelled cells in the nerves of enucleated animals were substantially increased compared to ONC animals at 32 days postoperative, afterwhich they fell to ONC levels.

Total Cell Counts

Total cell counts were performed on two separate sets of animals. Since the earlier counts were not done with BrdU labelling, they are not included here. However, the two sets of data were essentially identical.

In spite of the fact that the curves for proliferation in the nerve, tract and tectum are rather similar to each other, the corresponding curves for total cell counts differ considerably. The total cell counts in the optic tract showed a single peak which occurred at 14 days postoperative (Figs 5 and 6). Cell numbers then fell slowly to 60% of maximal values by 54 days after surgery . By contrast, in the optic tectum there was a bimodal curve with peaks at both

3 and 7 days postsurgery and a substantial dip in cell number at 5 days (Figs 7 and 8). The number of cells then dropped to approximately 35% of the maximal numbers by 32 days and remained at this level for the duration of these experiments. Finally, in the optic nerve there was again an early peak of cell number at 1 day postoperative, followed by a dip and then a rapid climb to a second peak at 7 days. After this point, the number of cells in the nerve remained at a plateau for a short period and then began to climb slowly to reach its final peak value at 32 days. There was then a gradual fall to approximately 75% of maximal values and this level persisted at least until 91 days postoperative (Figs 9 and 10).

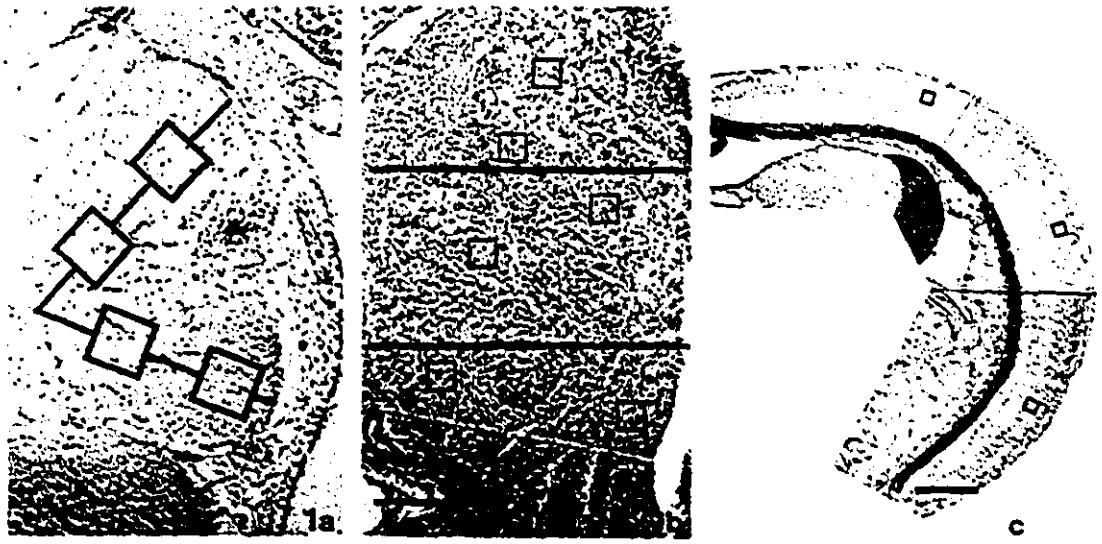
In all instances, the graphs for total cell counts in the optic nerve, tract, and tectum were similar for both ONC and enucleated animals at early time points. In the optic tectum the two curves are nearly superimposable for their entire lengths except that the second peak at 7 days was more than twice as large in the enucleated animals (Fig 7). In the optic tract the two curves separated after 14 days postoperative with the enucleated values falling off much more rapidly than the ONC values (Fig 5). In the optic nerve, the values for enucleated animals failed to reach ONC values at 7 days and remained substantially below them for the duration of the experiment (Fig 9).

Discussion

In the present study, patterns of cell proliferation and changes in total cell number were characterized in the visual system of goldfish following an optic nerve crush or optic enucleation. In general, following an optic nerve

Figure 1. The arrangement of counting bins used in this study. All sections are stained with Harris' hematoxylin and the counting bins are drawn approximately to scale. **a.** A cross-section of the optic tract with a line bisecting the dorsal and ventral halves of the tract. Two counting bins were placed on each line. **b.** A longitudinal section of the optic nerve: the crush lies just above the frame of the photo. The six small bins were used for total cell counts while labelled cells were counted in the segment between the horizontal lines. **c.** A cross section of the rostral optic tectum showing the position of the three counting bins that were used for total cell counts in the *stratum fibrosum et griseum superficiale (sfgs).* Scale bar for **a** and **b** = 100 µm; **c** = 270 µm.

Figure 2a. BrdU positive cell counts in the optic tract. Values for enucleated animals rise faster than ONC values until they both peak at the same magnitude at 7 days postinjury. Differences in values between ONC and ER optic tract at each timepoint were compared using the Student's *t* -test, *$p<0.05$; **$p<0.01$; ***$p<0.001$; **b.** BrdU incorporation in a cross-section of the optic tract 7 days following optic nerve crush. Representative labelled cells are marked with arrows. Note that the cells are distributed evenly throughout the tract. Scale bar = 100µm.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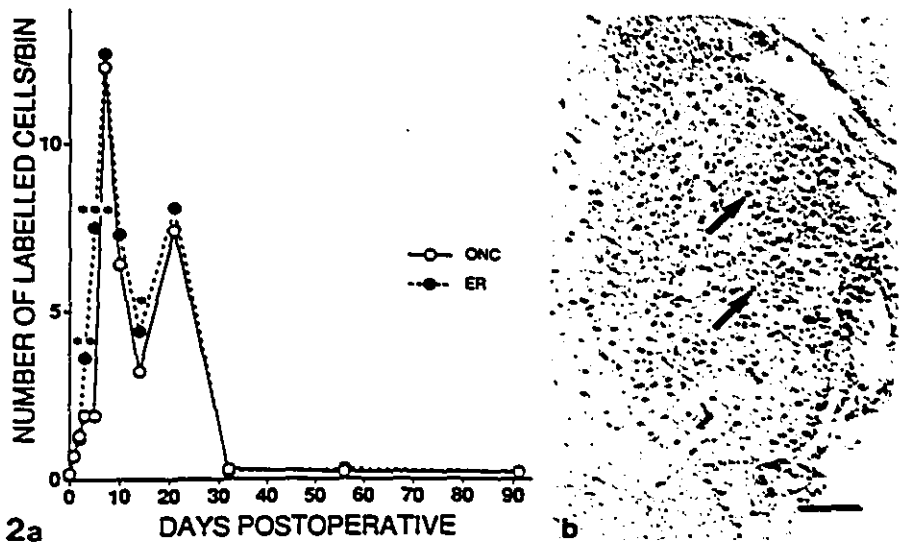
NUMBER OF LABELLED CELLS/BIN
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10
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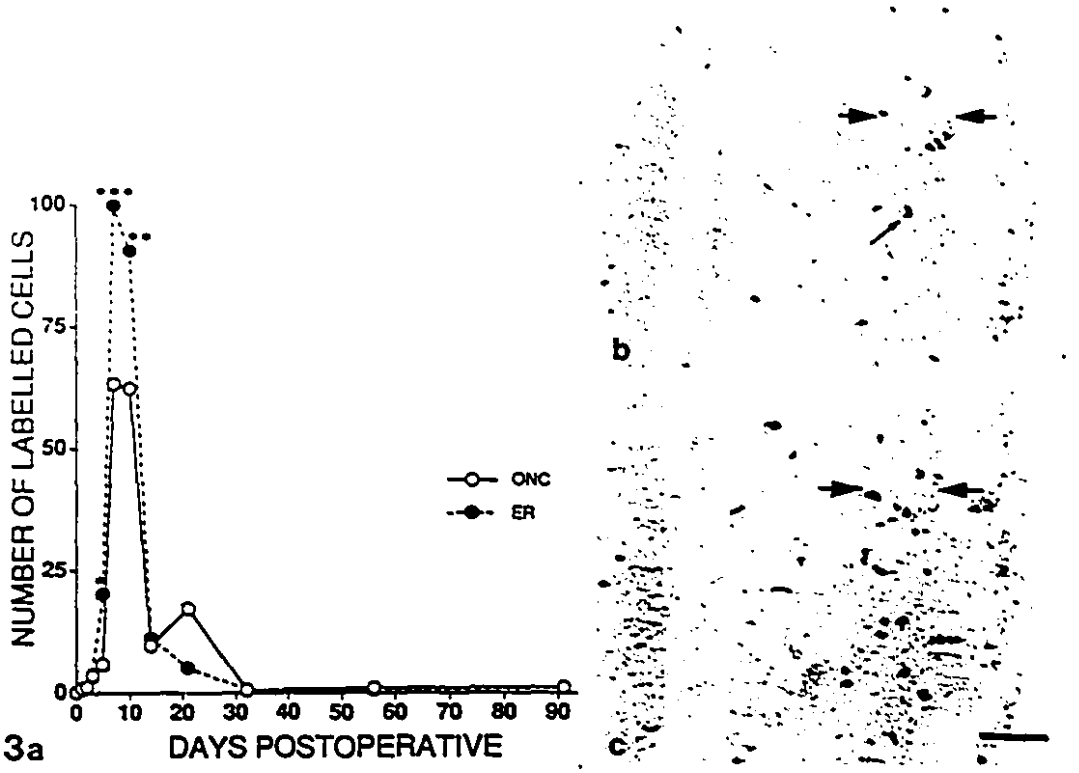
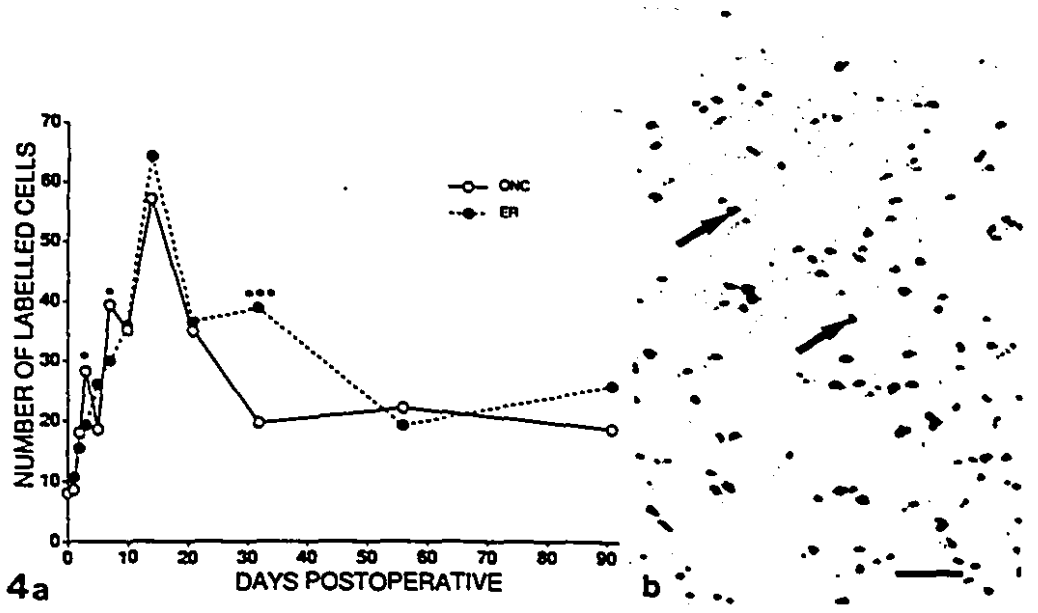
90
DAYS POSTOPERATIVE
ONC
ER
2a
b

Figure 3a. BrdU positive cell counts in the optic tectum. The magnitude of cell labelling in ONC and ER animals is similar except at 7 to 10 days postoperative where maximum values of ER animals are significantly elevated over ONC values. Asterisks as in Figure 2; **b** and **c**, cross-section of the rostral portion of the optic tectum showing labelled cells. The large arrows indicate the width of the *sfgs* **b.** Seven days after a nerve crush. The small arrow indicates a representative labelled cell. **c.** Seven days after enucleation. Note the increase in the number of labelled cells in the *sfgs* compared to the ONC optic tectum. Scale bar = 60μm.

Figure 4a. BrdU positive cell counts in the optic nerve. The time course of cell labelling is similar between ONC and ER animals. Note however, the persistence of labelled cells in ER animals at 32 days as compared to ONC values. **b.** A longitudinal section of the optic nerve 14 days postcrush showing maximum labelling. The representative labelled cells are marked with the arrows and the crush lies to the left of the photo. Scale bar = 60 μm.


NUMBER OF LABELLED CELLS
100
75
50
25
0
0 10 20 30 40 50 60 70 80 90
DAYS POSTOPERATIVE
ONC
ER
b
c

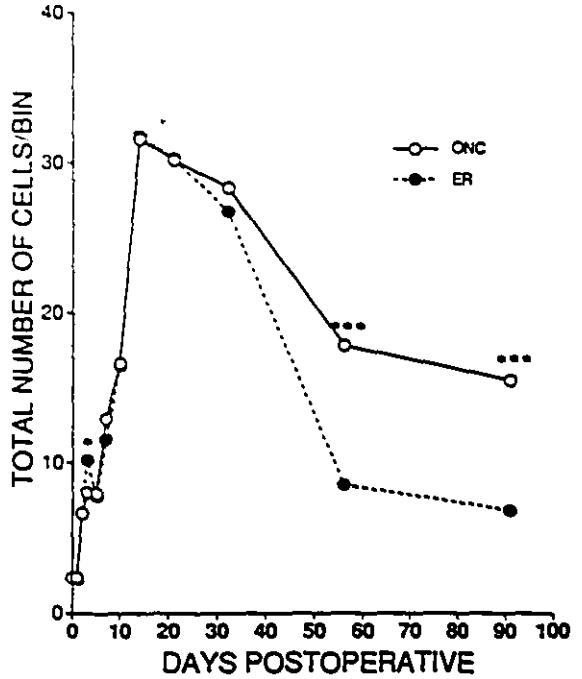
3a


NUMBER OF LABELLED CELLS
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90
DAYS POSTOPERATIVE
ONC
ER
b

4a

Figure 5. Total cell counts in the optic tract. Both experimental sets of animals show a similar pattern of cellularity until 56 days postinjury when ER values fall significantly below the ONC values. Asterisks as in Figure 2

Figure 6. Changes in cellularity of the optic tract following optic nerve crush. All sections are stained with Harris' hematoxylin; **a.** A cross-section of a normal optic tract. **b.** Seven days following optic nerve crush; the tract is hypercellular compared to the normal tract. **c.** Fourteen days following optic nerve crush: the tract shows maximal hyperplasia. Scale bar = 100μm.


TOTAL NUMBER OF CELLS/BIN
40
30
20
10
0
ONC
ER
0 10 20 30 40 50 60 70 80 90 100
DAYS POSTOPERATIVE

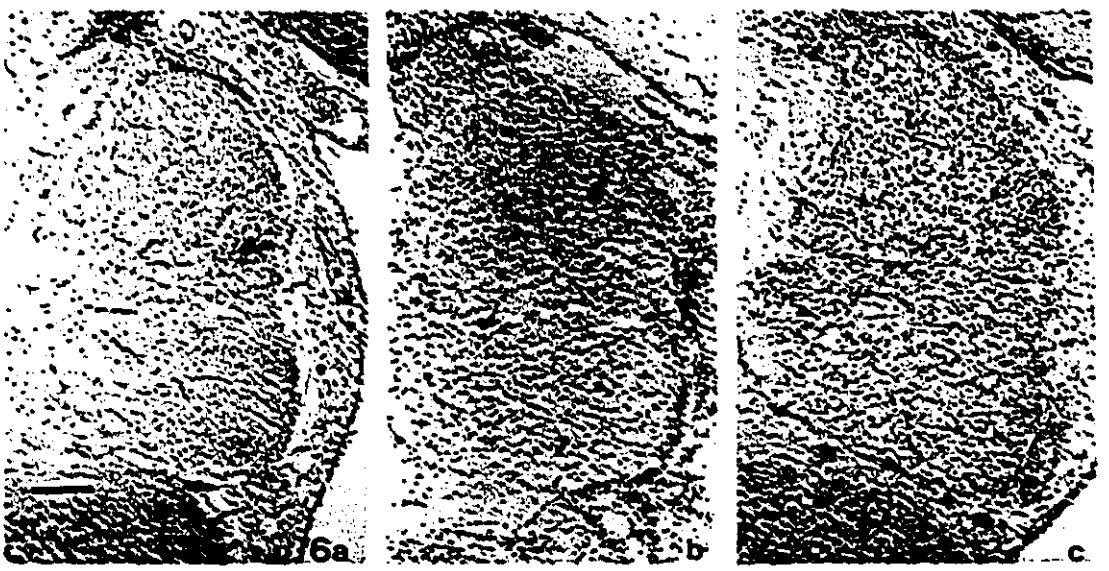
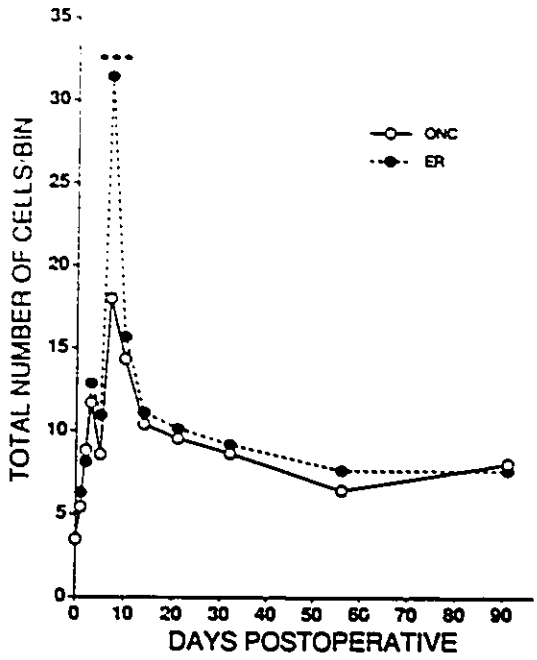

6a
b
c

Figure 7. Total cell counts in the optic tectum. The ONC and ER patterns are very similar except that maximum cell numbers at 7 days which is elevated in ER animals. Asterisks as in Figure 2.

Figure 8. Harris' hematoxylin stained cross-section of the rostral portion of the optic tectum . The arrowheads mark indicate the width of the *sfgs* . **a.** Normal optic tectum. **b.** Seven days following optic nerve crush. **c.** Seven days following enucleation. Note that the ER animal shows an increase in the amount of cellularity in the *sfgs* as compared to the ONC animal. Scale bar = 60μm


TOTAL NUMBER OF CELLS/BIN
DAYS POSTOPERATIVE
ONC
ER
0
5
10
15
20
25
30
35
0
10
20
30
40
50
60
70
80
90

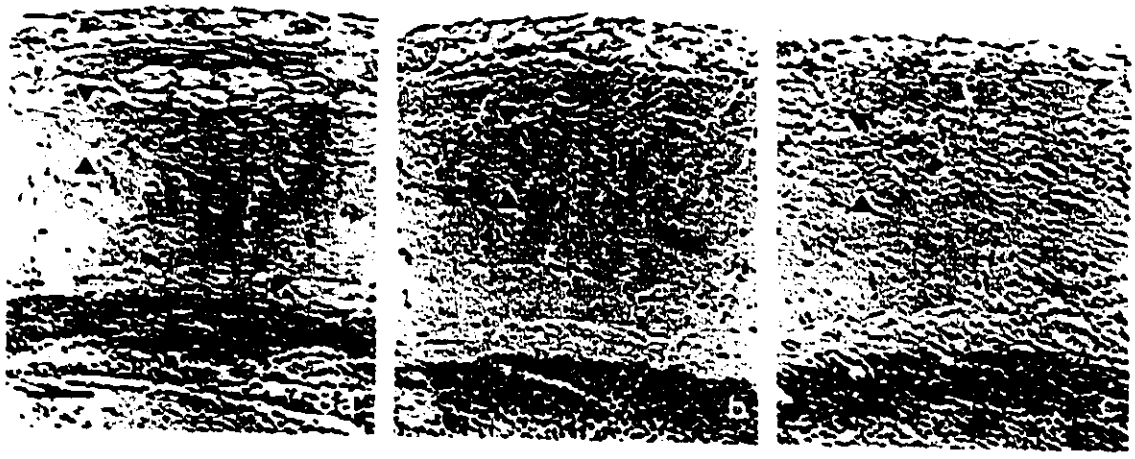
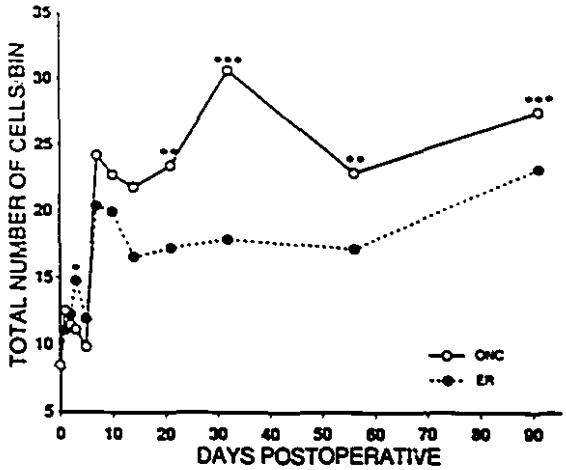
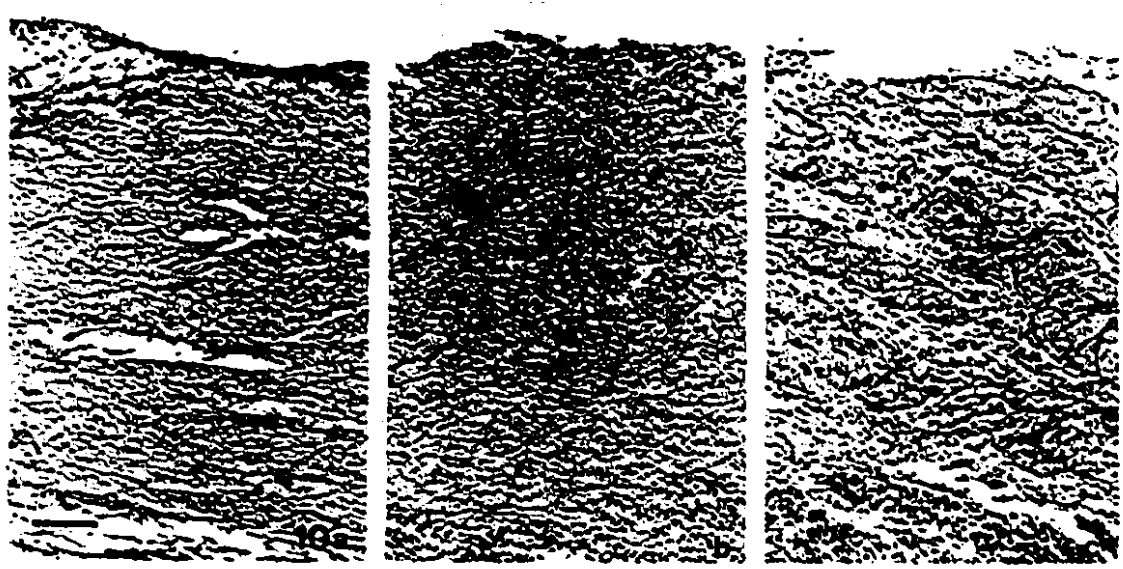


Figure 9. Total cell counts the optic nerve. Optic nerve crush and enucleation patterns are similar except that ONC values peak later and at a greater magnitude than ER values. Asterisks as in Figure 2

Figure 10. Harris' hematoxylin stained longitudinal sections of the optic nerve. **a.** Normal optic nerve. **b.**Thirty-two days following an optic nerve crush. There is increased cellularity in the astrocytic domains compared to the normal nerve. The lesion is located to the left of the photo **c.** Seven days after enucleation: note that maximum cellularity in the ER nerve is lower than maximum cellularity in the ONC nerve. Scale bar = 60μm.


TOTAL NUMBER OF CELLS/BIN
DAYS POSTOPERATIVE
ONC
ER



crush, all parts of the visual system showed increased proliferation which had resolved by 32 days postoperative. By contrast, the pattern of changes in cell number often involved several peaks of hyperplasia with cell numbers remaining elevated in all cases, even at the latest timepoint examined. Similar results have previously been reported in the goldfish using a different lesion model (Levine, 1982) or different methods of analysis (Stevenson and Yoon, 1978; Springer and Wilson, 1989; Battisti et al, 1995). In contrast to these data, quantitative investigations in the mouse (Lawson et al, 1994) have revealed several differences between the cellular responses of the mammalian CNS to Wallerian degeneration and those which have been demonstrated in the fish. The initial response in the goldfish optic nerve is much more rapid than that seen in the mouse, i.e., the first increase in either total cellularity or cells of mononuclear lineage is seen by 1 day postoperative in the fish (Fig. 10) (Battisti et al, 1995) but does not begin until after 3 days in the mouse. Conversely, the time taken to reach peak numbers of both total cells and cells of the mononuclear lineage is more protracted in the fish, i.e., 2-4 weeks (Fig. 10) (Battisti et al, 1995), than it is in the mouse where peak numbers are reached within 1 week after surgery. Thus, in the mammalian optic nerve, there is a delay in the initiation of the cellular response to Wallerian degeneration and the peak response is only short lived. Interestingly, a similar phenomenon is also seen in the mammalian PNS where the cellular response (ascertained using monoclonal antibodies against mononuclear cells) is also delayed until 2-3 days postoperative (Brown et al, 1991). By contrast, in the fish CNS, the cellular response begins almost immediately after surgery and the number of reactive cells continues to rise during the period when the mammalian response has already begun to diminish.

An important question that arises in this study is the identity of the cells

we observed proliferating in the visual paths following axonal damage. We can exclude astrocytes as possible participants in the optic tract and optic synaptic layers of the tectum because the astrocytes of the fish brain are radial glia with their cell bodies at the ventricular lining and their processes extending into the CNS parenchyma (Levine, 1989, 1995). Consequently, there are no free astrocytes in the tract or the tectum and they cannot account for the hyperplasia seen in those structures. In the optic nerve, on the other hand, there are local astrocytes which could contribute to the responses we saw (Levine, 1989, 1995; Dowding et al, 1991; Battisti et al, 1995). Although there have been no studies demonstrating that astrocytes proliferate after CNS injury in lower vertebrates, it has been shown that they do so in mammals (Reier et al, 1983; Miller et al, 1986; Miyake et al, 1988; Carbonell et al, 1991). It is therefore likely that astrocytes are active participants in the hyperplasia of the optic nerve in our animals and might account for the continued elevation of cell numbers in the nerve, particularly that seen at late postoperative times.

Several studies in fish and amphibians have yielded similar results to ours and have demonstrated that microglia or macrophages play a major role in the post-traumatic optic path hyperplasia seen following an optic nerve lesion. There is no marker available at present to identify cells of mononuclear origin in the goldfish but we are now working on a method to identify proliferating phagocytic cells to confirm the identity of the cells observed in this study. In other studies, Goodbrand and Gaze (1991) used a monoclonal antibody to label microglia in *Xenopus laevis* tadpoles after optic nerve injury. They found an increase in the number of active microglia which peaked at 5 days postinjury in the optic tract and tectum and then declined until at 32 days levels it had returned to normal values. Similar observations were made by Springer and Wilson (1989), who used cobaltous lysine to label degenerating

axons in goldfish which had undergone optic enucleation. Phagocytic cells were labelled in these studies through their ingestion of labelled axonal debris. Dowding et al (1991), used a monoclonal antibody which recognized the leukocyte common antigen (CD45) of Cichlid fish, to demonstrate an increase in the number of microglia/macrophages in the visual system at early timepoints following optic nerve crush. Most recently, Battisti and colleagues (1995) used a monoclonal antibody (OX-42) directed against a mammalian complement receptor to label cells of monocyte lineage in the goldfish. We have tried to use this antibody in our lab to label mononuclear cells without success. Using this antibody, as well as electron microscopy to identify labelled cells, they showed that following crush injury to the optic nerve there was an elevation of monocyte derived cells in the nerve which peaked 4 weeks following injury. These studies, together with similar observations in mammals (Graeber et al, 1988; Giulian et al, 1989; Stoll, et al, 1989; Morsshead and Van Der Kooy, 1990; Griffin et al, 1992; Perry et al, 1993; Lawson et al, 1994), make it likely that microglia and/or macrophages are the predominant cells which are responding in our study, particularly in the optic tract and tectum where there are no free astrocytes.

Another question that must be addressed concerns the origin of the cells which participate in the cellular response after optic axotomy since the increases observed may represent either proliferation of resident cells or the invasion of the visual paths by peripheral mononuclear cells (Miller et al, 1986; Perry et al, 1987; Stoll et al, 1989; Carbonell et al, 1991; Battisti et al, 1995). Although our data do not directly address this problem, we do find evidence for cell recruitment shortly after injury in both the optic nerve and tectum. In both of these structures, we observed an early transient rise in cell number (at 1 day postoperative in the nerve and at 3 days in the tectum) at a time when

proliferation values were still at the minimal control levels. Thus, proliferation of indigenous cells cannot explain these results and we believe they represent an early invasion of the affected structures by peripheral cells. (A similar transient rise in cellularity was also seen in the optic tract but it was associated with increased local proliferation). This is typical of the response of the mammalian PNS (Perry et al. 1987; Stoll et al . 1990a.) or neonatal CNS (Milligan et al. 1991) to injury but is very different from what has been described in the adult mammalian CNS (Milligan et al. 1991; Rao and Lund. 1993; Lawson et al. 1994) where the cellular response is mediated by indigenous microglia. In this regard, it appears that the goldfish CNS responds to damage in a similar manner to the mammalian PNS or neonatal CNS, both systems where axonal growth can occur (Fawcet and Keynes, 1990; Kirkin et al. 1993).

The possibility that there is an early, transient mononuclear invasion of the visual paths in the goldfish following optic axotomy is particularly interesting because it has been shown that early invading cells in the mammalian PNS drive the proliferation of Schwann cells (Beuche and Friede. 1984) and secrete interleukin-1 (Giulian et al. 1986). Interleukin-1 is known to induce the non-neuronal cells of the nerve to secrete nerve growth factor which is necessary for successful sensory axon regeneration (Heumann et al. 1987; Brown et al. 1991). If similar events were occurring in the goldfish brain, the later and much larger bursts of proliferation in the visual system might be due to the effects of the initial invading cells. In addition, invasion by these cells might drive the secretion of growth factors which are necessary for successful optic fiber regeneration. These possibilities may be examined by using methods to prevent the invasion of the CNS by peripheral mononuclear cells after optic axotomy, e.g. silica dust injection or X-irradiation of the

haemopöetic organs.

The possibility that regrowing axons have an effect on the non-neuronal cell response was investigated by performing optic enucleation. Animals with an optic enucleation displayed enhanced proliferation in the optic tract and tectum, as compared to the ONC animals, at early postoperative times. This effect was seen in the tract between 3 and 5 days postoperative and in the tectum between 7 and 10 days. Given that regrowing axons would not have reached either the tract or tectum at these early postoperative times (Lowenger and Levine, 1988), direct interaction with the regenerating axons cannot account for these early differences between the two sets of animals. It may be that enucleation is more traumatic to the visual system than optic nerve crush and therefore elicits a brisker cellular response. This idea is supported by observations in mammals that Wallerian degeneration occurs more rapidly following transection as opposed to a crushing lesion (Lunn et al, 1990) and that the cellular response to axotomy is in turn correlated with the rate of Wallerian degeneration (Perry et al, 1990; Perry et al, 1991; Lawson et al, 1994). Thus, it is reasonable to suggest that the differences between the ONC and ER animals in the present study are due to similar effects in the goldfish CNS. Interestingly, Goodbrand and Gaze (1991), working In *Xenopus laevis* tadpoles, showed that the microglial response to injury was the same following either an optic nerve crush, cut or optic enucleation. However, they did not quantify the microglial response and so it is possible that differences of the sort we describe were also present in their animals but escaped detection.

Our conclusion that regenerating axons do not play a major role in driving the cellular response to optic nerve damage in the goldfish seems to be in contradiction to the results of other workers. Stevenson and Yoon (1978) examined goldfish after crushing the optic nerve and found that there was a

late increase of proliferation in radial glial (periependymal) cells deployed along the inner surface of the *stratum periventriculare*, which did not occur in the absence of regenerating axons, i.e., after enucleation. However, it should be noted that this effect was quite small (the maximal number of labelled cells per tectal section was 4), it occurred at 35 days after optic nerve crush when Wallerian degeneration is for the most part over, and no mention was made of axonal effects in the optic synaptic layers where our counts were done. Similarly, Johns et al (1977) found that regenerating optic axons produced an enhancement of proliferation in the germinal zone at the caudal edge of the tectum. Again, this zone falls outside of the regions we investigated and, since it contains no optic fibers, would not be expected to undergo Wallerian degeneration. Thus, although these workers present clear evidence that regenerating optic axons have an effect on cells in the tectum, they present no evidence that they affect cells directly involved in Wallerian degeneration. In the present study, the only time at which there might have been such an axonal effect was at 32 days postoperative, where animals without an eye showed a persistent elevation of proliferation in the optic nerve at a time when ONC animals did not (see Fig 10). Otherwise, the late (>10d) patterns of proliferation did not differ significantly between ER and ONC animals. Thus, despite the fact that central axons regenerate in the brain in the goldfish, the non-neuronal response to CNS damage appears, for the most part, to be regulated by local factors which are not influenced by the presence or absence of regenerating axons.

Finally, the hyperplastic and proliferative response seen throughout the visual system during the first two weeks after optic nerve crush or optic enucleation, is mirrored by a decrease in both proliferation and total cellularity after this time. A similar response decrement has been reported in other studies

of the cellular response to CNS damage as well (Stevenson and Yoon, 1978; Levine, 1982; Giulian et al, 1989; Springer and Wilson, 1989; Lawson et al, 1994; Battisti et al, 1995). In ONC animals we found that cell numbers in the optic tectum fell to levels which were comparable to normal, while in the optic tract and nerve they remained somewhat elevated for the duration of the study. In addition, in the optic tract and nerve, ER values decreased considerably more than ONC values at the long term timepoints, an observation which may be related to the extreme atrophy seen in the absence of regenerating axons (Levine, 1993). These decreases are presumably mediated by cell death or the emigration of cells out of the visual paths. Indeed, several studies, in both fish and mammals, have shown that reactive cells in damaged CNS eventually exit the parenchyma by entering capillaries, the ventricles or the subarachnoid space (Giulian et al, 1989;Springer and Wilson, 1989; Griffin et al, 1992).

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Chapter II

Introduction

Circadian rhythms represent precisely regulated, endogenous physiological cycles which are used to measure periodic changes in the environment such as the time of day or the season of the year. They are found in every eukaryotic organism and typically have a period of one day but there are a number of examples with a period of a few hours or less (for review see Rapp, 1979). They control a vast array of processes ranging from reproduction (Turek and Campbell, 1979), metabolism of glycogen (Ehret et al, 1977), to regulation of opsin mRNA levels in photoreceptors (Korenbrot and Fernald, 1989). There are many factors that regulate circadian rhythms which are known as zeitgebers: periodic signals from the environment that keep a circadian rhythms synchronized with the environment. (Aschoff et al, 1982). The most critical zeitgeber is generally held to be photoperiodism which is the daily light-dark cycle. (Moore-Ede and Moline, 1985).

In mammals, the pineal gland serves a central role in photoperiodism as much of its biochemical synthesis, metabolism and secretion is light dependent (Klein, 1979). The pineal hormone melatonin is considered the central pineal product as virtually all of the important functions of this gland relate to it (Arendt 1985; Smith, 1985; Bartness and Goldman, 1989). Melatonin production is known to be depressed during the light phase and elevated during the dark phase of the day (Bartness and Goldman, 1989) making it an ideal, although undoubtedly not exclusive, candidate for the transmission of photoperiod information to the body. In fact, removal of this gland renders some animals unresponsive to photoperiod information (Goldman and Elliott, 1989). Most of the research into the role of the pineal gland and melatonin has been conducted in the context of reproduction. An example of the control

photoperiod has on reproduction can be illustrated with animals that are short- or long-day breeders. Short-day breeders are those whose breeding season is triggered when daylight progressively decreases (fall) while long-day breeders have their breeding season triggered by progressively longer daylight (spring) (Moore-Ede and Moline, 1985). Reproduction is so tightly regulated to a critical amount of daylight that in hamsters, for example, anything less than 12.5 hours of light per 24 hours will result in testicular regression (Gaston and Menaker, 1967). However, it is also evident that what is possibly more important than the duration of the light is the phase of the circadian rhythm with which it coincides. For example, in hamsters with regressed testes, recrudescence will occur using a 6 hours light interval as long as it falls on a critical phase of the circadian rhythm (Stetson et al, 1975). Also, in female hamsters, two 1 hour exposures to light separated by 12 hours of darkness will mimic a 14 hour photoperiod and allow the maintenance of ovarian cycles (Goldman and Darrow, 1983).

The are several target sites for melatonin in mammals. These include the suprachiasmatic nuclei (SCN), the median eminence/pars tuberalis, a small part of the choroid plexus at the caudal end of the fourth ventricle, the anterior pituitary and the retina (Vanecek et al, 1987; Vanecek, 1988; Reppert et al, 1988). The SCN, located ventrally in the anterior hypothalamus just above the optic chiasm, is believed to be the 'pacemaker' of circadian rhythms for two reasons. First, removal or destruction of the SCN results in a loss of circadian rhythms (Rusak and Zucker, 1979; Moore, 1983) and second, transplantation of fetal or neonatal hypothalamus containing the SCN in arrhythmic animals restores circadian rhythmicity (Drucker-Colin et al, 1984; Sawaski et al, 1984: Lehman et al, 1987)

In lower vertebrates such as teleost fish, circadian rhythms are also

prevalent. One such example is the daily rhythm of rod precursor cell proliferation in the retina of the African cichlid fish *Haplochromis burtoni* where elevated proliferation is observed during the nighttime and depressed proliferation during the day (Chui , et al 1995): a rhythmic cycle similar to that of melatonin. In fact, the pineal gland and melatonin are believed to be just as important in lower vertebrates as in mammals in conveying photoperiod information to the body. In the common carp, *Cyprinus carpio*, plasma melatonin levels are regulated in precisely the same manner as in mammals with elevated concentration in the dark and depressed concentrations in the light (Kezuka et al, 1988). This regulation is so well controlled with light that even when *C. carpio* are reared in unnatural photoperiods, plasma melatonin levels adjust accordingly.

Seasonality changes, as a result of changing photoperiods, in the control and production of a variety of hormones have also been demonstrated in fish. They include changes in plasma melatonin concentrations (Iigo and Aida, 1995); the growth hormone-releasing action of dopamine (Wong et al, 1993); the effects of neuropeptide-Y on growth hormone and gonadotropin-II (Peng et al, 1993); plasma steroids and gonadotropin levels (Okuzawa et al, 1989); the binding capacity of high affinity gonadotropin-releasing hormone binding sites(Andersson et al, 1992); and testosterone, 11-ketotestosterone, and estradiol-17β levels (Down et al, 1990). One difference in seasonality changes in fish as compared to mammals appears to be that environmental temperature is a zeitgeber for the fish (Iigo and Aida, 1995), although in much less of a capacity compared to photoperiod (Gwinner, 1986). An example of the importance of temperature and photoperiod on fish is demonstrated in gonadal maturation and spawning. There is a critical photoperiod and temperature required in order to induce both gonadal maturation and spawning (Peter,

1981; Razani et al, 1987; Okuzawa et al, 1989) ensuring that spawning will occur only once during the year and only during a specific season.

With the importance of circadian rhythms and photoperiodism evident in the regulation of a number processes in lower vertebrates, it was of interest to investigate whether they had any influence upon axonal regrowth and the non-neuronal cell response during axonal regeneration in the central nervous system (CNS) of goldfish. In addition, we noticed that the proliferative response to axonal damage varied depending on the time of the year the experiments were performed so it was of interest to investigate the possibility of a seasonal effect. In fact, there is previous evidence that suggests seasonal effects occur during CNS regeneration in the fish. Retinotectal reorganization following removal of the caudal portion of the tectum of goldfish results in compression of the retinotecal map only if the fish are operated on in the spring (Wye-Dvorak et al, 1979). Temperature, a zeitgeber in anamniotes, is also known to effect rates of axonal regeneration in the goldfish CNS. An exponential increase in growth rate between 20 and 30° C corresponding to a Q_{10} of 2.3 has been demonstrated(Springer and Agranoff, 1977). However, to date there has been no evidence to suggest that the non-neuronal cell response or axonal regrowth in any system is influenced by an environmental factor such as photoperiod. Here, evidence is provided to show that under autumn-like conditions, the proliferative response of non-neuronal cells and axonal regrowth occurs at a faster rate following optic nerve crush (ONC) in goldfish as compared to the response under spring-like conditions. The implications of this are that the success of failute of regeneration may depend on the time of the year that the animal is operated on.

Materials and Methods

Goldfish (*Carassius auratus*) 6-7 cm in body length from snout to base of tail were acclimatized for 2 weeks prior to surgeries in environmental chambers at the McGill Phytotron facilities. The following conditions were used to simulate spring and autumn conditions in the laboratory based on previous observations of changes in water temperature during the course of a year and simulating natural photoperiods: *spring:* the water temperature was kept constant at 18° C and a 13:11 hour light/dark cycle with a light intensity of 70 µmol was used; *autumn*: the water temperature was kept constant at 22° C and a 11:13 hour light/dark cycle with a light intensity of 70 µmol was used. Following acclimitization, the fish were anaethestized in MS222 (0.1% tricaine methane sulphonate; Sigma) and the right optic nerve was crushed behind the eye using watchmakers forceps with a 45° angle tip. The nerves were crushed for a count of 10 which produced an obvious separation of the nerve. The operated fish were kept in 52 litre tanks and allowed to survive for 1, 3, 5 , 7, 10, and 14 days (n=3-4 in each group) for the autumn conditions and for 5, 7, 10, and 14 days (n=3-4 in each group) for the spring conditions.

Bromodeoxyuridine (BrdU) Immunohistochemistry

Six hours before sacrifice, the fish were injected intraperitoneally with 0.1 mL of 5mg/ml BrdU. The whole brain and nerve were removed and fixed in Carnoy's fluid (10% acetic acid, 60% absolute ethanol, 30% chloroform) for approximately 18 hours. The tissues were then processed for paraffin embedding. Ten µm sections were cut and mounted on 3-

aminopropyltriethoxysilane coated slides and allowed to dry at 37° C overnight. Sections were treated with 2N HCL for 20 min at 37° C to fragment the DNA and then exposed to a monoclonal antibody against BrdU (Becton Dickinson, USA), at a dilution of 1:50. Labelled cells were visualized using an avidin-biotin peroxidase kit following the directions of the manufacturers (Vector Laboratories, USA), and counterstained with cresyl violet acetate.

Assessment of axonal regrowth using proline autoradiography

Eight day post crush fish (n=5 for each experimental group), acclimatized using the same conditions as above and independent of the fish used in the timecourse, were used to investigate axonal regrowth. Twenty hours before sacrifice, the fish were injected intraoccularly with 10 µCi ^{3}H-proline (Amersham) in 2-3µL. At sacrifice, the whole brain and nerve were removed and fixed in Carnoy's fluid (10% acetic acid, 60% absolute ethanol, 30% chloroform) for approximately 18 hours. The tissues were then processed for paraffin embedding. Ten µm sections were cut and mounted on gel subbed slides, dipped in photographic emulsion (Kodak NTB2) and developed 9 days later. Drawings of the nerves were made with a Leitz drawing tube and measurements of axonal regrowth were made from the crush site to the optic chiasm using a map measurer.

BrdU positive cell counts

BrdU positive cell counts were done using a dichroic blue filter, which substantially enhanced the contrast of the DAB labelled cells. All labelled cells in the *stratum fibrosum et griseum superficiale* (*sfgs*) in a cross section of the

rostral half of the optic tectum were counted under the 25x objective. To obtain control values, labelled cells in the unoperated *sfgs* were counted.

Results

General observations and morphology

In general, the operated optic nerve appeared swollen and hypercellular in both sets of experimental animals when compared to the unoperated side. The nerve appeared acellular in and directly around the crush site in the spring acclimatized fish, whereas the crush site in the autumn acclimatized fish was hypercellular as in the rest of the nerve. Interestingly, the crush site in the spring acclimatized fish showed very little signs of recovery from the nerve crush while autumn acclimatized fish appeared to be healing well.

Axonal regrowth in the nerve

The spring acclimatized fish exhibited a very slow rate of regrowth with an average axonal regrowth distance of 0.32mm from the crush site. In contrast, autumn acclimatized fish showed an enhanced rate of regrowth with an average axonal regrowth distance of 1.7mm from the crush site. (Figure 11).

BrdU positive cell counts

BrdU positive cell counts conducted in the spring acclimatized fish showed a nearly identical timecourse of proliferation as reported in Chapter I for fish which had been operated on in the spring. There was a rapid rise in proliferation beginning at 5 days postcrush and peaking between 7 and 10 days. This was then followed by a similarly rapid decline in proliferating cells with a return to control levels by 14 days postcrush. Autumn acclimatized fish

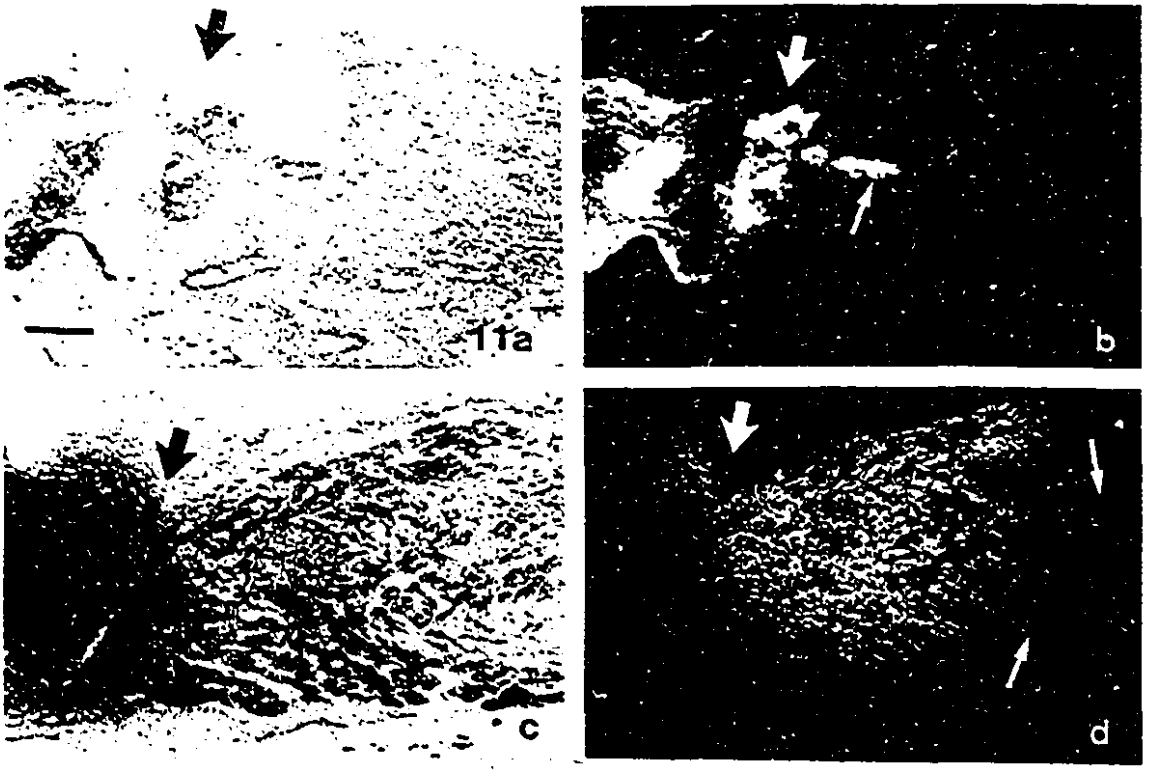
showed a much more rapid response following ONC but the overall timecourse pattern of the response was similar to what was seen in the spring fish. There was a rapid rise in proliferation beginning at 1 day postcrush, peaking between 3 and 5 days, and followed by a rapid decline to near control levels by 7 days postcrush (Figure 12).

Discussion

In the present chapter, seasonality influences of photoperiod and temperature on the non-neuronal cell response and axon regrowth following axonal damage in the goldfish have been demonstrated. Fish acclimatized in autumn conditions showed an enhanced rate of axonal regrowth and a more rapid initiation of a non-neuronal cell response compared to fish acclimatized in spring conditions. There are two variables which are contributing to the seasonality effect being observed: temperature and photoperiod. The effect temperature contributes to regeneration is expressed by the Q_{10} -- the factor by which a "reaction velocity" is increased for a 10^0 C rise in temperature (Cancalon, 1985). For regenerating goldfish optic nerve, the exponential increase in growth rate from 20 to 30^0 C corresponds to a Q_{10} of 2.3 (Springer and Agranoff, 1977), i.e., for every 10^0 C increment in temperature, a 2.3 fold increase in the rate of axonal elongation will be observed. It is expected then that over a 4^0 C temperature difference, axonal growth should differ by approximately 125μm . The fact that I observed at 1480μm difference in the rate of axonal elongation corresponding to a 5.3 fold increase over 4^0 C suggests that temperature is only playing a minor role in the seasonality

Figure 11. Autoradiographs from longitudinal sections of optic nerves 8 days postcrush demonstrating growth of regenerating axons. In all cases, the large arrow represents the crush site, small arrows represent labelled axons and the eye is to the reader's left. For darkfield images, labelled fibres appear white on a black background **a.** brightfield view of an optic nerve from a spring acclimatized fish. **b.** darkfield view of the same section demonstrating that regrowing axons have grown only slightly past the crush site. It is of interest to note that the regenerating axons have avoided the acellular region. **c.** brightfield view of an optic nerve from an autumn acclimatized fish. **d.** darkfield view of the same section demonstrating regenerating axons have grown well past the crush site. Scale bar = 160μm

Figure 12. BrdU positive cell counts in the optic tectum following optic nerve crush of spring (13:11 light:dark(LD); 18^0C) and autumn (11:13 LD; 22^0C) acclimatized fish. Both experimental sets of animals exhibit very similar cellular responses to axonal damage except that autumn acclimatized fish initiate their response much earlier than spring acclimatized fish.


11a
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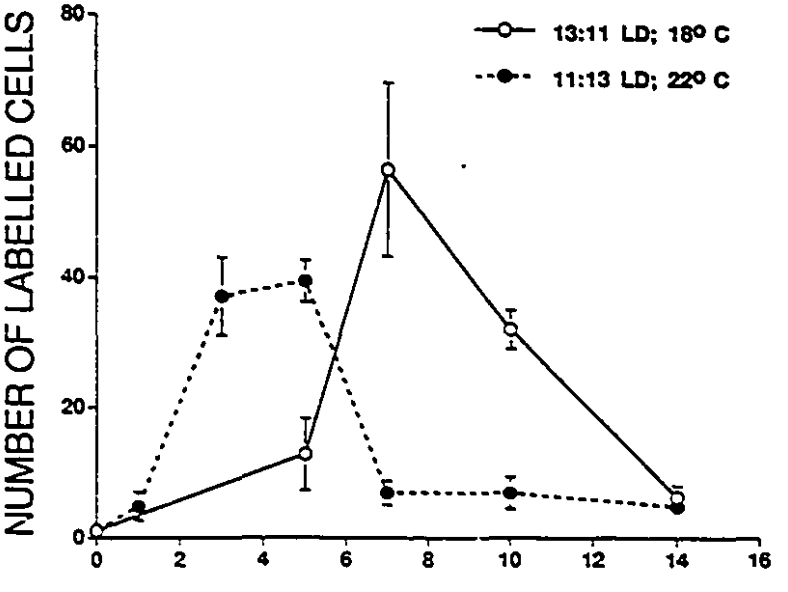
13:11 LD; 18° C
11:13 LD; 22° C
NUMBER OF LABELLED CELLS
0
20
40
60
80
0
2
4
6
8
10
12
14
16
DAYS POSTOPERATIVE

12

changes observed. Photoperiod thus appears to be the principal factor in conferring the seasonality changes observed.

Photoperiod is generally held as the most important zeitgeber effecting circadian rhythms (Moore-Ede and Moline, 1985) and there have been a number of studies done to determine the role photoperiod plays in controlling circadian rhythmicity. Resonance studies, which consist of fixed short light intervals coupled with long dark intervals (ex. 6:18 light:dark (LD), 6:30 LD) determined that what is probably more important than the duration of light is during what time of the circadian cycle does the light fall upon (Turek and Campbell, 1979). For example, in male hamsters with regressed testes, resumption of normal reproductive activity will occur if 6 hour light intervals fall on the critical phase of the rhythm (Stetson et al, 1975). However, if the timing of these 6 hour light intervals is out of phase, recrudence will not occur (Elliot et al, 1972). In rams, reproductive competence is restored following a 8:40 LD cycle but not a 8:28 LD cycle illustrating once again the importance of a critical phase in circadian rhythm (Almedia and Lincoln, 1982).

The importance of a critical phase in circadian rhythms has also been studied using skeleton photoperiods which are pulses of light ranging in time from a few seconds to one hour separated by a long duration of darkness. Male hamsters exposed to 15 min of light every 6 hours (for a total of 1 hour/24hours) will not undergo gonadal regression while hamsters exposed to 1 continuous hour of light in 24 hours regressed (Rudeen and Reiter, 1980). Also, male hamsters reared in a nonstimulatory light regime of 8:16 LD had gonadal regression prevented when exposed to 10 seconds of light every 2, 4, or 7 days during the dark phase of the 8:16 LD regime(Earnest and Turek, 1984).

The photoperiod effects discussed above illustrate the importance of

light in determining whether or not an organism will reproduce. Photoperiod is equally important in conveying seasonality changes and consequently appropriate spawning times in fish. Changes in hormone levels during spawning season, are brought on, in part, by lengthening days. In common carp and carp/goldfish hybrids, estradiol 17β, 11-ketotestosterone, and gonadotropin levels are elevated in the spring during their spawning season, (Down et al, 1990). In female carp and male and female hybrid carp, testosterone levels are also elevated during their spawning season compared to any other season. In the Cyprinid *Gnathopogon caerulescens*, which spawns from early April to early July, a long photoperiod (15:9 LD) is required to maintain gonadal activity, plasma gonadotropin and gonadal steroid levels: a 12:12 LD does not maintain these levels (Okuzawa et al, 1989).

Given the importance of photoperiod as a zeitgeber, the question arises as to what conveys the photoperiod information into circadian responses. Melatonin, the primary product of the pineal gland, but also produced by the retina in the frog *Xenopus laevis* (Cahill and Besharse, 1989), rainbow trout (Gern and Ralph, 1978), and goldfish (Grace et al, 1991) is the hormone generally believed to transmit photoperiod information to the body. Although the exact function of melatonin is unclear, it has been shown to modulate photoreceptor outer segment membrane turnover (Besharse and Dunis, 1983), light and dark-adaptive photoreceptor movements (Pierce and Besharse, 1985), and melanin pigment granule migration within the retinal pigment epithelium (Pang and Yew, 1979). In mammals, it is mainly targetted to the suprachiasmatic nuclei (Vanecek, 1988) which lie adjacent to the third ventricle just dorsal to the optic chiasm and receives a direct input from the retina (Sofroniew and Weindl, 1982). In goldfish, using a radioreceptor assay with 2-[^{125}I]iodomelatonin as the radioligand, target sites for melatonin were found (in

order of decreasing number of sites) in the: optic tectum-thalamus, hypothalamus, telencephalon, cerebellum, and medulla oblongata (Iigo et al, 1994).

It seems likely that a major physiological difference between the autumn acclimatized and spring acclimatized fish is that melatonin production would be expected to occur for a longer duration in autumn acclimatized fish which received 2 hours more darkness per day. This suggests that the enhanced axonal regrowth and faster initiation of the non-neuronal cell response may somehow be linked directly or indirectly to melatonin. Melatonin is known to convey seasonality changes in a number of organisms (Arendt, 1985) which are often characterized by seasonal changes in a variety of hormone levels (Okuzawa et al, 1989; Down et al, 1990; Wong et al, 1993). One class of hormones which is particularly relevant for this study is the gonadal steroids which are known to exhibit seasonal variation (Okuzawa et al, 1989; Down et al, 1990) and, at least in the case of testosterone, play a role in axonal regeneration (Jones, 1993;1994).

Testosterone has been shown to have trophic effects upon peripheral nerve regeneration in mammals, with increased testosterone levels accelerating functional recovery from nerve damage (Yu and Yu, 1983; Kujawa and Jones, 1990;1991). It appears that one way in which testosterone mediates these effects is through regulation of cytoskeletal proteins (Jones and Oblinger, 1994). Two days following axotomy of the hamster facial nerve, neuronal ßII tubulin mRNA levels were found to be significantly elevated when exogenous testosterone was present, but not after axotomy alone. In addition, it was demonstrated that neuronal rRNA which normally reaches peak levels 2 days following axotomy, could reach levels twice that amount in only 6 hours if exogenous testosterone was present following axotomy (Kinderman and

Jones, 1993). Since testosterone levels have been shown to be elevated in the autumn in the cyprinid, *Gnathopogon caerulescens* (Okuzawa et al, 1989), and in male carp and hybrid carp/goldfish of unknown sex (Down et al, 1990), it seems that this may be one factor to explain the enhanced axonal regrowth demonstrated in the autumn acclimatized fish in this study. It is important to note, however, that the male carp and hybrid carp/goldfish examined by Down et al (1990) also exhibited elevated testosterone levels in the spring at levels similar to those observed in the autumn. This elevation in testosterone also occurs naturally in the springtime in female carp which do not show an autumn increase (Down et al, 1990). In addition, under forced long photoperiods (15:9 LD) and increased water temperature (25^0C) in *G. caerulescens*, Okuzawa et al (1989) demonstrated increased testosterone levels in the spring. Thus, these two groups found evidence for elevated testosterone levels in both spring and autumn conditions. However, in the case of Okuzawa et al (1989), to achieve elevated testosterone levels in the spring, they had to subject the fish to unnatural conditions. At 20^0C, a natural springtime temperature, and a 15:9 LD cycle, elevated testosterone levels were not observed. Down et al (1990) did not state what temperature the fish were reared in, but from the work of Okuzawa et al (1989), this is clearly a factor that may have contributed to the elevated levels observed in the spring. Thus, the control of testosterone levels by circadian zeitgebers has not yet been resolved and although it is attractive to suggest that testosterone may be involved in the enhanced axonal regrowth and cellular response observed here, further studies will have to be performed to assess its precise role.

Summary

This thesis described the changes in proliferation and total cell number and environmental effects on the non-neuronal cell response following axon damage in the visual paths of goldfish. In general, it was found that following axonal damage, proliferation in all parts of the visual system peaked between 7 and 14 days and was resolved to normal or near normal levels by 32 days postoperative. Total cellularity also peaked between 7 and 14 days and with the exception of the nerve which remained elevated for the duration of the study, was resolved to normal or near normal levels by 32 days postoperative. In order to assess the effect regenerating axons have on the cellular response, optic enucleation was performed. Enucleation resulted in elevated proliferation in all parts of the visual system in at least one early timepoint but well before regrowing axons would have ever reached these structure. Thus, there is very little evidence to suggest that axons exert a major effect upon the cellular response following damage. Interestingly, there is evidence to suggest that there is recruitment of peripheral mononuclear cells to the optic nerve and tectum in the early days following surgery.

It has also been demonstrated that environmental factors have an effect the non-neuronal cell response and axonal regeneration following optic nerve crush. Fish acclimatized under autumn-like conditions initiated a faster cellular response and underwent enhanced axonal regrowth compared to fish acclimatized under spring-like conditions. It is believed that photoperiod is the factor primarily involved in the response and that temperature played only a minor role. It is postulated that the hormone melatonin is somehow linked directly or indirectly to the enhanced axonal regrowth and faster cellular response observed.

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