THE ROLE OF RETINAL GANGLION CELL AXONS IN THE REGULATION OF GLIAL CELL NUMBERS IN THE RODENT OPTIC NERVE

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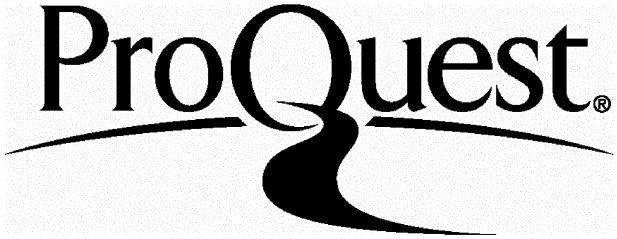

Abstract

The effect of increased optic nerve axon number on glial cell numbers was studied in a transgenic mouse that expresses the human *bcl-2* gene from a neuron-specific-enolase promoter. The normal postnatal loss of retinal ganglion cell (RGC) axons was decreased and, consequently, the number of axons in the adult optic nerve was 80% greater. The expression of human Bcl-2 in RGCs protected the cell body from programmed cell death when the axon was cut, but it did not protect the isolated axon from Wallerian degeneration, even though human Bcl-2 was present in the axon.

The numbers of both oligodendrocytes and astrocytes increased proportionally in the transgenic optic nerve. Unexpectedly, the transgene was expressed in both types of glia, but this was not responsible for the increased numbers of these cells. Oligodendrocytes increased because of a decrease in normal cell death, whereas astrocytes increased because of increased proliferation. To investigate whether astrocyte proliferation normally depends on axons I looked first at the effect of axon removal induced by Wallerian degeneration and found that this stopped astrocyte division. This was also the case in cut optic nerves in the Wld^S mutant mouse where the axons did not degenerate. I found that axonal transport, but not electrical activity in the axons was required for axons to stimulate astrocyte division, suggesting that the putative astrocyte mitogen must be continuously transported from the cell body to the axon. I showed that purified RGCs can stimulate astrocytes to synthesise DNA when RGCs were co-cultured with a mixed population of optic nerve cells; of all the signalling molecules tested, only bFGF could mimic this effect of the RGCs.

Finally I have found weak evidence that Wallerian degeneration of axons may utilise some of the proteases (caspases) that mediate programmed cell death.

Contents

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Finally, I am especially grateful to Martin Raff whose experience, wisdom and infectious enthusiasm have been instrumental in the writing of this thesis.

To Rachel b. 12 January 1997.

Abbreviations

Arac	Cytosine arabinoside
bFGF	Basic fibroblast growth factor
B-S	Bottenstein-Sato
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
BAF	Boc-Asp(O-Me)-FMK
CNTF	Ciliary neurotrophic factor
CDK	Cyclin-dependent kinase
CHX	Cycloheximide
DMEM	Dulbecco's Minimal Eagles Medium
DRG	Dorsal root ganglion
E	Embryonic day
EBSS	Earl's balanced salts solution
EGF	Epidermal growth factor
FCS	Fetal calf serum
G anti-MIg Fl	Fluorescein-coupled goat anti-mouse immunoglobulin
G anti-MIg TR	Texas Red-coupled goat anti-mouse immunoglobulin
G anti-MIg G1 Fl	Fluorescein-coupled goat anti-mouse G1 immunoglobulin
G anti-RIg Fl	Fluorescein-coupled goat anti-rabbit immunoglobulin
GGF	Glial growth factor
GFAP	Glial fibrillary acidic protein
GC	Galactocerebroside
ICE	Interleukin-1β converting enzyme
IGF-1	Insulin-like growth factor

LGN	Lateral geniculate nucleus
NT-3	Neurotrophin-3
NSE	Neuron-specific-enolase
NGF	Nerve growth factor
NCAM	Neural cell adhesion molecule
MEM	Minimal Eagles Medium
MBP	Myelin basic protein
or	Ocular retardation
PI	Propidium iodide
PCD	Programmed cell death
PDL	Poly-D-lysine
PDGF	Platelet derived growth factor
PBS	Phosphate buffered saline
P	Postnatal day
RGC	Retinal ganglion cell
Sh anti-RIg Fl	Fluorescein-coupled sheep anti-rabbit immunoglobulin
Sh anti-RIg TR	Texas Red-coupled sheep anti-rabbit immunoglobulin
Shh	Sonic hedgehog
s.e.m	Standard error of the mean
SC	Superior colliculus
TBLS	Tris buffered lysine saline
TGF	Transforming growth factor
TTX	Tetrodotoxin
Wld^s	Wallerian degeneration deficient strain
zVAD	Z-Val-Ala-Asp(O-Me)-CH_2F

CHAPTER 1

GENERAL INTRODUCTION

Size Control in animals

The control of tissue growth *in vivo* is still a relatively unexplored field of biology. The final size of an adult tissue depends on several factors, including the size of individual cells, the amount of extracellular matrix, and, especially, the total number of cells. In general larger animals are larger because they have more cells. Although the control of cell numbers is of fundamental importance in animal development, it has received surprisingly little attention (Raff, 1996).

Historically animal tissues have been classified according to their mitotic potential. An early classification divided tissues into three broad groups: renewing, expanding, and static (Goss, 1978). The renewing tissues are those in which cells are generated and lost throughout life: examples are gut, blood, and skin. Expanding tissues remain static in adult animals but retain the ability to expand if required: examples are the liver and kidney. The adult liver is unusual in that it can regenerate its original mass by compensatory hyperplasia if most of it is lost. Although an adult kidney can enlarge if its mate is removed, this is largely the result of hypertrophy rather than hyperplasia, although some hyperplasia occurs (Karp et al., 1971).

Static tissues are those where the cell number is determined during development and cannot be changed in the adult. The neurons in neural tissues fall into this group, as they are generally postmitotic and cannot be replaced if lost. There are exceptions, however. Olfactory neurons are constantly turned over in adult vertebrates: they die throughout life and are regenerated from dividing precursors, both in the olfactory epithelium and in the olfactory bulb (Lois et al., 1996).

Other examples of static tissues are muscle and adipose tissue. Muscle and fat can enlarge in adults, but they do not proliferate. In the case of muscle, however, satellite cells in adult muscle can divide to give rise to myoblasts, which can then fuse with existing myotubes to replace lost muscle cells.

In summary, with the exception of the liver, the majority of adult tissues retain some capacity for growth achieved mainly by hypertrophy, but they have little ability to grow by cell proliferation. Most hyperplastic growth occurs during development, where the challenge is to understand how the growth of an organ is controlled and co-ordinated with the growth of other organs and tissues.

The control of tissue size during development

During development the size of a tissue is the product of cell proliferation, cell migration, and cell survival. Cell survival is increasingly recognised as being fundamentally important in the control of cell numbers. Tissue size also depends on the amount of extracellular matrix, but in only a few tissues does the matrix make a major contribution to the tissue; bone and cartilage are examples.

In all tissues cells divide at some stage during development. In higher animals at least, these cell divisions occur in response to extracellular signals–so called mitogens. By making cells dependent on signals from other cells for division, animals ensure that their cells only divide when new cells are needed–mainly for growth or to replace lost cells. Such signals can be endocrine, paracrine or autocrine. The growth of the liver and kidney, for example, seems to be controlled mostly systemically (Goss, 1978), whereas the growth of the gut seems to be mainly under local control. A gut transplanted from an embryo to a postnatal

animal, for instance, will continue to grow as if it were still in the embryo (Zinar et al., 1971).

Cell division is also controlled at the intracellular level. Cells choose to ignore or respond to extracellular mitogens, depending on the regulated expression of specific mitogen receptors, as well as on the intracellular signalling pathways that the receptors activate. Ultimately, the signalling pathways converge on the cell cycle control system. The progression from G1 to S-phase of the cell cycle is the main point in the cell cycle where mitogen regulation occurs. It is regulated by a series of cyclin and cyclin-dependent kinase (CDK) complexes, the activities of which are regulated by phosphorylation and dephosphorylation and by two families of inhibitory proteins called CDK inhibitors that can arrest the cell cycle in G1 when present at a high enough concentration (reviewed by Sherr, 1994). An example of such an inhibitor is $p27^{kip1}$ (p27), which has recently been knocked-out in mice by three groups, who report similar findings (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). They have shown that the disruption of the function of p27 results in enhanced organ growth, which is due to increased cell proliferation. All organs in these animals contain more cells than normal suggesting that p27 is normally involved in limiting cell proliferation, and thus size, in many organs. P27 is normally degraded in the cell by the ubiquitin–dependent proteasome pathway, and it is possible that the levels of p27, and thereby proliferation controls, depend on controls that regulate this degradation pathway (Pagano et al., 1995).

Some cells are highly motile during development and can be produced in one location while destined for another. Neurons in the vertebrate central nervous system originate as progenitor cells in the ventricular and subventricular zones and differentiate after they migrate

to their final destinations. In neonatal rats, cells from the subventricular zone of the lateral ventricle migrate to the olfactory bulb and differentiate into olfactory neurons (Luskin, 1993). Even in adults, large numbers of these cells migrate into the olfactory bulb, thereby maintaining the size of the organ, despite continuous loss of cells from the bulb. Another example of a migratory cell type is the oligodendrocyte precursor cell. They arise from germinal zones and the migrate throughout the CNS, eventually differentiating into oligodendrocytes (Pringle and Richardson, 1993). Thus, controls on oligodendrocyte precursor cell migration play an important part in determining the final size of the oligodendrocyte population in each region of the CNS.

Programmed cell death (PCD), occurs widely in normal development. It is an active process that occurs by an intracellular mechanism that has been conserved throughout evolution in all animal systems studied. The proteins required for PCD are expressed constitutively in all nucleated mammalian cells (Jacobson et al., 1994; Weil et al., 1996). During the process of PCD, cells undergo a characteristic series of changes, called apoptosis, which includes the condensation of nuclear chromatin and the shrinking of the cell. Early on in the process, the dying cell is phagocytosed by neighbouring cells or macrophages (reviewed by Wyllie et al., 1980).

The function of PCD is to remove unwanted cells, which are usually perfectly healthy. It is involved in the sculpting of structures such as the fingers and toes, and it has recently been shown to be involved in the formation of the pre-amniotic cavity of the mouse embryo (Coucouvanis and Martin, 1995). It removes cells that end up in the wrong location: mis-routed retinal ganglion cells, which send their axons from one eye to the

other rather than to their correct location in the mid-brain, are eliminated in this way (Lam et al., 1982).

The idea that PCD plays a major role in the control of cell numbers during development originated in studies of the nervous system, where the generation of neurons is often followed by large scale cell death (reviewed in Oppenheim, 1991). This massive death is thought to be a mechanism for matching the number of neurons to the number of target cells they innervate. This would facilitate development and speed up evolution.

What determines whether a cell dies or survives during development? For the most part the answer is not known. In some cases cells die because they fail to get the extracellular survival signals they need. Developing neurons, for example, need neurotrophic factors from the targets they innervate; about half get enough to survive, while the rest do not and undergo PCD (Hamburger and Levi-Montalcini, 1949). In the case of sympathetic neurons, the target–derived survival signal has been identified as nerve growth factor (NGF) (reviewed in Barde, 1989).

It has recently been proposed that all cells have a requirement for extracellular survival signals to avoid PCD (Raff, 1992). The survival signal can come from other cells of a different type, or be autocrine, as in lens and cartilage (Ishizaki et al., 1993; Ishizaki et al., 1994).

A second mechanism responsible for some cell deaths in normal development depends on extracellular signals that activate the death programme. A recent study, for example, has implicated bone morphogenetic protein (BMP) as a PCD–inducing signal responsible for the death of cells between the developing digits of the chick hind limb (Zou and Niswander, 1996). Interestingly, the same study reported that

ducks may have webbed feet because they do not express BMP in the areas where interdigit cell death occurs in the chick. In some cases, PCD–inducing signals act systemically rather than locally: the reabsorption of the amphibian tadpole tail during metamorphosis is known to involve PCD that is activated by an increase in the levels of thyroid hormone in the blood (Kerr et al., 1974). In some cases PCD in development seems to be controlled by a combination of these two mechanisms. For example, in the mouse proamniotic cavity, one signal from an outer layer of endoderm cells creates a cavity by promoting PCD in the inner ectodermal cells, whilst a second signal from the basement membrane promotes the survival of the cells that line the cavity by protecting them from the PCD–activating signal (Coucouvanis and Martin, 1995).

The development of the rodent optic nerve

In this study I have used the rodent optic nerve as an example of a simple and well-defined organ to examine the factors that influence the number of glial cells in the nerve. The glial cells of the optic nerve have been widely studied because they are exposed to a limited number of influences since the nerve contains no neuronal cell bodies or synapses. The rodent optic nerve also has the advantages that it can be isolated in its entirety for biochemical analysis and for tissue culture studies, and it can easily be experimentally manipulated in the animal with relatively little disruption of other brain centres.

The optic nerve is an example of an organ where size largely reflects cell number. The adult rat optic nerve is larger than the mouse optic nerve because it contains more cells than the mouse nerve: it contains 600,000 cells (Barres et al., 1992), whereas the adult mouse contains 150,000 cells

(Burne et al., 1996); there are no discernible differences in cell sizes between the two species.

The rodent eye and optic nerve develop from the neural tube like other parts of the CNS. They form from an extrusion of the diencephalon, which forms the optic vesicle. This structure then expands laterally, retaining a connection with the brain via the optic stalk. Next, the optic vesicle undergoes a series of invaginations that transforms it into the double–walled eye cup. The first involutions involve only the eye, but later they continue along the underside of the optic stalk and generate a groove called the optic fissure. RGCs arise in the rat retina at embryonic day (E)11 (Young, 1984) and their axons grow out into the optic stalk by E14. Axonal outgrowth is preceded by a period of neuroepithelial cell death that clears a path for the outgrowing axons in both the rat (Horsburgh and Sefton, 1986) and the mouse (Silver and Sidman, 1980). At this stage the optic stalk is made up entirely of neuroepithelial cells and undifferentiated glial cells. The pathway that pioneering RGCs growth cones follow as they grow back to the brain is entirely cellular. By E15 in the rat, the axons cross the mid-line of the diencephalon, forming the optic chiasm. In the normal rat, all the retinal ganglion cell axons cross to the contralateral side at the chiasm and project to the superior colliculus. This can be demonstrated by injecting horse radish peroxidase into the superior colliculus and following its transport back into the eye (Linden and Perry, 1983). It is presumed that the arrangement of the mouse visual system is the same, although this has not been demonstrated directly.

Neuronal cell death and the loss of axons during the development of the rodent optic nerve

RGCs, like most other neurons in the mammalian nervous system, are produced in greater numbers than will be required in the adult animal. Using retrograde labelling with horse radish peroxidase, there is a 35% reduction in the number of rat RGCs projecting to the brain during the first few postnatal days (Potts et al., 1982). Quantification of the axon numbers in the newborn rat optic nerve compared to the adult rat reveals a 60% loss of axons (Lam et al., 1982). The numbers of axons in the rat optic nerve changes from around 400 at E15 to 240,000 at birth (Sefton and Lam, 1984), and 100,000 at postnatal day (P) 5. An equally large loss of axons is seen in the mouse optic nerve (Williams et al., 1990). These axon losses can be attributed to the death of a large number of RGCs during development and are believed to reflect a competition for target–derived survival factors.

The development of optic nerve glial cells

The macroglial cells of the adult optic nerve are of two main types, astrocytes and oligodendrocytes. The only other glial cells found in the adult optic nerve in any numbers are microglial cells, the macrophages of the CNS. There are also a small number of progenitor cells belonging to the oligodendrocyte lineage, and possibly some belonging to the astrocyte lineage.

The oligodendrocytes and astrocytes arise from different cell lineages. The astrocytes develop from the neuroepithelial cells of the optic stalk, while oligodendrocytes develop from progenitors that migrate into the nerve from germinal centres in the brain (Small et al., 1987). Dividing cells that contain recognisable glial filaments and stain with antibodies

against glial fibrillary acidic protein (GFAP), an astrocyte marker, can be found as early as E16 and their numbers increase for the next few weeks. Oligodendrocyte precursors first migrate into the rat optic nerve at around E15, but the first differentiated, postmitotic oligodendrocytes, which express the oligodendrocyte marker galactocerebroside (GC), are not seen until around birth, and their numbers increase for the next 6 weeks (Miller et al., 1985). Oligodendrocyte progenitor cells are like many other precursor cells in that they divide a limited number of times before they stop dividing and terminally differentiate. The timing of oligodendrocyte progenitor differentiation is thought to depend on an intrinsic clock that somehow measures time, and dictates when the cell stops dividing and differentiates along a default path to become an oligodendrocyte (Raff et al., 1985; Temple and Raff, 1986). There is evidence that the clock has two components: a timing component that measures time and seems to involve an accumulation of p27 (Durand et al., 1997), and an effector component that stops the cell cycle when time is up and depends on lipid soluble signals such as thyroid hormone (Barres et al., 1994).

Astrocytes in the optic nerve perform various functions. They partition axons, form the subpial glia limitans, probably induce endothelial cells to form the blood brain barrier (Janzer and Raff, 1987), and surround nodes of Ranvier, the functional significance of which is unknown (Hildebrand and Waxman, 1984). Astrocytes produce various growth factors that are critical for normal development, including platelet derived growth factor alpha (PDGF-AA, Richardson et al., 1988), insulin-like growth factor (IGF-1), and neurotrophin-3 (NT-3) (reviewed in McMillian et al., 1994). Oligodendrocytes, by contrast, seem to have only one main role, which is to myelinate axons, increasing the rate and efficiency of action potential propagation.

Objectives

It remains a major challenge to understand how the numbers of cells in an organ or organism are determined. The optic nerve is an attractive organ in which to address this question, being one of the simplest and most accessible parts of the central nervous system.

Since the RGC axons are in place before the glial cells are formed, it seems likely that they would play a crucial role in glial cell development. This would appear to be supported by studies on the mutant *ocular retardation* (*or*) mouse, whose RGC axons fail to enter the optic stalk: glial cells fail to develop in these mice and there are no optic nerves (Silver and Robb, 1979).

I initially studied a mouse where the situation in the optic nerve is opposite to that in the *or* nerve: in this mouse the optic nerve contains more axons than normal. This transgenic mouse, which expresses the human *bcl-2* gene under the control of the neuron-specific-enolase promoter (Martinou et al., 1994), presented the ideal opportunity to study the role of RGC axons in the control of glial cell numbers during optic nerve development. Once I had established that the optic nerves from these animals contained not only more axons, but also more astrocytes and oligodendrocytes, I examined how the numbers of each of these cell types were controlled. In chapter 2 I discuss my results on the control of oligodendrocyte numbers, while in chapter 3 I discuss my results on the control of astrocyte numbers.

The *bcl-2* proto-oncogene, first isolated in human B cell lymphomas, has the ability to inhibit PCD. In my studies of the *bcl-2* transgenic mouse, I observed that the over-expression of Bcl-2 did not prevent Wallerian degeneration in RGC axons, although it did protect the

RGC cell bodies from dying when their axons were cut. This raised the general question of whether Wallerian degeneration involves a death programme that is similar to PCD. I discuss some preliminary experiments that I have done to address this question in chapter 4.

CHAPTER 2

THE CONTROL OF OLIGODENDROCYTE NUMBERS

Introduction

To study the role of RGC axons in the regulation of rodent optic nerve glial cell numbers I first examined the axons themselves.

It has been shown there is a massive amount of cell death in the rat RGC population during the first postnatal week (Lam et al., 1982), and this results in an equally large loss of axons from the optic nerve (Potts et al., 1982; Sefton and Lam, 1984). The death of RGCs during development shows the morphological characteristics of apoptosis, indicating that they die by PCD. PCD can be delayed in many different types of cells, including neurons, by overexpressing genes such as the *bcl-2* proto-oncogene, but the molecular basis of the protective effect of the Bcl-2 protein is unknown. It has been possible to inhibit the molecular pathway that leads to PCD in neurons either by direct micro-injection of a DNA expression vector consisting of *bcl-2* and a neuron-specific-enolase (NSE) promoter (Garcia et al., 1992) or by the generation of transgenic animals using similar constructs. The model system that I have used in this study is the optic nerve of a transgenic mouse that expresses a human *bcl-2* transgene controlled by the NSE promoter (Dubois-Dauphin et al., 1994). This animal is apparently normal, apart from having a slightly larger brain as a result of increased numbers of various types of neuron, including RGCs, due to a decrease in the normal cell death of these cells (Martinou et al., 1994).

The initial aim of this study was to quantify the increase in the number of RGC axons found in adult and newborn transgenic optic nerves compared to wild-type, as well as to establish the normal time course of axon loss from wild-type optic nerves. To study the effect of axon numbers on the glial cell population it was also important to establish whether the individual axons present in transgenic nerves were different in calibre or

morphology from those found in wild-type mice. In this study I provide evidence that the optic nerves of adult transgenic mice have twice the diameter and contain 80% more axons than wild-type nerves. I also show that the size of individual axons in transgenic optic nerves is the same as in wild-type mice, and a normal proportion of them are myelinated.

Previous studies have revealed several ways that the number of oligodendrocytes in the mammalian optic nerve can be controlled. The oligodendrocytes found in the optic nerve develop from precursor cells that migrate into the nerve from the brain (Small et al., 1987). As oligodendrocytes are normally postmitotic (Gard and Pfeiffer, 1989; Hardy and Reynolds, 1991), while their precursor cells proliferate extensively (Temple and Raff, 1986), their final number depends on how many precursors migrate into the nerve and how many times each divides before differentiating. Normal cell death also plays a major role in controlling oligodendrocyte numbers, as at least half of the oligodendrocytes in the nerve undergo PCD soon after they develop (Barres et al., 1992).

Optic nerve axons influence oligodendrocyte numbers in several ways: they promote precursor cell proliferation and survival (Barres and Raff, 1993), and they promote oligodendrocyte survival (Barres et al., 1993b). Barres et al. (1992) proposed that normal oligodendrocyte death might help match the number of oligodendrocytes to the number of axons requiring myelination, just as normal neuronal death is thought to help match the number of neurons to the number of target cells the neurons innervate (Cowan et al., 1984; Oppenheim, 1991): it was suggested that newly formed oligodendrocytes require a signal(s) from axons to avoid PCD, and only about half manage to receive enough signal to survive.

To test the idea that oligodendrocyte numbers are matched to axon numbers by PCD, in a competition for limiting amounts of axon-dependent survival signals I studied oligodendrocyte numbers in the *bcl-2* transgenic mice that have an increased number of axons in their optic nerve. I discovered that the adult nerves from these animals contain not only about twice the number of axons but also glial cells, including astrocytes, microglial cells and oligodendrocytes. The increased number of oligodendrocytes apparently results predominantly from a decrease in oligodendrocyte death. I also show that the *bcl-2* transgene is unexpectedly expressed in oligodendrocytes, but this is unlikely to be responsible for the increase in glial cell numbers.

Results

The heterozygous transgenic mouse line 73 expresses a human *bcl-2* gene in many CNS neurons, including RGCs, and, as a result, the number of neurons and the sizes of the brain and optic nerves are increased compared to wild-type animals. In most other respects the transgenic mice appear normal, apart from a problem of sterility in the female mice homozygous for the transgene. I crossed male transgenic mice with female C57BL/6J mice, and stained retinal cells from each member of the litters with an anti-human Bcl-2 antibody to identify the transgenic and nontransgenic (wild-type) progeny. As expected, I found that about 50% of the progeny expressed the *bcl-2* transgene. No cells were stained by the antibody in C57BL/6J retinae, attesting to its specificity.

Effect of Bcl-2 on optic nerve axons

To examine the optic nerve axons I prepared photomontages of cross-sections from both optic nerves from three adult transgenic mice and three adult wild-type siblings, as described in the Materials and Methods. As shown in Table 2.1, the average surface area of the optic nerves in transgenic mice was about twice that of nerves in wild-type littermates, and the number of axons was about 80% greater. The number of axons in wild-type mice is in good agreement with those reported by Williams et al. (1990) for the same strain of mice. The average diameter and area of individual axons were about the same in transgenic and wild-type optic nerves. I also found that the majority of axons were myelinated, both in wild-type and transgenic nerves.

The average length of the optic nerves measured in six month old animals was also not significantly different in the two types of mice — 4.7 ± 0.3 mm in transgenic mice versus 4.9 ± 0.2 mm in wild-type mice (mean ± standard error of the mean, (s.e.m.), n = 10 and 6, respectively).

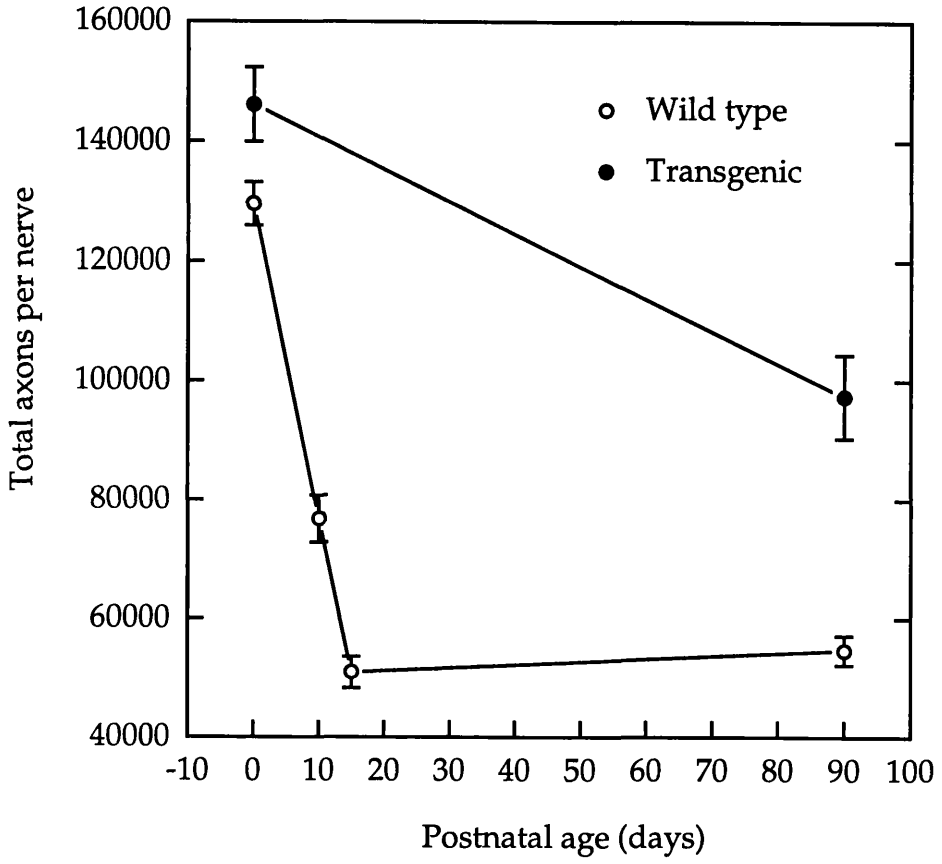
As shown in Figure 2.1, the average number of axons present at birth was a little greater in transgenic optic nerves than in wild-type nerves, but this difference was not statistically significant. As expected, in both cases the numbers of axons decreased postnatally, presumably reflecting the normal death of RGCs, but the decrease was much greater in wild-type nerves than in transgenic nerves.

Table 2.1

	Wild type	Transgenic
Axon number	54,666 ± 2,471	97,400 ± 7,124
Nerve area (cross section)	103 ± 2 x10^3 μm^2	200 ± 20 x10^3 μm^2
Axon area (cross section)	0.34 ± 0.008 μm^2	0.35 ± 0.007 μm^2
Myelinated axons (%)	96.3 ± 0.96	96.1 ± 0.88

Axons in adult optic nerves. Counts and measurements were made on electron micrographs of transverse sections of optic nerves from 3-month-old wild-type and transgenic mice (line 73), as described in the Materials and Methods. The nerve area and axon counts were made on one section from each of a pair of optic nerves taken from 3 individual animals, giving a total of 6 sections per group. About 1,400 individual axons areas were measured per group. About 1,200 axons taken from 3 different animals per group were assessed for myelination; an axon was counted as myelinated if it had at least one complete wrap of myelin. Results are presented as the mean ± s.e.m.

Figure 2.1



Axon numbers in optic nerves of wild-type and transgenic (line 73) mice from various ages. The results are expressed as mean ± s.e.m. of counts from 1 nerve from 3 animals at each age, except for adults, where both nerves were counted from 3 animals. The differences between the transgenic and wild-type nerves at P0 are not statistically different when analysed by Student's t-test ($p>0.05$).

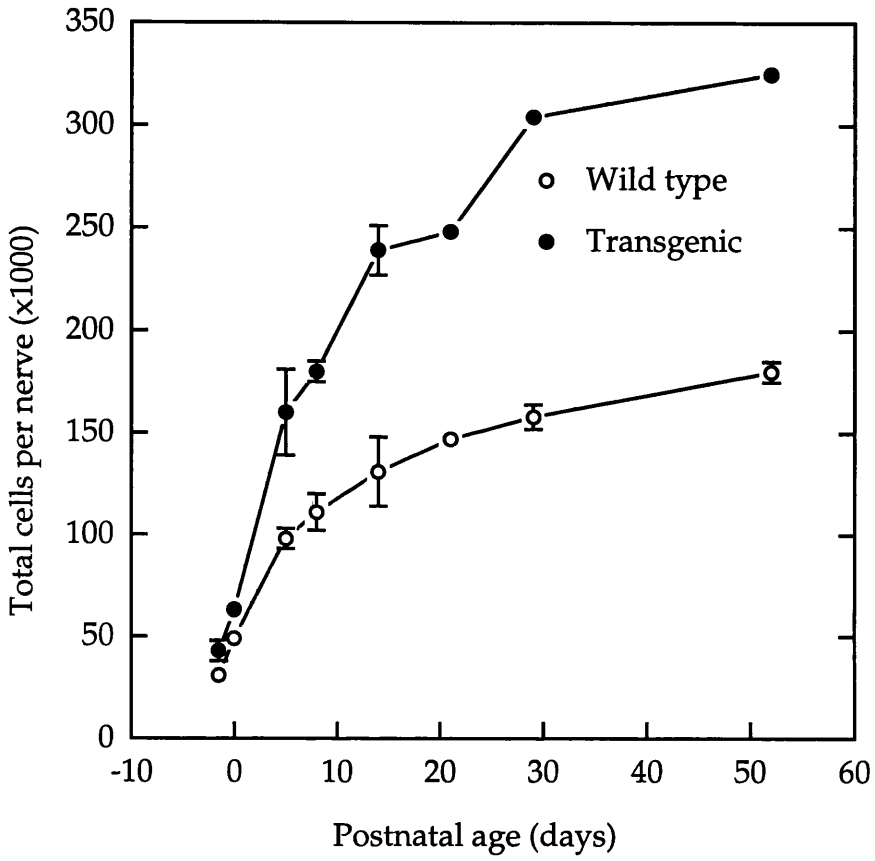
Optic nerve glial cells

To compare the total numbers of glial cells in the optic nerves of transgenic and wild-type mice, I measured the total amount of DNA in the nerves and divided the values by the amount of DNA per mouse diploid cell. As shown in Figure 2.2, there was no significant difference in the total number of cells at E17.5 between transgenic and wild-type littermates; there was a small but significant difference at birth, and this difference steadily increased until about P30, when there were almost twice as many cells in transgenic nerves than in wild-type nerves, and this difference persisted at P50.

To determine the number of each glial cell type, I examined three pairs of nerves from three month old transgenic mice and three pairs from their wild-type littermates by electron microscopy. I examined two transverse sections from the central third of the nerve, at least 100 μm apart. I only counted cells within the boundary of the glial limiting membrane, and I did not count endothelial cells. Mature oligodendrocytes and astrocytes were easy to identify (Peters et al., 1991): oligodendrocytes had a dark cytoplasm, dark nucleoplasm with clumped chromatin, many free and membrane bound ribosomes, abundant microtubules, a well-defined Golgi apparatus, and an endoplasmic reticulum often arranged in stacks; astrocytes, by contrast, had a lighter cytoplasm and nucleoplasm, frequently an irregularly shaped nucleus, and numerous intermediate filaments, which were often in bundles. Microglial cells were more difficult to identify with certainty, but typical ones contained clumped chromatin, long, winding endoplasmic reticulum, and often phagocytosed debris. A previous study defined the identifying characteristics of oligodendrocyte precursor cells (Fulton et al., 1992): they were small, with little cytoplasm, no intermediate filaments, clumped chromatin at the

periphery of the nucleus, and a density intermediate between oligodendrocytes and astrocytes. I was unable to classify about 3% of the cells. As shown in Table 2.2, there were about twice as many oligodendrocytes, astrocytes, microglial cells and oligodendrocyte precursor cells in nerves from *bcl-2* transgenic mice compared to nerves from their wild-type littermates. There were no obvious differences in glial cell size in the two types of nerves, consistent with the finding that both total cell number and nerve volume were about doubled.

Figure 2.2



The increase in total cells in the optic nerves of wild-type and transgenic (line 73) littermates during development. Cell numbers were determined by measuring the total DNA in the nerve and dividing by the amount of DNA per cell. Results are expressed as mean ± s.e.m. of single nerves from 3-10 animals per group.

Table 2.2

	Wild type	Transgenic
Oligodendrocytes	91 ± 4	204 ± 4
Astrocytes	50 ± 4	116 ± 9
Oligodendrocyte precursors	6 ± 4	11 ± 1
Microglia	3 ± 2	7 ± 1

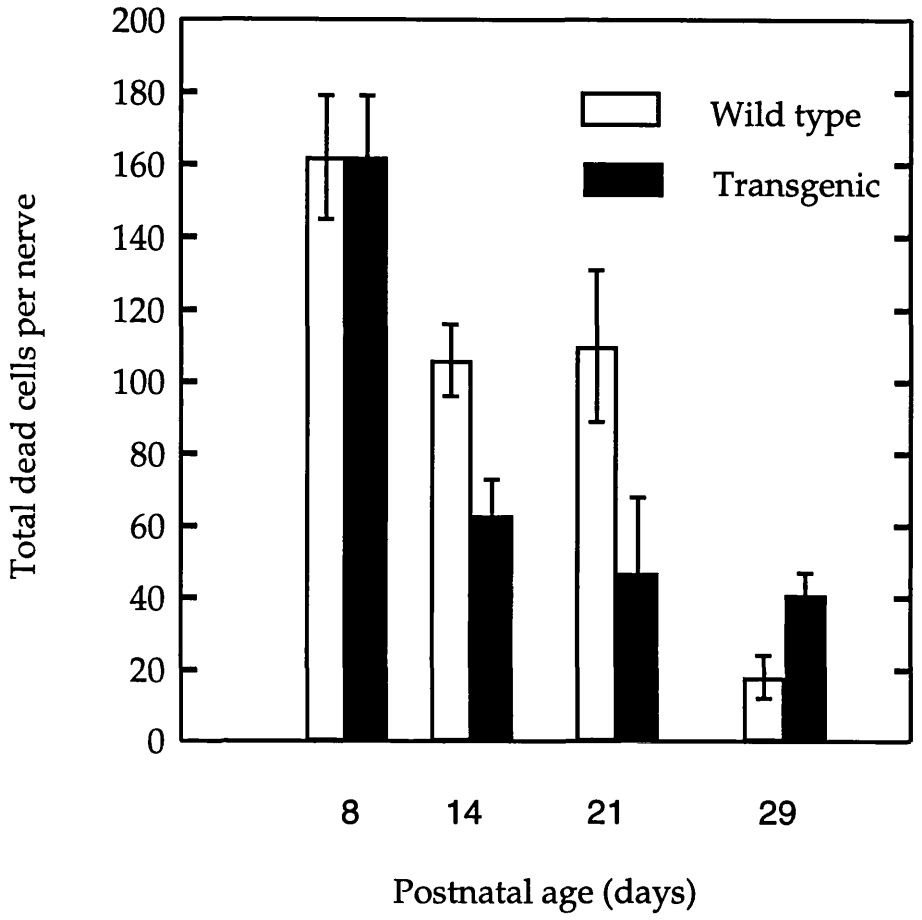
Identity of glial cells in electron micrographs of adult optic nerves. Cell counts were made on electron micrographs of transverse sections of optic nerves from 3-month-old adult transgenic (line 73) and wild-type mice. Two sections were taken, at least 100 μm apart, from the central third of both nerves from 3 animals in each group. The results are expressed as the meam ± s.e.m. of 12 sections per group.

Cell death

To compare the amount of glial cell death in transgenic and wild-type optic nerves, I stained serial longitudinal frozen sections of nerves from mice of different ages with propidium iodide (PI) to identify normal and pyknotic nuclei and counted the total number of pyknotic nuclei in each nerve. The total numbers of dead cells were significantly lower in transgenic nerves than in wild-type nerves at P14 and P21 but were not significantly different at P8 or P29 (Figure 2.3a). Moreover, the proportion of dead cells was also lower in the transgenic nerves at P7, P14 and P21 than in wild-type nerves at these ages (Figure 2.3b).

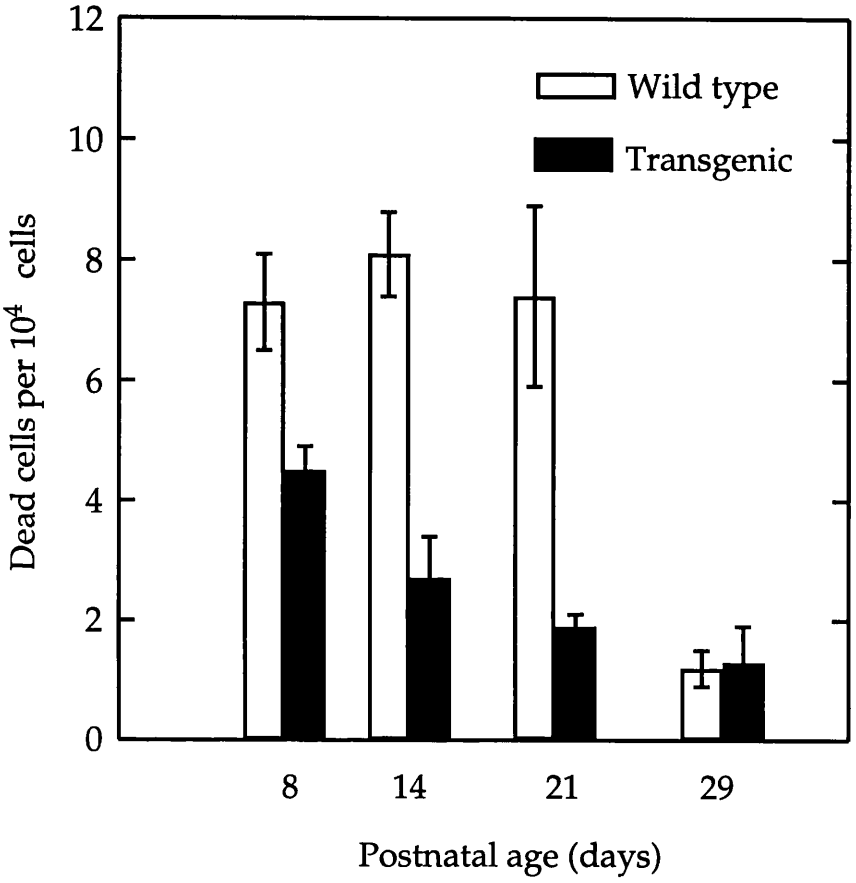
It has been shown that all cell death in the postnatal rat optic nerve is confined to the oligodendrocyte cell lineage (Barres et al., 1992). To help determine if this is also the case in transgenic and wild-type mouse optic nerves, I double-labelled frozen sections of these nerves with PI to identify dead cells and anti-GFAP antibodies to identify astrocytes. None of the dead cells were $GFAP^+$, as shown previously in the rat optic nerve (Barres et al., 1992). Thus it is likely that most of the dead cells in the postnatal mouse optic nerve belong to the oligodendrocyte lineage, and at least some of the increase in oligodendrocyte number in the transgenic optic nerve reflects a decrease in cell death.

Figure 2.3a



Cell death in the optic nerves of wild-type and transgenic (line 73) mice of various ages. All of the dead cells were counted in PI-stained, serial frozen sections of single nerves from 3-animals at each age. The results are expressed as mean ± s.e.m.

Figure 2.3b

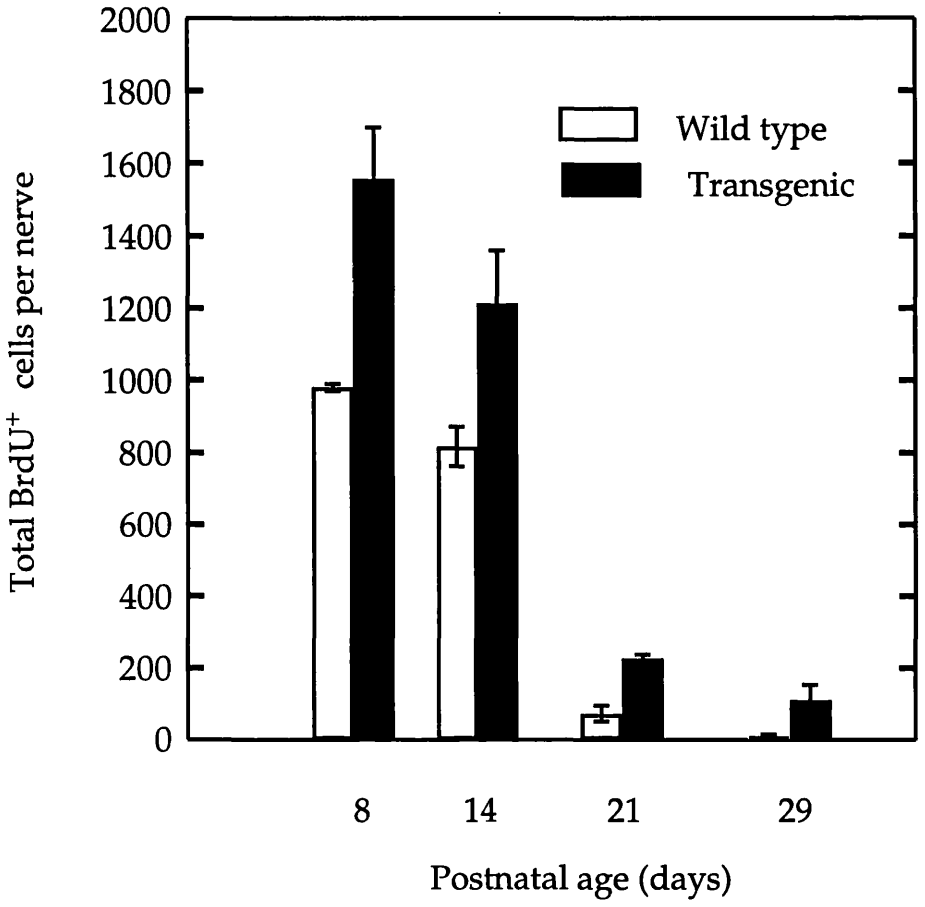


Proportion of dead cells in the optic nerves of wild-type and transgenic (line 73) mice of various ages. All of the dead cells were counted in PI-stained, serial frozen sections of single nerves from 3-animals at each age and the result divided by the total number of cells at each age from Figure 2.2. The results are expressed as mean ± s.e.m.

Cell proliferation

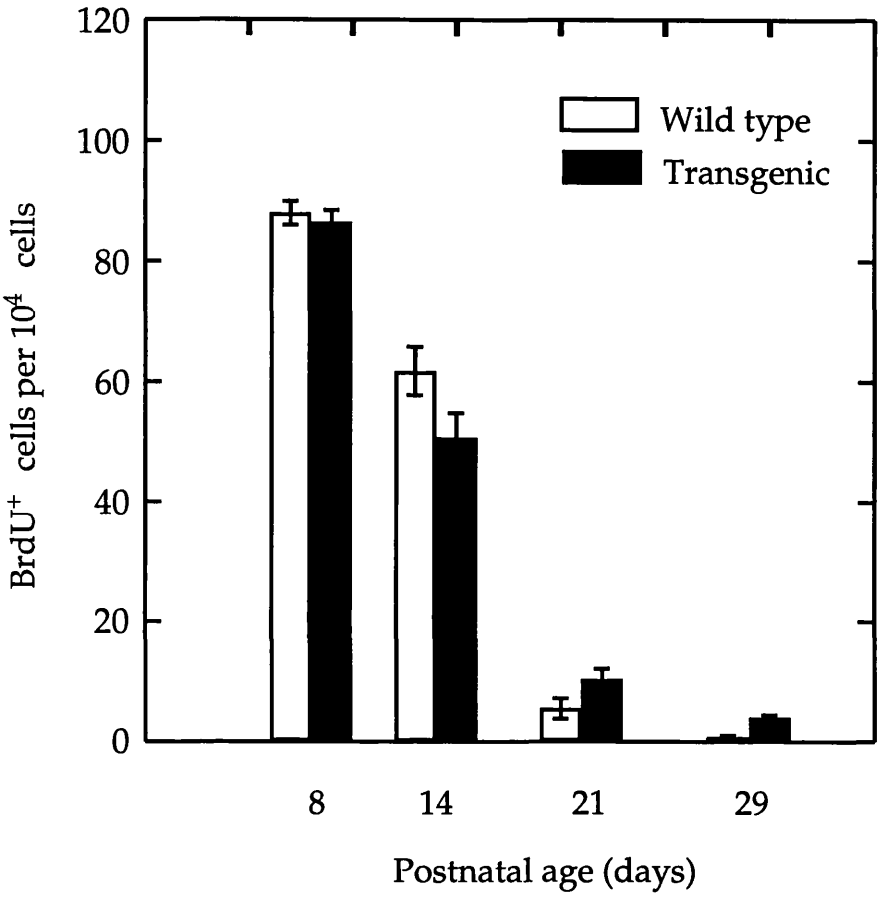
To compare the numbers of dividing cells in the optic nerves of transgenic and wild-type mice, I injected bromodeoxyuridine (BrdU) into the peritoneum of P8, P14, P21, and P29 mice. One hour later the mice were killed and serial frozen sections of their optic nerves were stained with anti-BrdU antibody, and all the $BrdU^+$ cells in each nerve were counted. As shown in Figure 2.4a, the numbers of $BrdU^+$ cells were increased in transgenic compared to wild-type nerves at all four ages. However, if the data is presented as the proportion of all cells that were dividing at any one time a different picture emerges; as shown in Figure 2.4b, there were no significant differences in the proportion of cells that had incorpotrated BrdU at any age except P29. As most of the increase in astrocyte numbers in rodent optic nerve occurs in the first postnatal week while most of the increase in oligodendrocyte numbers occurs after this time (Skoff, 1990; Barres et al., 1992), some of the BrdU-labelled cells at P8 in our experiments would be expected to be astrocytes, while most of the labelled cells at later ages would be expected to be oligodendrocyte precursor cells.

Figure 2.4a



Cell proliferation in the optic nerves of wild-type and transgenic (line 73) optic nerves at various ages. Mice were injected twice with BrdU 2 hours apart. Two hours after the final injection, serial frozen sections of the optic nerve were cut and stained with anti-BrdU antibody. All of the $BrdU^+$ cells were counted in single nerves from 3-4 animals at each age. The results are expressed as means ± s.e.m.

Figure 2.4b



Proportion of proliferating cells in the optic nerves of wild-type and transgenic (line 73) optic nerves at various ages. Mice were injected twice with BrdU 2 hours apart. Two hours after the final injection, serial frozen sections of the optic nerve were cut and stained with anti-BrdU antibody. All of the $BrdU^+$ cells were counted in single nerves from 3-4 animals at each age and the result divided by the total number of cell at each age from Figure 2.2. The results are expressed as means ± s.e.m.

Bcl-2 transgene expression in optic nerve glial cells

In the CNS, NSE is thought to be expressed only in neurons and neuroendocrine cells (Schmechel and Marangos, 1983). This is also reported to be the case for *lac Z* transgenes controlled by an NSE promoter, where transgene expression was detected by β-galactosidase staining (Forss-Petter et al., 1990). To test whether glial cells in the optic nerve of the *bcl-2* transgenic mice express the transgene, I stained cells isolated from the optic nerve with an anti-human Bcl-2 monoclonal antibody, together with cell-type-specific antibodies to identify the glial cells. To my surprise, many optic nerve cells isolated from transgenic mice from line 73 expressed the human Bcl-2 protein, including all the myelin basic protein (MBP)$^+$ oligodendrocytes and almost all the GFAP$^+$ astrocytes (Table 2.3); none of the cells isolated from wild-type littermates did so. Microglial cells, identified by their Fc receptors (Raff et al., 1979), and GFAP$^-$ flat cells (presumptive meningeal cells) in cultures prepared from transgenic nerves did not express detectable human Bcl-2, although some microglial cells were weakly stained (not shown). When frozen sections of adult optic nerves were stained with anti-human Bcl-2 antibody, most of the glial cells in transgenic nerves were weakly labelled, as apparently were the axons, whereas there was no staining of wild-type nerves (not shown).

Table 2.3

Mouse line	Age	% Human Bcl-2$^+$ oligodendrocytes	% Human Bcl-2$^+$ astrocytes
Line 73	P7	100	97 ± 4
Line 73	P14	100	99 ± 2
Line 71	P7	28 ± 4	17 ± 3
Line 71	P14	30 ± 2	10 ± 1

Expression of human Bcl-2 protein in optic nerve glial cells. Cells dissociated from optic nerve were allowed to adhere to a coverslip for 20 minutes and then double-stained for human Bcl-2 and for cell-type, as described in Materials and Methods. At least 160 cells were assessed on each coverslip. The results are expressed as means ± s.e.m. of 3 separate experiments.

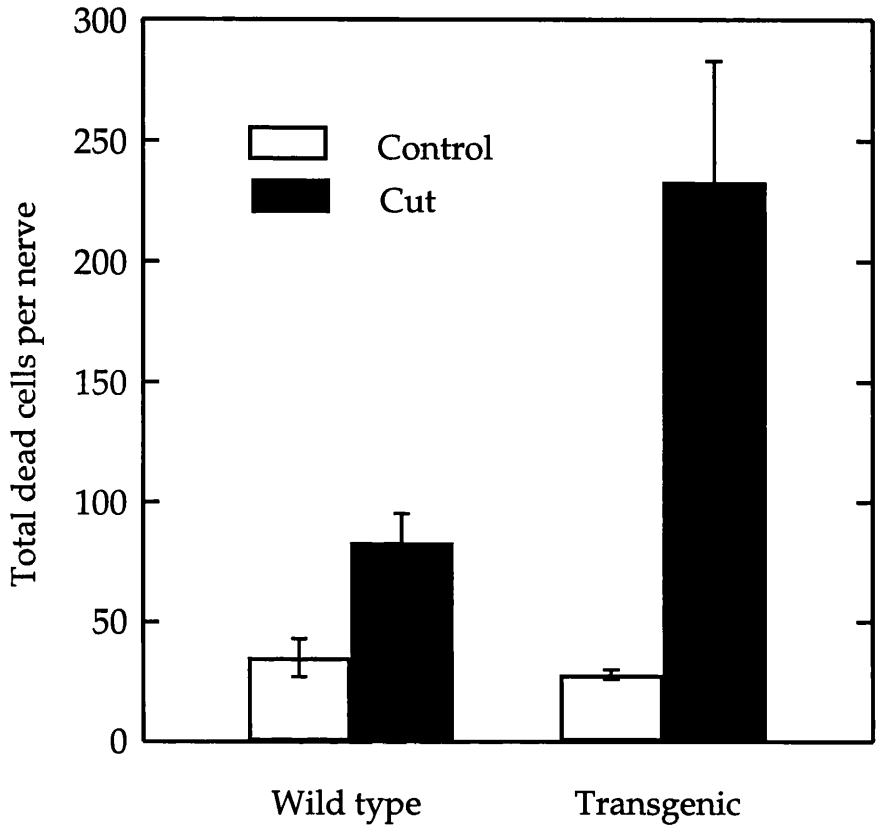
Effect of transgene expression on oligodendrocyte survival in culture

To determine whether the expression of the *bcl-2* transgene could protect optic nerve oligodendrocytes from PCD when cultured without added survival signals I dissociated cells from P8 optic nerve and cultured them at low density in Dulbecco's Minimal Eagles Medium (DMEM). Cells derived from wild-type and transgenic animals were cultured together so that their survival could be directly compared in the same environment. After 1 day and 3 days cultures were stained for GC and human Bcl-2. After 1 day there were many viable GC^+ oligodendrocytes, and 78 ± 1% of them were Bcl-2^+ (mean ± s.e.m. of 3 cultures), consistent with the fact that 4/6 of the mice used to prepare the optic nerve cells expressed the *bcl-2* transgene. After 3 days, the great majority of GC^+ cells were apoptotic, but of those that were alive 82 ± 3% were Bcl-2^+ (mean ± s.e.m. of 3 cultures). Thus the expression of the transgene failed to protect oligodendrocytes from PCD in culture in the absence of added survival factors.

Effect of optic nerve transection

Normally, when the developing optic nerve is cut behind the eye, RGCs undergo PCD (Snider et al., 1993); their disconnected axons rapidly degenerate, and oligodendrocytes in the nerve die (David et al., 1984) by PCD (Barres et al., 1993b). To determine if the expression of the *bcl-2* transgene in oligodendrocytes protects the cells from this fate following transection, I cut the optic nerve of P18 transgenic and wild-type mice and examined the nerves after 4 days. I stained frozen sections with the RT97 monoclonal anti-neurofilament antibody (Wood and Anderton, 1981) to visualise the axons and with PI to identify pyknotic glial cell nuclei (described in chapter 4). The number of dead glial cells was at least 3-fold greater in the cut transgenic nerve than in the cut wild-type nerve (Figure 2.5). None of the dead cells were $GFAP^+$ (not shown), consistent with the possibility that they were oligodendrocytes, as shown previously in transected rat optic nerves. Thus *bcl-2* expression in oligodendrocytes does not appear to protect them from PCD following optic nerve transection.

Figure 2.5

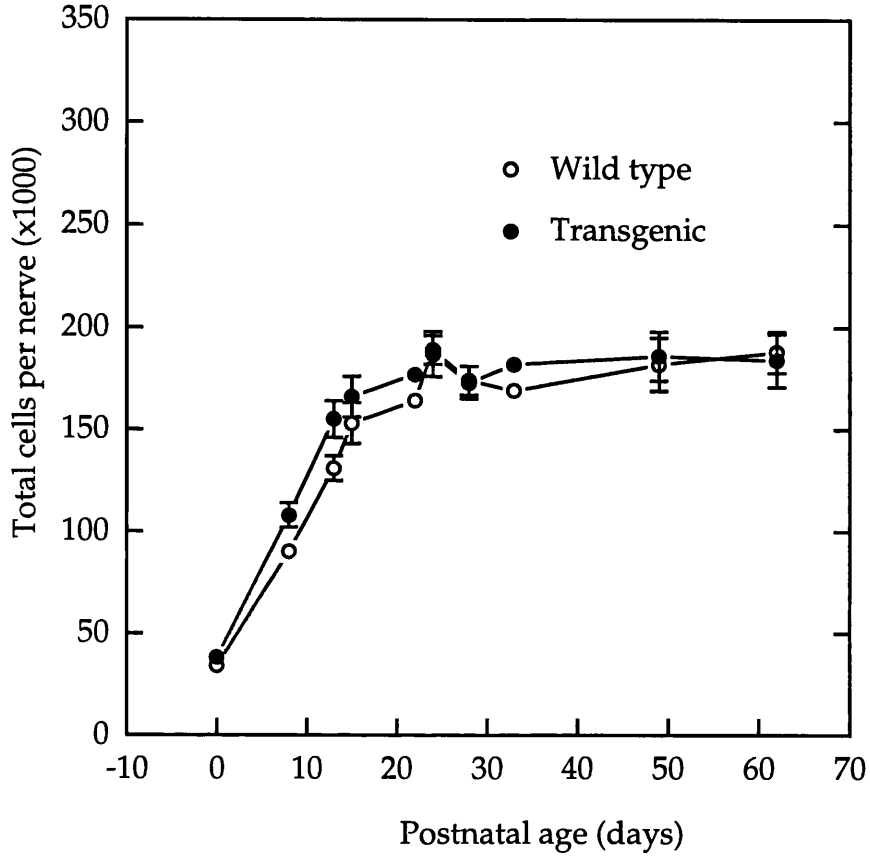


The effect of nerve transection on the numbers of dead cells in wild-type and transgenic (line 73) nerves 4 days after transection. The experiments were carried out and analysed as in Figure 2.3a. Cells in 3 nerves from each group were counted, and expressed as mean ± s.e.m.

A different bcl-2 transgenic mouse line

To help determine whether the increase in astrocytes and oligodendrocytes seen in the *bcl-2* transgenic mouse line 73 was directly related to the expression of the transgene in the glial cells themselves (rather than indirectly related to the increased number of axons), I studied a second transgenic mouse line expressing the same transgene from the same promoter. In this mouse line, however, the transgene is not expressed in RGCs and the optic nerves are normal in size (Martinou et al., 1994). As shown in Figure 2.6, the number of glial cells in the optic nerves of these mice was not significantly different from their wild-type littermates at various ages. I also found that there was no significant difference in the total number of dead cells seen at P14, an age when I found a greatly reduced number of dead cells in optic nerves from line 73 animals compared to wild-type littermates: the total number of PI-stained pyknotic nuclei in P14 transgenic line 71 nerves was 97 ± 13, compared to 90 ± 9 in wild-type nerves, (mean ± s.e.m. of 3 nerves in both cases). Nonetheless, many of the glial cells, including both astrocytes and oligodendrocytes, expressed the human Bcl-2 protein (Table 2.3). Although the proportion of glial cells that expressed the transgene was less in line 71, the amount of Bcl-2 protein expressed in the positive cells was at least as great, if not greater, when quantified in a confocal fluorescence microscope: in human Bcl-2^+ glial cells from the 73 line the mean number of arbitrary units was 41 ± 4, while in human Bcl-2^+ glial cells from the 71 line the mean number of arbitrary units was 50.3 ± 10 (n = 25 cells in both cases). In both lines, there were no significant differences in the proportions of oligodendrocytes that expressed human Bcl-2 at P7 and P14 (Table 2.3).

Figure 2.6



The increase in total cells in the optic nerves of wild-type and transgenic (line 71) littermates during development. The experiments were carried out and analysed as in Figure 2.2. Results are expressed as mean ± s.e.m. of single nerves from 3-8 animals per group.

Disussion

The number and calibre of axons in transgenic optic nerves

I found that optic nerves from the *bcl-2* transgenic mouse line 73 (Martinou et al., 1994) contained 80% more axons than wild-type nerves. This was the result of a decrease in the normal postnatal loss of RGC axons: in wild-type optic nerves 60% of the axons are lost between birth and P14, whereas only 35% are lost in the transgenic nerves.

The expression of Bcl-2 varies in different neuronal populations in this mouse and whereas some neuronal populations are increased compared to wild-type animals, others are not. All RGCs present in the transgenic retina expressed human *bcl-2* transgene about equally, as assessed by immunofluoresence staining of newborn retinal sections (not shown). Bcl-2 expression did not therefore guarantee survival, since not all Bcl-2^+ RGCs present at birth survived to adulthood. What determines how many RGC neurons survive in this mouse? One factor may be the size of the target field. Mouse RGC axons project to the lateral geniculate nucleus (LGN) via the superior colliculus (SC, Linden and Perry, 1983). During normal mouse development about 30% of the dorsal LGN neurons die (Heumann and Rabinowicz, 1980). Since human Bcl-2 is expressed in LGN neurons in line 73 mice (Martinou et al., 1994), there may be a greater number of LGN neurons in the transgenic mouse due to a suppression of normal cell deaths, and the same may be true of the SC. A greater number of target neurons could supply a greater amount of trophic survival factor than in wild-type animals and thereby support a greater number of RGCs. The size of the LGN and SC in this transgenic mouse has yet to be determined.

I found that there was no difference in the calibre of individual axons in transgenic and wild-type optic nerves. The size of the superior colliculus could also be important in this context. It has been shown in the peripheral nervous system that an increase or decrease in target size will cause a corresponding change in the calibre of innervating axons (Voyvodic, 1989). My results would predict a proportional increase in the size of the target field in the transgenic mouse if the same rules apply in the CNS.

Oligodendrocytes

The majority of axons in the *bcl-2* transgenic optic nerves are myelinated (Table 2.1), suggesting that the oligodendrocyte population has either increased in absolute numbers or each oligodendrocyte had increased the number of axons it myelinated. I find that the former is the case (Table 2.2). Using intracellular dye injection and confocal microscopy, it has been shown that mature oligodendrocytes have 15-20 longitudinal processes (Butt et al., 1994). The spatial distribution of axons myelinated by an oligodendrocyte is tightly constrained to a radial field with a diameter of approximately 30 μm. It would be interesting to establish whether these restrictions apply when oligodendrocytes are presented with a greater number of axons than normal as in the transgenic mouse, but this has not been determined.

It has been shown in the developing rat optic nerve that axons are required for oligodendrocyte survival (Barres et al., 1993b), and that 50% of the oligodendrocytes produced in the rat optic nerve normally die within 2-3 days after they develop (Barres et al., 1992). It was suggested that this massive death reflects a competition for axon-dependent survival signals (Barres et al., 1992). Forcing newly-formed oligodendrocytes to compete for

limiting amounts of axon-dependent signals may help match the final number of oligodendrocytes to the number of axons requiring myelination (Barres et al., 1992). A prediction of this hypothesis is that an increase in axon numbers should decrease oligodendrocyte death.

My findings are consistent with this prediction. The number of oligodendrocytes in the transgenic optic nerve is twice that in the wild-type optic nerve, and much of the increase seems to result from a decrease in normal oligodendrocyte death. During the first three postnatal weeks, when oligodendrocyte death would be expected to be maximal (Barres et al., 1992), the proportion of dead cells in transgenic optic nerves is significantly less than in nontransgenic nerves. It was shown that 90% of the dead cells in the postnatal rat optic nerve are oligodendrocytes and 10% are oligodendrocyte precursor cells (Barres et al., 1993b). Although I have not directly identified the dead cells in the mouse optic nerve, it seems likely that most of them belong to the oligodendrocyte lineage, as none of them express the astrocyte marker GFAP.

Three lines of evidence suggest that the decrease in normal oligodendrocyte death that occurs in the transgenic optic nerve is mainly secondary to the increase in axons, astrocytes, or both, rather than directly related to the expression of the *bcl-2* transgene in oligodendrocytes. First, transection of the trangenic nerve at P18 leads to increased glial cell death, which is proportionally even greater than that seen following transection of wild-type nerves. As none of the dead cells in the cut nerve are $GFAP^+$, and, in the rat, the increased cell death in transected optic nerves is confined to oligodendrocytes (Barres et al., 1993b), it seems likely that the increased cell death in the cut mouse optic nerve is also mainly oligodendrocyte death. Thus the expression of the *bcl-2* transgene in oligodendrocytes seems not to protect them from PCD when they are

deprived of axonal signals *in vivo*. Second, when deprived of survival signals *in vitro*, transgenic oligodendrocytes do not survive better than wild-type oligodendrocytes. Third, a different *bcl-2* transgenic mouse line (line 71), in which the transgene is not expressed in RGCs (Martinou et al., 1994) but is expressed in 30% of the oligodendrocytes and 15% of the astrocytes in the optic nerve, has a normal number of optic nerve glial cells (assessed at various ages) and a normal number of dead cells (assessed at P14). Moreover, the proportion of oligodendrocytes expressing the transgene in this line does not increase from P8 to P14, suggesting that expression of the transgene does not confer a selective survival advantage on these oligodendrocytes. As the amount of human Bcl-2 protein expressed in individual glial cells is at least as great in line 71 as in line 73, it seems unlikely that the expression of the *bcl-2* transgene in oligodendrocytes in line 73 protects these cells from normal cell death.

The assessment of cell proliferation of oligodendrocyte precursor cells in the developing optic nerves is complicated by the fact that unlike cell death, where only oligodendrocyte lineage cells are involved both astrocyte and oligodendrocyte lineage cells proliferate in the developing nerve. An investigation of the proportion of astrocytes that incorporated BrdU at P5 revealed that more were in S-phase in transgenic nerves at this age when compared to wild-type (Table 3.1). This means that during the first postnatal week more astrocytes were being generated in the transgenic nerves. As most of the increase in astrocyte numbers in rodent optic nerves occurs earlier than the increase in oligodendrocyte numbers (Barres et al., 1992; Skoff, 1990), many of the BrdU-labelled cells at later ages would be expected to be oligodendrocyte precursor cells. If one considers the proportion of all cells that incorporated BrdU in transgenic and wild-type nerves at P8 and P14 no differences were revealed (Figure 2.4b). The

implication of this observation is that, unlike for astrocytes at P5, there was no increase in the proportion of oligodendrocyte lineage cells dividing at any of the times examined.

Axons normally promote the proliferation and survival of oligodendrocyte precursor cells in the developing optic nerve (Barres and Raff, 1993), but my findings suggest that the rate at which these cells proliferate is not increased by the presence of a greater number of axons. However, there are greater numbers of oligodendrocyte progenitor cells in adult transgenic optic nerves than in adult wild-type nerves (Table 2.2), raising the possibility that the normal death of oligodendrocyte precursor cells (Barres et al., 1992) is decreased in the transgenic optic nerve. Consistent with this possibility, it was previously shown that transection of the neonatal rat optic nerve results in a decrease in the numbers of oligodendrocyte and their precursors (David et al., 1984), without a change in the proportions of the cells that incorporated tritiated thymidine. These findings and mine suggest that axons promote the survival of oligodendrocyte precursor cells in the developing optic nerve.

Microglia

Microglia also seem to be increased about 2-fold in the transgenic compared to wild-type optic nerves. The mechanism(s) underlying this apparent increase is unknown as little is known about how the number of microglial cells in the nerve is normally controlled. These cells migrate into the developing nerve via the blood vessels and pia-arachnoid, which are presumably increased in the transgenic nerves; this increase by itself might be enough to account for the increase in microglial cells. As cultured astrocytes produce factors that are mitogenic for cultured microglial cells (Shafit-Zagardo et al., 1993; Lee et al., 1994), it is also

possible that the increase in microglia is secondary to the increase in astrocytes.

Materials and Methods

Chemicals

All chemicals were from Sigma, unless otherwise stated.

Animals

Transgenic mice expressing human bcl-2 under the control of the NSE promoter were kindly provided by Dr. J-C. Martinou (Martinou et al., 1994). The mouse line 73 that was used in this study were descendants of one founder. Transgenic male mice were mated with normal C57BL/6J females. The day of birth was designated as P0. Offspring were screened for the transgene by immunofluorescence staining with a mouse monoclonal anti-human Bcl-2 antibody (Pezzella et al., 1990), using either frozen sections of retinae or freshly dissociated retinal cells, as described below.

Staining for transgene expression

Frozen sections of retina or optic nerves were postfixed in methanol at - 20°C for 10 minutes, washed, and incubated over night at 4°C with a monoclonal anti-human Bcl-2 antibody (hybridoma supernatant, diluted 1:1 in Tris buffered saline, containing 1% bovine serum albumin (BSA) and 10 mM L-lysine, TBLS). The antibody was visualised with a fluorescein-coupled goat anti-mouse immunoglobulin (G anti-MIg-Fl, Jackson Laboratories, diluted 1:100 in TBLS). Where dissociated retinal cells were tested, retinae were dissociated without enzymes and the cells allowed to adhere to poly-D-lysine (PDL, 0.01 mg/ml)-coated cover-slips for 20 minutes at room temperature in Minimal Eagles Medium (MEM, Gibco BRL), containing 25 mM Hepes buffer and 1% BSA. Cells were fixed with methanol and stained as described above, except that the incubations were for 30 minutes at room temperature. All slides and cover-slips were

washed in buffer, mounted in Citifluor (Citifluor UKC, UK), and examined in a Zeiss Axioskop fluorescence microscope.

Frozen sections

Mice were anaesthetised with a lethal injection of Sagatal. They were then perfused through the heart with 0.1 M phosphate buffer (pH 7.4) to remove blood cells, followed by 4% paraformaldehyde in the same buffer. Retinae or optic nerves were removed and immersed in the same fixative over-night at 4°C. They were then transferred to 1 M sucrose in phosphate buffer until equilibrated, embedded in OCT compound (Miles), and frozen in liquid nitrogen. Frozen sections were cut at 10 μm and collected onto glass microscope slides that had been previously coated with 1% gelatine. Sections were either stained immediately or stored at - 20°C until use.

Electron microscopy and axon counting

Mice were perfused with 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The optic nerves were removed, immersed in the same fixative over-night at 4°C, and post-fixed in 1% osmium tetroxide in the same buffer for 1 hour. After washing three times, the nerves were dehydrated in a graded series of alcohols and embedded in epoxy resin (EPON). Thin transverse sections were cut with an LKB ultramicrotome and mounted on Formvar-coated single slot grids, counter-stained with uranyl acetate and lead citrate and examined in a JOEL 100 CX II electron microscope at 80 KV. Optic nerves from three transgenic mice and three wild-type littermates were studied at each age. Both of the nerves from each animal were studied in the case of adults.

A low-power micrograph (magnification of either 300 or 380) was taken of each entire transverse section of the nerve. This was used to measure the area of the nerve, using a 'Summer Sketch' graphics digitizing drawing tablet (Summagraphics) and the CIGAL 2-D drawing programme (J.T. Voyvodic unpublished) with suitable calibration. A second series of micrographs was taken at higher magnification (6,350 for P0, P10, P15 nerves; 2,900 for adult nerves). The first micrograph of each series was taken at the pial surface, and serial ones were taken across the nerve until the other pial surface was reached. The micrographs were put together to create a continuous picture, representing a ribbon of tissue from one side of the nerve to the other. I counted the total number of axons in this montage and measured the area of the montage with the drawing tablet. I then calculated the fraction of the nerve area that the montage represented (which was between 5 and 10%) and multiplied this fraction by the number of axons counted to estimate the number of axons in the whole nerve.

I measured the area and circumference of axons in adult nerves, using the drawing tablet and a line drawn through the centre of the micrographs to sample the axons; only axons crossed by this line were measured. The number of axons measured in each sample was around 400. The results from 3 transgenic nerves and from 3 wild-type nerves were pooled for comparison.

Electron microscopy and cell counting

The thin transverse sections used to perform cell counts were prepared in the same way as described above for axon counting. The optic nerves used were taken from six 12 week old animals, three transgenic and three wild-type littermates. The central third of both nerves from each

mouse was used. Two sections, at least 100 μm apart, were prepared from each of the nerves, to make a total of 12 sections per group.

To count the numbers of each type of glial cell, cells were located at a magnification of 2,900, and then the magnification was increased to 10,000 to examine the cellular ultrastructure. The entire section was systematically scanned for every cell with a visible nucleus.

Measurement of DNA

The total amount of DNA in the optic nerve was measured as previously described (Barres et al., 1992). Pairs of nerves were dissected by initially cutting behind the eye and then removing the brain with the optic nerves still attached. Both optic nerves were then cut at the chiasm and placed in digestion buffer containing 10 mM Tris HCL, 50 mM EDTA, 0.1% SDS and 200 μg/ml proteinase K. The nerves were minced with scissors and incubated at 55°C for 48 hours. After digestion the final volume was measured and the DNA assayed using a fluorimetric method (Labarca and Paigen, 1980) that measures the amount of Hoechst 33258 dye that binds to DNA, using a Perkin-Elmer LS-5 luminescence spectrometer. The amount of DNA was translated into cell number by assuming 5.8 pg of DNA per diploid cell (Ausubel et al., 1991). DNA standard curves were prepared using the same percentage of SDS that was present in the tissue samples. No correction was made for the number of cells in S and G2 phases of the cell cycle, which contain more than 2N DNA.

Staining of dead cells in optic nerve sections

Frozen sections (10 μm) of optic nerves were prepared after perfusion fixation as described above. To determine the number of dead cells in optic nerves, frozen sections were stained with propidium iodide

(PI) (Barres et al., 1992). Sections were first postfixed with 70% ethanol at -20°C for 10 minutes. They were then incubated in PI and DNAase-free RNAase A (100 ug/ml) for 15 min at 37°C (Rodriguez-Tarduchy et al., 1990). The slides were then washed in buffer, mounted in Citifluor and examined as for immunofluorescence staining described above.

The total number of dead cells in a nerve was determined by counting all the pyknotic cells in all of the sections prepared from the entire nerve. Condensed or fragmented nuclei, which were also phase dark, were counted as pyknotic, and only cells within the boundary of the glial limiting membrane were considered. No correction for section thickness was made.

To determine if any of the dead cells were astrocytes, I prepared sections of P7 optic nerves, post-fixed them in methanol, and incubated them over-night in a rabbit antiserum against GFAP (Pruss, 1979), diluted 1:100 in TBLS. The antibodies were visualised with sheep anti-rabbit fluorescein (Sh anti-RIg Fl, Jackson Laboratories diluted, 1:100). The sections were then stained with PI as described.

Other sections of optic nerves were prepared and assessed for numbers of dead cells in the same way as described except that they were taken from P22 animals four days after one optic nerve had been transected, as desribed in chapter 3. Three nerves that had been cut and three control nerves were taken from both transgenic and wild-type littermates.

BrdU incorporation

To assess the number of proliferating cells in the developing optic nerve, BrdU (0.1 mg/g), was injected into the peritoneum of mice of various ages 1 hour before sacrifice. Frozen sections of optic nerves were prepared as described above, except that sections were collected on 3-aminopropyltriethoxy-silane-coated slides. The sections were treated with 50% HCl (together with 1% Triton-X 100) for 20 min at room temperature to denature the DNA. After washing in buffer, the BrdU incorporated into DNA was visualised with a fluorescein-conjugated mouse monoclonal anti-BrdU antibody (diluted 1:3 in TBLS, Beckton Dickinson). All of the sections from each nerve were stained, and the total number of BrdU $^{+}$ cells within the boundary of the glial limiting membrane were counted.

Staining for transgene expression in optic nerve glial cells

Optic nerves were removed and dissociated with papain (30 U/ml, Worthington) in MEM/Hepes, containing L-cysteine (0.4 mg/ml) and DNAse (0.04%). The nerves were cut into small pieces and incubated in papain solution at 37°C for 75 minutes for P7 nerves and 90 minutes for P14 nerves. Cells were dissociated by passing the nerve fragments through a 21 and then 23 gauge needle in medium containing ovomucoid (2 mg/ml) and DNAse (0.04%). After washing, the cells were plated onto PDL-coated cover slips in 20 µl of MEM Hepes containing 1% BSA, allowed to adhere for 20 minutes and then fixed with methanol. The cells were stained with a rabbit antiserum against MBP (a gift from D. Colman) diluted 1:100 in TBLS) to identify oligodendrocytes, or with a rabbit antiserum against GFAP to identify astrocytes. The antibodies were visualized with sheep anti-rabbit immunoglobulin conjugated to Texas Red (Sh anti-RIg TR, Jackson Laboratories, diluted 1:100). The cells were

then stained with the monoclonal antibody against human Bcl-2 as described above. The G anti-MIg Fl used to visualise the anti-Bcl-2 antibody did not recognise the rabbit antibodies.

To determine if the *bcl-2* transgene was expressed in microglial cells, P7 optic nerve cells were cultured for 3 days in DMEM containing 10% FCS. The cells were then labelled with normal rabbit serum (diluted 1:100), followed by Sh anti-RIg TR, to localise Fc receptors on microglial cells (Raff et al., 1979). The cells were then fixed with methanol and stained with anti-human Bcl-2 antibody as described.

To compare the brightness of the immunofluorescence staining in Bcl-2$^+$ cells from transgenic lines 71 and 73, stained cells were viewed on a Bio-Rad MRC 1000 confocal, laser scanning, fluorescence microscope. Individual Bcl-2$^+$ cells were selected at random, and the 'area' command was used to collect brightness readings. Twenty frames were collected for each image and the average fluorescence intensities were converted into numerical readings of arbitrary values.

Survival of dissociated optic nerve glial cells in culture

To determine whether the expression of human Bcl-2 in oligodendrocytes could protect them when they are deprived of survival signals in culture, I dissociated P8 optic nerve cells using 0.125% trypsin for 30 minutes in Earle's balanced salts (EBSS, Gibco BRL). After dissociation, the cells were washed in 30% fetal calf serum (FCS) and cultured at low density (10,000 cell per coverslip) in DMEM. Both wild-type and transgenic cells were mixed together in these cultures so that they were exposed to the same environment. After 1 day and 3 days in culture cells were stained with a monoclonal anti-GC antibody (Ranscht et al., 1982): ascites fluid diluted 1:100 in TBLS), followed by Texas-Red-coupled goat anti-mouse

immunoglobulin (G anti-MIg TR, Jackson Laboratories, diluted 1:100 in TBLS) to identify the oligodendrocytes. The cells were then fixed with methanol and stained with anti-human Bcl-2 as above, except that the antibody was visualised with a class-specific, fluorescein-coupled, goat anti-mouse IgG1 (G anti-MIgG1 Fl, Nordic, diluted 1:50 in TBLS). I then counted the proportion of GC^+ oligodendrocytes that were Bcl-2^+.

CHAPTER 3

THE CONTROL OF ASTROCYTE NUMBERS

Introduction

In the previous chapter I focused on how oligodendrocyte numbers are controlled the rodent optic nerve. In this chapter I investigate how astrocyte numbers are controlled.

Astrocytes develop from the neuroepithelial cells that form the optic stalk, the primordium of the optic nerve (Small et al., 1987), Using GFAP as a marker for astrocytes (Bignami et al., 1972), it has been shown that astrocytes first appear in the developing rat optic nerve at E16 (Miller et al., 1985) and then increase in number until 6 weeks postnatally (Barres et al., 1992). As normal cell death does not seem to play a part in adjusting astrocyte numbers in the rodent optic nerve, at least postnatally (Barres et al., 1992, and this study) I have studied how astrocyte proliferation is controlled.

I described previously that transgenic mice that express a human *bcl-2* transgene controlled by a NSE promoter (Martinou et al., 1994) contain 80% more axons in the adult optic nerve (Chapter 2) and also contain a correspondingly increased number of glial cells, including astrocytes (Table 2.2). In this chapter I show that the increase in astrocyte number in the transgenic optic nerve results from an increase in cell proliferation in the astrocyte lineage during the first postnatal week, suggesting that axon numbers may regulate the proliferation of developing astrocytes or their precursor cells.

Most astrocytes in the rodent optic nerve are born in the first postnatal week (Skoff, 1990), so I have focused on this period of development. I provide direct evidence that axons normally drive the proliferation of astrocytes in the postnatal rodent optic nerve. I show that astrocyte division halts when the neonatal nerve is cut or when

colchicine, but not tetrodotoxin, is injected into the eye, suggesting that the mitogenic influence of axons depends on axonal transport and but not on axonal electrical activity. I also show that RGCs can stimulate DNA synthesis in astrocytes in optic nerve cultures and that this effect is not mimicked by neuregulin, platelet derived growth factor (PDGF), Sonic hedgehog (Shh), epidermal growth factor (EGF) or transforming growth factor alpha (TGFα). However, basic fibroblast growth factor (bFGF) is mitogenic for optic nerve astrocytes.

Results

Optic nerve glial cells in *bcl-2* transgenic mice

In the electron microscopic study of adult optic nerve described in Chapter 2, I found (Table 2.2) that there were about twice as many astrocytes (as well as oligodendrocytes, microglial cells and oligodendrocyte lineage cells) in nerves from *bcl-2* transgenic mice compared to nerves from their wild-type littermates. There were no obvious differences in astrocyte cell size in the two types of nerves, consistent with the finding that both total cell number and nerve volume were approximately doubled.

Cell death in *bcl-2* transgenic optic nerve

As described in Chapter 2, I compared the amount of glial cell death in transgenic and wild-type optic nerves, and found that it was significantly lower in transgenic nerves than in wild-type nerves at P14 and P21 (Figure 2.3a).

To establish whether any of the dead cells found in either wild-type or transgenic nerves were astrocytes, I double-labelled frozen sections of these nerves with PI to identify dead cells and anti-GFAP antibodies to identify astrocytes. None of the dead cells were $GFAP^+$, as shown previously in the rat optic nerve (Barres et al., 1992). It is possible, however, that astrocytes that undergo PCD lose their GFAP. To test this possibility I treated optic nerve explants from P7 animals with a high concentration (1 μM) of the protein kinase inhibitor staurosporine for 3 days to induce PCD. Frozen sections were then cut and stained with PI and anti-GFAP antibodies: many pyknotic cells were seen to be brightly $GFAP^+$, indicating that astrocytes do not necessarily lose their GFAP when they undergo PCD. Thus it is likely that most of the dead cells in the postnatal mouse optic nerve belong to the oligodendrocyte lineage, as previously reported for the rat optic nerve (Barres et al., 1992). Astrocytes also failed to die when the transgenic optic nerve was transected.

Thus it is unlikely that a decrease in astrocyte death contributed to the increase in astrocyte numbers seen in the transgenic optic nerve.

Cell proliferation in *bcl-2* optic nerve

The numbers of dividing cells in the optic nerves of transgenic and wild-type mice were compared at several ages (Chapter 2). As shown in Figure 2.4a, the numbers of $BrdU^+$ cells were increased in transgenic compared to wild-type nerves at all four ages. As most of the increase in astrocyte numbers in rodent optic nerve occurs in the first two postnatal weeks while most of the increase in oligodendrocyte numbers occurs after this time (Skoff, 1990; Barres et al., 1992) some of the BrdU-labelled cells at P8 in my experiments would be expected to be astrocytes, while most of the labelled cells at later ages would be expected to be oligodendrocyte precursor cells.

As astrocytes were increased as much as oligodendrocytes in adult transgenic optic nerves (see Table 2.2), and astrocytes seem not to undergo normal cell death in the optic nerve, it was expected that the proliferation of astrocytes or their precursors would be greater in transgenic nerves than in wild-type nerves. To confirm this I looked at BrdU incorporation into astrocytes in P5 nerves. I injected BrdU twice, 2 hours apart, and killed the mice 2 hours after the last injection. In some mice I labelled serial frozen sections of optic nerve with anti-BrdU antibody, counted the total number of $BrdU^+$ cells in the nerves and calculated the proportion of labelled cells in each: 0.8% ± 0.1 in wild type mice compared to 1.1% ± 0.1 in transgenics (mean ± s.e.m., n=3). In others, I prepared cells from the optic nerve, cultured them overnight, and double-labelled them for BrdU and GFAP. Although the proportion of $BrdU^+$ cells in transgenic nerves was not very different from that in wild-type nerves, about 30% of the $BrdU^+$ cells were $GFAP^+$ in the cultures of transgenic cells compared to about 15% in cultures of wild-type cells (Table 3.1), suggesting that astrocyte proliferation was greater in the transgenic nerves. Moreover, the increased number of

total cells in the transgenic nerves compared to wild-type nerves at P5 could be entirely accounted for by the increased number of GFAP$^+$ astrocytes: Figure 2.2 shows that there were about 60,000 more cells in the transgenic nerves at P5 and, if one uses the numbers for the proportions of GFAP$^+$ cells from Table 3.1 to calculate the total numbers of astrocytes in the nerves at P5, there were about 60,000 more astrocytes.

These findings suggest that the difference in astrocyte numbers between transgenic and wild-type optic nerves mainly reflects an increase in the proliferation of astrocytes and/or their precursors.

Table 3.1

	Wild type	Transgenic
% GFAP$^+$ cells	42 ± 7	64 ± 8
% Brdu$^+$ cells expressing GFAP	15 ± 2	30 ± 4

Astrocyte proliferation in overnight cultures of P5 optic nerve cells. P5 transgenic (line 73) mice received 2 intraperitoneal injections of BrdU 2 hours apart and were sacrificed 2 hours after the last injection. Cell suspensions were prepared from the optic nerves and cultured overnight. The cells were stained for both GFAP and BrdU, and at least 1,000 cells were assessed. The results are expressed as means ± s.e.m. of 3 separate experiments.

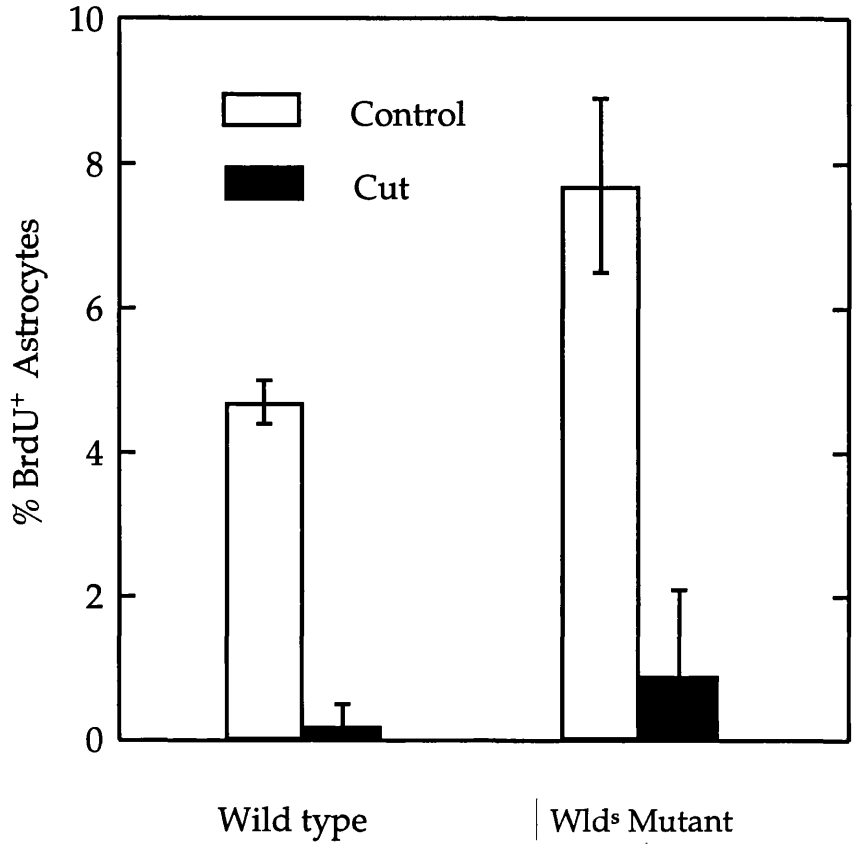
BrdU incorporation into astrocytes in transected optic nerves

To determine if axons are required for normal astrocyte proliferation in the developing optic nerve I cut the left optic nerve just behind the eye in P2 mice, allowed 4 days for the axons to degenerate, and then gave the mice 2 injections of BrdU 2 hours apart. Two hours after the last injection, the mice were killed, the cells in each nerve were dissociated in trypsin and cultured overnight, before they were fixed and stained for GFAP and BrdU. As shown in Figure 3.1, transection caused a dramatic reduction in the proportion of $GFAP^+$ astrocytes that incorporated BrdU.

The same result was obtained when the optic nerve of the Wallerian-degeneration-deficient Wld^S/C57BL (Wld^S) mouse was transected (Figure 3.1), even though, as expected, the cut axons remained intact in these mutant mice (not shown). This finding indicates that RGC axons that have been disconnected from their cell bodies, even though they are intact and can conduct action potentials (Perry et al., 1990), cannot maintain the proliferation of astrocytes in the developing optic nerve.

The same results were obtained when cell suspensions were prepared with papain, and the cells were stained immediately, instead of culturing them overnight. In one experiment, for example, 3% of the $GFAP^+$ astrocytes released from uncut, P6 wild-type nerves incorporated BrdU, whereas only 0.2% of the $GFAP^+$ astrocytes released from P6 nerves cut at P2 incorporated BrdU. In Wld^S nerves, the comparable values in this experiment were 5% and 0.4%.

Figure 3.1



The effect of nerve transection on astrocyte proliferation in the optic nerves of wild-type and Wld^S mutant optic nerves. One optic nerve was transected at P2, and mice were injected twice with BrdU 2 hours apart on day 6. Two hours after the final injection, the mice were killed and optic nerve cells were prepared, and cultured over-night, before they were fixed and stained with antibodies against BrdU and GFAP. At least 500 astrocytes were assessed in each experiment, and the results are expressed as means ± s.e.m. of three separate experiments.

Effect of intraocular tetrodotoxin on astrocyte proliferation

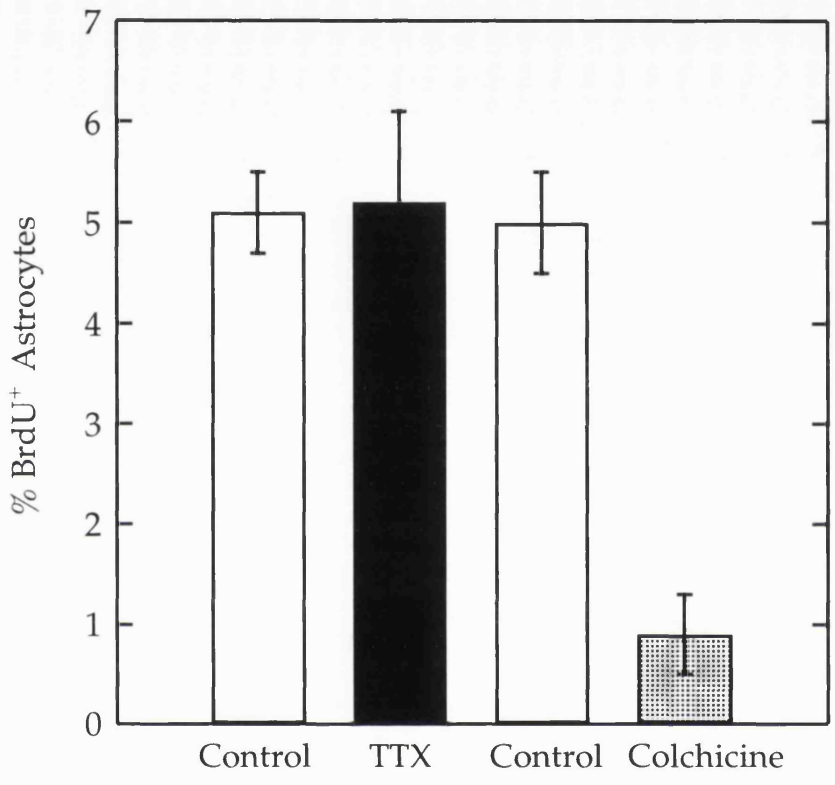
One possible explanation for the failure of cut Wld^S axons to promote astrocyte proliferation is that electrical activity may be required for the mitogenic affect of axons, as has been shown to be the case for the influence of axons on oligodendrocyte precursor cells in the optic nerve (Barres and Raff, 1993); even though the eyes are not open in the first postnatal week, RGCs are spontaneously electrically active at this time (Galli and Maffei, 1988). To test this possibility I injected tetrodotoxin (TTX) into the vitreous of the left eye of P3 rats. Two days later I gave the rats 2 injections of BrdU 2 hours apart. Two hours after the last injection, I killed the rats, dissociated their optic nerve cells, cultured the cells overnight and stained them for GFAP and BrdU. As shown in Figure 3.2, the TTX injections did not influence the proportion of astrocytes that incorporated BrdU, suggesting that electrical activity in the axons is not required for their mitogenic influence on astrocytes in the developing optic nerve.

Effect of intraocular colchicine on astrocyte proliferation

Another possible explanation for the failure of cut Wld^S axons to promote astrocyte proliferation is that the mitogenic affect of axons may depend on short-lived molecules that are continuously transported down the axon from the cell body. To test this possibility I injected a small amount of colchicine (0.1 μg) into the vitreous of the left eye of P3 rats; this dose blocks axonal transport, but leaves the RGCs ultrastructurally unchanged (Matthews et al., 1982). Two days later I injected the rats with BrdU and determined the proportion of astrocytes that incorporated BrdU, as described above. As shown in Figure 3.2, the intraocular injection of colchicine markedly decreased the proportion of astrocytes that incorporated BrdU compared to control injections of vehicle alone, suggesting that the mitogenic influence of axons depends on axonal transport.

To exclude the possibility that the injected colchicine was diffusing from the eye in to the optic nerve and affecting astrocytes directly I cut longitudinal frozen sections of optic nerves from P5 rats that had received intraocular colchicine 2 days earlier, stained them with PI and counted the total number of mitotic figures per nerve. If colchicine had diffused into the nerve one might expect it to trap dividing cells in mitosis and therefore increase the number of mitotic figures. In fact, there were fewer mitotic figures in the nerves from colchicine-treated rats: there were 166 ± 18 in vehicle-injected rats, compared to 99 ± 20 in colchicine-treated ones (mean ±sem, n=3).

Figure 3.2

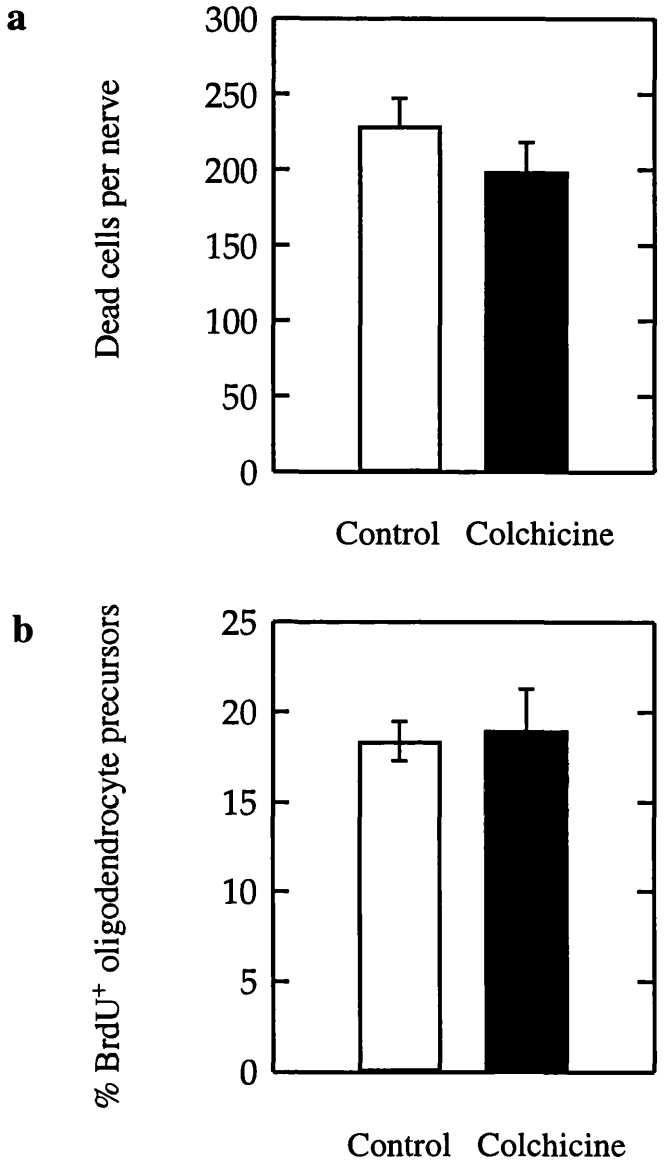


The effect of intraocular injection of TTX or colchicine on astrocyte proliferation in postnatal rat optic nerves. Injections were carried out at P3, and the rats were given BrdU at P5, before they were killed and their optic nerve cells assayed as in Figure 3.1. At least 500 astrocytes were assessed per experiment, and the results are expressed as means ± s.e.m. of three separate experiments.

Effect of intraocular colchicine on oligodendrocyte lineage cells

RGC axons have been previously shown to promote the survival of newly-formed oligodendrocytes (Barres et al., 1993b) and the proliferation or survival of oligodendrocyte precursor cells in the developing optic nerve (Barres and Raff, 1993). To determine whether these influences also depend on axonal transport I injected colchicine intraocularly as described above. Two days later I either cut longitudinal sections of the optic nerves, stained them with PI, and counted the total number of dead cells per nerve (all of which have been shown to be oligodendrocyte lineage cells- Barres et al., 1992) or injected BrdU, dissociated the optic nerve cells, cultured them overnight, and then stained them with both anti-BrdU antibody and with the A2B5 monoclonal antibody to identify the oligodendrocyte precursor cells (Raff et al., 1983). As shown in Figure 3.3, an intraocular injection of colchicine had no effect compared vehicle alone on either the number of dead cells (Figure 3.3a) or the proportion of oligodendrocyte precursor cells that incorporated BrdU (Figure 3.3b), suggesting that the axonal influences on oligodendrocyte lineage cells do not depend on axonal transport.

Figure 3.3



Rats were injected with colchicine or vehicle at P3 and killed at P5. In (a), serial frozen sections of the optic nerves were stained with PI, and the total numbers of pyknotic cells were counted. In (b), BrdU was injected twice 4 hours before the rats were killed; optic nerve cells were cultured overnight and stained for A2B5 (to identify oligodendrocyte precursor cells) and BrdU. The results are expressed as means ± s.e.m. of three nerves (a) or three separate experiments (b).

Effect of nerve transection on astrocyte numbers

As rat optic nerve astrocytes do not die during normal postnatal development (Barres et al., 1993, and this study), or after optic nerve transection (Barres et al., 1993b), I could directly quantify the influence of axons on the proliferation of astrocytes lineage cells in the developing rat optic nerve by determining what happens to total astrocyte numbers when the nerve is cut. To determine astrocyte numbers accurately I first measured the amount of DNA in the nerve and translated this value into the total cell number (Barres et al., 1992). I then determined the proportion of astrocytes in the nerve by simultaneously staining semithin frozen sections with anti-GFAP and anti-S-100β antibodies, to visualise all the astrocytes, and with PI, to visualise all cell nuclei (Figure 3.4). Finally, I multiplied the proportion of astrocytes by the total number of cells to obtain the total number of astrocytes in the nerve.

When the optic nerve was cut at P2, the number of astrocytes did not change 4 days later, whereas astrocyte numbers increased in the uncut control nerve as expected (Figure 3.5). Similar results were obtained in the Wld^S mutant mice (Figure 3.5). These findings suggest that the proliferation of astrocyte lineage cells in the neonatal optic nerve is absolutely dependent on axons. The up-regulation of GFAP in response to nerve transection was only seen in the wild-type nerves and not in the Wld^S mutant nerves, suggesting that the axons in the Wld^S nerve were still intact. However, in cut nerves from both mouse lines the astrocytes had moved to the centre of the nerve (Figure 3.4).

Figure 3.4 (Overleaf)

Immunofluoresence micrographs of semi-thin frozen sections of normal (A, B, C, D) or cut (E,F,G,H) optic nerves from P6 (A,B,E,F,G,H) or P2 (C-D) wild-type (A-F), or Wld^S (G-H) mice. The cut optic nerves were studied 4 days after transection. The sections were stained with PI (A, C, E, G) or for anti-GFAP and S-100B (B, D, F, H). Scale bar = 100 μm.

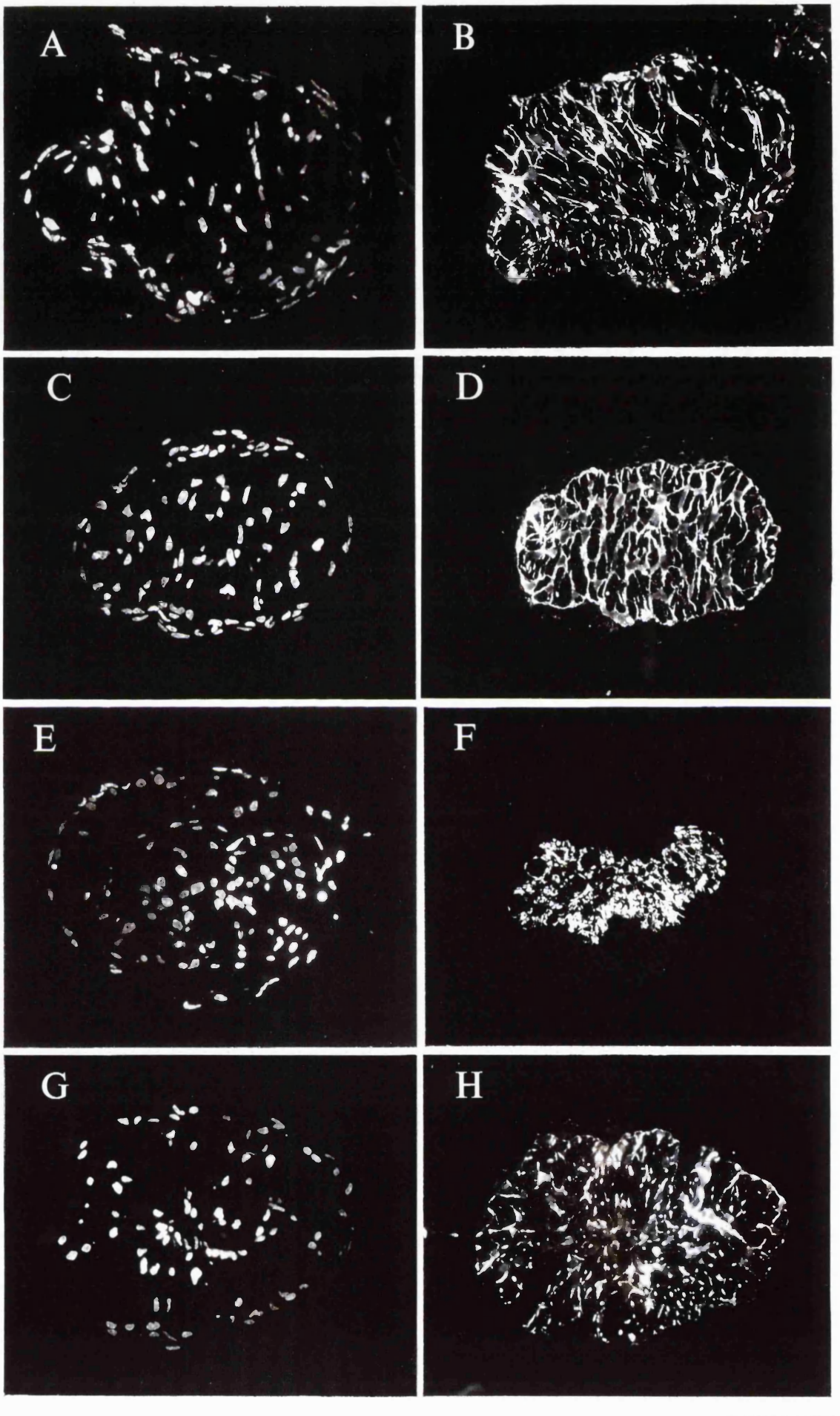
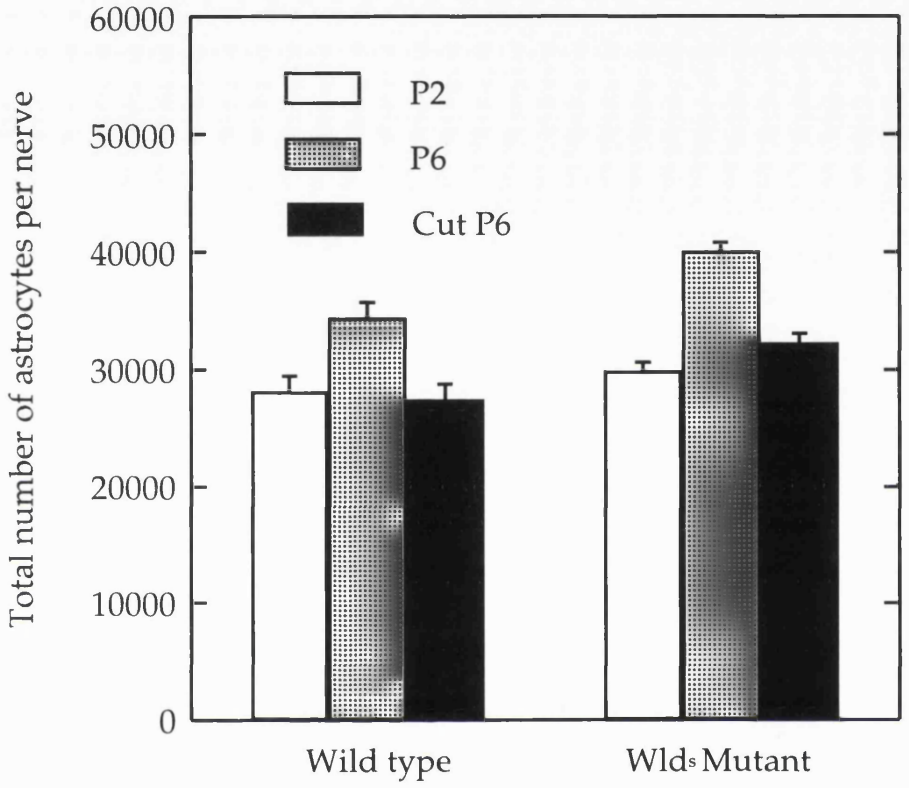

A
B
C
D
E
F
G
H

Figure 3.5



The effect of nerve transection on total astrocyte numbers in the optic nerves of wild-type and Wld^S mutant optic nerves. One optic nerve was transected at P2, and the mice were killed at P6. Cell numbers were determined by measuring the total DNA in the nerve and dividing by the amount of DNA per cell (DNA was measured in 8 nerves per group). The proportion of astrocytes was determined from semi-thin frozen sections stained simultaneously for S-100 β and GFAP (see Figure 3.4). Counts of total PI$^+$ cells and of astrocytes were made on at least 3 sections from each of 3 nerves per group. The total number of astrocytes was obtained by multiplying the mean total cell number by the mean proportion of astrocytes in single nerves from 3 animals per group. The results are expressed as means ± s.e.m.

Effect of RGCs on Astrocyte Proliferation in Culture

To demonstrate directly that RGCs are mitogenic for developing optic nerve astrocytes I co-cultured cells dissociated from newborn rat optic nerve with purified rat RGCs. The optic nerve cells were plated at low density in a small volume of serum-free medium and were left for 6 days so that most of them were no longer dividing. After 6 days I added purified RGCs; the co-cultures were maintained in the presence of laminin, ciliary neurotrophic factor (CNTF) and N-acety-L-cysteine, as these have been shown to promote the survival of purified RGCs (Meyer-Franke et al., 1995) . I did not add forskolin, which also promotes RGC survival (Meyer-Franke et al., 1995), as it inhibited astrocyte proliferation (not shown). About 18 hr after the RGCs were added I added BrdU for 6 hours and then fixed and stained the co-cultures for BrdU and GFAP (Figure 3.6). As shown in Table 3.2, the proportion of astrocytes that incorporated BrdU was 20-fold greater in the presence of RGCs than in their absence; bFGF and 10% FCS had an even larger effect, but ovomucoid, which was added in small amounts with the RGCs, had no effect on its own.

Figure 3.6 (Overleaf)

Immunofluoresence micrograph of co-culture of newborn optic nerve cells and RGCs stained for GFAP (A) and BrdU (C). The same field is shown in phase contrast (B). Note that one of the two astrocytes is $BrdU^+$ and there is a slight cross of GFAP staining to one of the RGC axons. Scale bar = 50 μm.

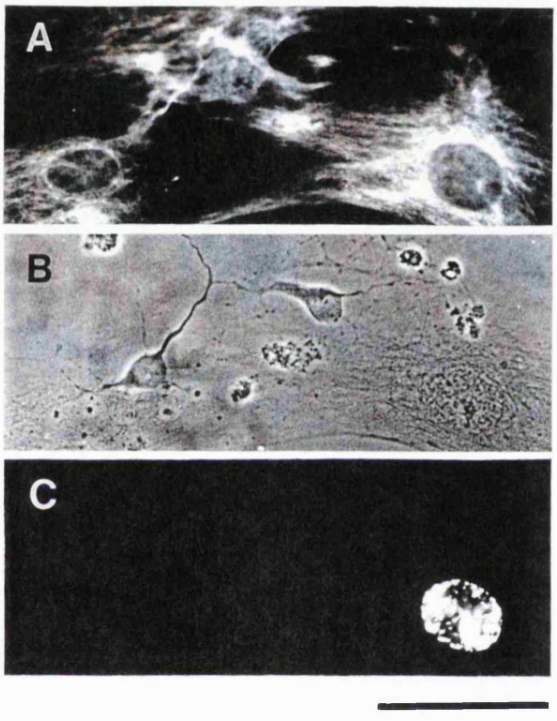

A
B
C

Table 3.2

Additives	% BrdU$^+$ Astrocytes
Control	1.1 ± 0.5
RGCs	22.6 ± 1.6
bFGF	28.2 ± 2.3
10% FCS	65.4 ± 1.6

P0 rat optic nerve cells were cultured at low density in serum-free medium for 6 days. After this time various additives were introduced, followed by BrdU the next day. After 6 hours the cells were fixed and stained for BrdU and GFAP. About 50-200 GFAP$^+$ astrocytes were assessed per coverslip, and 5 coverslips were examined per group. The addition of ovomucoid (40 µg/ml), which was added with the RGCs, had no effect when added on its own. Results of one representative experiment is shown as mean ± s.e.m. The experiment was repeated 3 times with similar results.

Effect of Growth Factors on Astrocyte Proliferation in Culture

As neuregulins have been shown to be made by RGCs (Meyer and Birchmeier, 1994; Bermingham-McDonogh et al., 1996) and to stimulate the proliferation of astrocytes isolated from the rat corpus callosum (Brockes et al., 1980), I tested whether the neuregulin glial growth factor 2 (GGF-2) could stimulate BrdU incorporation into astrocytes in rat optic nerve cell cultures, prepared as described above. The addition of GGF-2 did not stimulate BrdU incorporation in astrocytes, either in the presence or absence of 0.5% FCS, suggesting that the mitogenic affect of RGCs for optic nerve astrocytes, in culture at least, is not mediated by neuregulins. I also tested PDGF AA, EGF, TGFα, Shh, and bFGF to see if they would elicit a mitogenic response, either alone or in the combinations described in the Materials and Methods, and I found that only bFGF did so (Table 3.2).

Discussion

Astrocytes are increased as much as oligodendrocytes in the *bcl-2* transgenic optic nerve. As astrocytes apparently do not die in the postnatal rodent optic nerve during development (Barres et al., 1992 and this study), it seems likely that the increase reflects increased proliferation of astrocytes and/or their precursors in the transgenic nerve. Although the proportions of cells synthesising DNA in the transgenic and wild-type optic nerves at P5 are not very different, twice as many of these cells are $GFAP^+$ astrocytes in overnight cultures of transgenic compared to wild-type nerve, suggesting that proliferation of astrocyte lineage cells is greater in the transgenic nerve at P5. Moreover, there are about 60,000 more glial cells in transgenic optic nerves than in wild-type nerves at P5, and all of this increase can be accounted for by the increase in astrocyte numbers.

Although the *bcl-2* transgene is expressed in almost all the astrocytes in transgenic optic nerves, it is unlikely that the transgene is directly responsible for their increased proliferation, as over-expression of *bcl-2* has not been reported to increase cell proliferation. It is also unlikely that the expression of *bcl-2* directly influences the number of neuroepithelial cells capable of giving rise to astrocytes in the transgenic optic nerve since there was no significant difference in the total number of cells found at birth in transgenic and wild-type nerves (Figure 2.2). It seems more likely that the increase in axons in the transgenic optic nerve is either directly or indirectly responsible for the increased astrocyte proliferation, a conclusion supported by the previous finding that there are half as many astrocytes in rat optic nerves that were cut at birth and studied 15 days later, compared to uncut nerves (David et al., 1984).

I have provided four lines of evidence to suggest that RGC axons drive the proliferation of astrocytes in the developing postnatal rodent optic nerve. First, I show that astrocyte proliferation is increased in the developing optic nerve of *bcl-2* transgenic mice that have 80% more RGC axons, suggesting that axons are normally limiting for astrocyte proliferation. Second, when the mouse optic nerve is cut at P2 and BrdU is injected 4 days later, the proportion of astrocytes that incorporate the BrdU is greatly decreased compared to that in the uncut control nerve. Third, if the nerve is cut at P2, the number of astrocytes in the nerve 4 days later is unchanged, whereas the number increases in the uncut control nerve. As astrocyte cell death seems not to occur in the postnatal rodent optic nerve, neither during development (Barres et al., 1992 and this study), nor after transection (Barres et al., 1993b and this study), this finding strongly suggests that proliferation in the astrocyte lineage in the neonatal rodent optic nerve absolutely depends on RGC axons. Fourth, when purified RGCs are co-cultured with newborn optic nerve cells, they stimulate the incorporation of BrdU into astrocytes. It seems likely that axons also stimulate the proliferation of astrocyte precursor cells in the embryonic optic nerve as, in the *ocular retardation* mouse, where RGC axons fail to enter the optic stalk, glial cells in the optic nerve–including astrocytes, fail to develop (Silver and Robb, 1979).

A relationship between axons and glial proliferation has also been reported in a study of the effect of eye removal on the superior colliculus of the mouse (DeLong and Sidman, 1962). Here it was found that there was not only a large amount of neuronal cell death in the superior colliculus after the eye was removed on the day of birth, but there was also a significant reduction in tritiated thymidine incorporation into neuroglia. Interestingly, the effect on neuroglial cells was clearly seen during the

period of about 4 days before neuronal degeneration was recognisable; unfortunately, the phenotypes of the neuroglial cells was not established. However, the stimulation of astrocyte proliferation by neurons during CNS development may not be a general rule. It has been reported that some factors that are made by axons, such as neural cell adhesion molecule, (N-CAM), are inhibitory for astrocyte division, but this may only apply to adult animals where there is a need to keep astrocytes in a resting state (Krushel et al., 1995). Others have reported that neurons can inhibit astrocyte proliferation in cultures of developing hippocampal (Gasser and Hatten, 1990) and cerebellar cells (Hatten and Shelanski, 1988). As the optic nerve is entirely white matter and contains no neuronal cell bodies, it may be that my findings are more applicable to astrocytes in developing white than grey matter.

My results provide several clues as to how RGC axons stimulate astrocytes to divide in the developing optic nerve. One is that transected axons of the Wld^S mutant mouse are unable to maintain astrocyte proliferation in the optic nerve. This is not because electrical activity is required for axons to stimulate optic nerve astrocyte proliferation: an intraocular injection of TTX, which electrically silences the RGCs and their axons, does not affect astrocyte proliferation in the developing nerve, although it has been shown previously to decrease the number of dividing oligodendrocyte precursor cells in the optic nerve (Barres and Raff, 1993). The reason cut Wld^S axons are not mitogenic for the astrocytes seems to reflect a requirement for short-lived molecules that are antrogradely transported along the axon from the cell body: when a low dose of colchicine is injected into the eye, astrocyte proliferation in the developing optic nerve halts, although there is no effect on either the survival of oligodendrocytes or the proliferation of oligodendrocyte precursor cells.

Two lines of evidence suggested that neuregulins might be at least partly responsible for the mitogenic influence of RGC axons on developing optic nerve astrocytes. First, RGCs have been shown to make neuregulins (Meyer and Birchmeier, 1994; Bermingham-McDonogh et al., 1996). Second, neuregulins have been shown to be mitogenic for cultured astrocytes from the corpus callosum in the presence of FCS (Brockes et al., 1980). I show here, however, that the neuregulin GGF-2 is not able to stimulate the division of optic nerve astrocytes, either in the presence or absence of FCS. As all neuregulins are thought to be able to activate all type of neuregulin receptors (reviewed in Lemke, 1996), my finding makes it unlikely that the mitogenic effect of RGCs on optic nerve astrocytes is mediated by neuregulins. It may be that neuregulins are involved in other aspects of astrocyte development, such as maturation or survival (Pinkas-Kramarski et al., 1994). I have tested a number of other signalling molecules known to be made by RGCs, including PDGF-AA (Mudhar et al., 1993), bFGF (de-longh and McAvoy, 1992), and Shh (Jensen and Wallace, 1997), also EGF and TGFα that might be involved (L. Lillien, personal communication). I find that of these factors, used alone or in combination, only bFGF is mitogenic for optic nerve astrocytes in serum free, very low density cultures. It remains to be determined whether bFGF, or any other members of the FGF family, mediates the mitogenic interaction between RGCs and optic nerve astrocytes.

In summary, I have shown that RGC axons drive the proliferation of astrocyte lineage cells in the developing postnatal rodent optic nerve. The mitogenic effect of axons depends on axonal transport but not on electrical activity, and it might be mediated in part by a FGF family member.

Materials and Methods

Animals

Transgenic line 73 mice expressing human *bcl-2* under the control of the NSE promoter were bred and screened for transgene expression as described in Chapter 2.

Wld^S/C57BL (Wld^S) mutant mice (Perry et al., 1990) and C57Bl/6J wild-type controls were purchased from Harlan-Olac and bred in the University College London (UCL) animal facility. Sprague/Dawley rats were obtained from the UCL breeding colony.

Staining of dead cells in optic nerve sections

The preparation and staining of optic nerve sections with PI were carried out as described in Chapter 2. Also as described in Chapter 2, the total number of dead cells in a nerve was determined by counting all the pyknotic cells in all of the sections prepared from the entire nerve. In some experiments sections were first stained overnight with rabbit anti-GFAP antiserum (diluted 1: 100 in TBLS) in establish whether any of the dead cells were astrocytes; in these cases sections were post-fixed with cold methanol for 10 minutes and the anti-GFAP was detected with Sh anti-RIg Fl. The sections were then stained with PI as described. To determine whether astrocytes that undergo PCD maintain their GFAP, I cultured P8 rat optic nerves as explants on polycarbonate filters (Nuclepore, 0.8 μm pore size) for 5 days floating in DMEM. I induced PCD with a high concentration (1 μM) of the protein kinase inhibitor staurosporine, which induces PCD in all nucleated cell types we have studied (Raff et al., 1993). I then sectioned and stained the nerves with anti-GFAP antibodies and PI.

BrdU incorporation

To determine what proportion of the dividing cells in P5 *bcl-2* transgenic and wild-type nerves were astrocytes, I injected littermates with BrdU. Two injections of 0.1 mg/g BrdU were given 2 hours apart. Optic nerves were removed and dissociated with papain (30 U/ml, Worthington) in MEM/Hepes, containing L-cysteine (0.4 mg/ml) and DNAse (0.04%). The nerves were cut into small pieces and incubated in papain solution at 37°C for 30 minutes. Cells were dissociated by passing the nerve fragments through a 21 and then 23 gauge needle in medium containing ovomucoid (2 mg/ml) and DNAse (0.04%) and cultured overnight on PDL-coated coverslips (30,000 cells per cover-slip) in a modified Bottenstein-Sato (BS) medium (Bottenstein and Sato, 1979), containing insulin (10 µg/ml) and N-acetyl-L-cysteine (25 µM) with 0.5% FCS. The cells were fixed with cold methanol, stained for GFAP and BrdU as described in Chapter 2, except that the Bu20a anti-BrdU monoclonal antibody (Magaud et al., 1989) was used as hybridoma supernatant diluted 1:1 with TBLS, the antibody was visualised with G anti-MIg Fl, and the cells were pre-incubated in 2 M HCl for 10 minutes, followed 0.1 M sodium borate for 10 minutes to denature the DNA.

To determine the proportion of astrocytes dividing in the optic nerve after injections in the eye or optic nerve transection I injected mice with the same regime of BrdU but in these cases optic nerves were dissociated with trypsin and then cultured overnight on PDL-coated coverslips as previously described. Susequent staining the next day for BrdU and GFAP was as described above. In some experiments the cells were stained on their surface with A2B5 monoclonal antibody (Eisenbart et al., 1979) followed by G anti-MIg TR, (diluted 1:100 in TBLS) to identify the

oligodendrocyte precursor cells (Raff et al., 1983) and then fixed in methanol and labelled for BrdU.

Optic nerve transection and injection into the eye

P2 mice were anaethetised by cooling to 4°C, and an incision was made in the left eyelid so that the eyeball could be gently retracted. The optic nerve was cut with microscissors behind the globe, and the incision was closed with 10-0 suture. Successful transection was confirmed by visual inspection when the mouse was sacrificed 4 days later.

P3 rats were anaesthetised by cooling to 4°C. P7 rats were anaesthetised with a mixture of Hypnorm and Diazepam. A small incision was made in the eyelid with scissors, and injections were made just posterior to the corneoscleral junction. A volume of 0.5 µl of fluid containing colchicine ($5x10^{-4}$ M in sterile saline), tetrodotoxin [10^{-4} M in phosphate buffered saline (PBS)], or vehicle alone was slowly injected into the vitreous of the left eye through a 34-gauge needle attached to a 5 µl Hamilton syringe.

Measurement of DNA

The total amount of DNA in optic nerves was measured as previously described (Barres et al., 1992) and in Chapter 2.

Frozen sections

Conventional frozen sections of optic nerve were prepared from P5 rats as described in chapter 2.

Semi-thin frozen sections of optic nerve were prepared from P2 or P6 mice. The mice were killed with a lethal injection of Sagatal and the optic nerves were removed. The nerves were embedded in 2% followed by

10% gelatine, cryoprotected with 2.3 M sucrose, and 0.5 µm transverse sections were cut at -90°C on a Reichert-Jung FC 4E cryo-microtome. Sections were washed with PBS, fixed with methanol, and then stained with a combination of rabbit anti-S-100β (East Acres Biologicals; 1:200) and rabbit anti-GFAP ((Pruss, 1979), diluted 1:200) over-night at 4°C. The primary antibodies were visualised with fluorescein-conjugated goat anti-rabbit immunoglobulin (G anti-RIg Fl, Jackson Laboratories; 1:100). Sections were then counter-stained with PI to visualise all nuclei. I then counted the total number of cells and the total number of astrocytes (fluorescein-labelled cells) in each section.

Co-culture of retinal ganglion cells and optic nerve cells

To determine if RGCs stimulate DNA synthesis in optic nerve astrocytes in culture the two cell types were cultured together, pulsed with BrdU and then stained for BrdU and GFAP. First, newborn rat optic nerve cells were cultured at low density (500 cells per 6 mm PDL-coated cover-slip) in a serum-free Neurobasal medium (Gibco BRL), containing N-acetyl-L-cysteine (60 µg/ml) and penicillin-streptomycin (50 U/ml). The cover-slips were in a 96-well plate (Falcon) in a volume of 50 µl. The medium was replaced after 3 days, laminin (200 µg/ml) was added after 5 days, and the cells were used after 6 days, by which time approximately 70% of the cells present were GFAP$^+$ astrocytes.

Retinal ganglion cells were purified by sequential immuno-panning from newborn rat retinae, following the procedure of Meyer-Franke et al. (1995). The only modification was that after the RGCs were removed from the final panning dish they were washed twice with ovomucoid (2 mg/ml; Boehringer-Mannheim) rather than with FCS, so that at no stage were the RGCs exposed to serum. More than 95% of the cells were Thy-1$^+$, a marker

of RGCs (Barnstable and Drager, 1984). A total of 5,000 purified RGCs were added to the 6 day-old cultures of optic nerve cells. Before the RGCs were added, the laminin was washed out. The RGCs were added in 50 µl of the medium described above, except that it now also contained 50 ng/ml CNTF. In some cases 10% FCS, ovomucoid (40 µg/ml), or human recombinant GGF-2 (50 ng/ml; Cambridge Neuroscience), either with or without 0.5% FCS, was added instead of the RGCs. Other wells recieved PDGF-AA, TGFα (both from Pepro Tech, 50 ng/ml), EGF (Sigma, 50 ng/ml), Shh (a gift from H. Roelink and T. Jessell, 3 µg/ml), or bFGF (Pepro Teck, 0.5 ng/ml) to see if they would elicit a mitogenic response. Some wells recieved a combination of PDGF+EGF, PDGF+bFGF, EGF+bFGF or PDGF+bFGF+EGF at the concentrations above apart from bFGF that was administered at 50 ng/ml under these conditions. The next day BrdU (50 µM) was added to all wells; after 6 hours the cells were fixed with cold methanol, stained with antibodies against BrdU and GFAP, and the proportion of astrocytes that had incorporated BrdU was determined.

CHAPTER 4

DO AXONS HAVE A DEATH PROGRAMME?

Introduction

The morphology displayed by the cell bodies of RGCs that die during development is the same as that seen when RGCs die as a result of axotomy (Snider et al., 1993; Rabacchhi et al., 1994) and has the characteristics of apoptosis or PCD. Since the over-expression of human *bcl-2* is able to inhibit these naturally–occurring cell deaths during development, I have investigated whether this is also true after axotomy. I have used a retinal explant culture system where RGCs are deprived of both axons and trophic factors and is therefore equivalent to axotomy *in vivo*. I show that over-expression of human *bcl-2*, protects RGCs from axotomy-induced PCD. This raised the question of whether human *bcl-2* could also protect the cut RGC axons from Wallerian degeneration (Waller, 1850). Interestingly, I found that a cut axon, unlike the cell body, is not protected by *bcl-2* .

There are several aspects of Wallerian degeneration that suggest that axons may have a form of death programme, even though it is not blocked by *bcl-2*. Several electron-microscopic studies have reported that, despite the scale of axon loss from the rodent optic nerve during the first postnatal week, it is usually not possible to recognise degenerating axons (Sefton and Lam, 1984). I have also found this to be true in both *bcl-2* transgenic and wild-type optic nerves. This implies that the degenerated axons are cleared very rapidly during development just as cells that die by PCD are, consistent with the possibility that the axon degeneration is an active process that triggers rapid phagocytosis much as PCD does. Evidence that axon degeneration is not a passive process comes also from studies of the Wld^S mutant mouse, where Wallerian degeneration of both CNS and PNS axons is delayed in cut axons both *in-vivo* (Perry et al., 1990) and *in-vitro* (Glass et al., 1993). The same phenomenon occurs if NGF-withdrawal

is used to induced the death of cultured sympathetic neurons from this mouse (Deckwerth and Johnson, 1994).

As Wallerian degeneration occurs without the cell body, it presumably involves a mechanism that is intrinsic to the axon. PCD can also occur in cells from which the nucleus has been removed–so called cytoplasts, indicating that a nucleus is not required for PCD (Jacobson et al., 1994). However, a comparison of PCD in cytoplasts and Wallerian degeneration in axons should be made with caution because cytoplasts still contain a greater variety of organelles than do axons. Further evidence that many of the components of the PCD pathway are located in the cytosol and can be activated without a nucleus has come from studies of cell-free systems and from these there is increasing evidence that PCD is mediated by a family of interleukin-1β (IL-1β) converting enzyme (ICE)-like cysteine proteases (reviewed in Martin and Green, 1995). These proteases, now called caspases (Alnemri et al., 1996), participate in a proteolytic cascade and cut key proteins in the cell to kill it neatly and quickly. In this chapter in addition to asking whether Wallerian degeneration can be blocked by *bcl-2*, as described above, I also ask if caspases are involved in Wallerian degeneration. I have used a culture system of dorsal root ganglia (DRG) neurons to investigate this question. I show that the presence of caspase inhibitors can inhibit the degeneration of neurofilaments in cut DRG axons, at least in the short term, suggesting that caspases may be involved in Wallerian degeneration.

I have also used the Wld^S mutant mouse to address the question of whether Wallerian degeneration resembles PCD. The gene that is mutated in the Wld^S mouse has yet to be identified, but it is thought to act locally in the axon to control Wallerian degeneration. The molecular machinery required for Wallerian degeneration is apparently present in mutant

axons, since the axons are capable of rapid degeneration in some circumstances (Glass et al., 1994; George et al., 1995); it seems that the regulation of the programme is somehow abnormal in the mutant. I have used DRG neurons from Wld^S mice to determine whether a high concentration of staurosporine, which is a broad-spectrum kinase inhibitor that has been shown to induce PCD in all nucleated cell tested, would induce Wallerian degeneration in cut axons. These experiments could not be done on wild-type axons, as these rapidly undergo Wallerian degeneration spontaneously. I show that the presence of a high concentration of staurosporine does not trigger rapid neurofilament degeneration in cut Wld^S axons.

Results

The effects of Bcl-2 on RGC survival following axotomy

Normally, when the developing optic nerve is cut behind the eye, RGCs undergo PCD (Snider et al., 1993), and their disconnected axons rapidly degenerate. To determine whether the expression of the *bcl-2* transgene would protect the soma of RGCs from axotomy-induced PCD, I cultured explants of adult retina from transgenic and wild-type mice and, after 4 days in culture, stained them with an antiserum that labels PGP 9.5, a cytosolic enzyme in RGCs. I counted the numbers of RGC somata remaining in the ganglion cell layer of measured lengths of retinal sections and compared them to those in uncultured adult retina. Whereas only 30% of the RGC somata survived in explants of wild-type retina, 90% survived in explants of transgenic retina, indicating that the *bcl-2* transgene protects the RGC soma from axotomy-induced PCD.

The effect of Bcl-2 on RGC axons following axotomy

To determine if the expression of the *bcl-2* transgene protects axons from Wallerian degeneration following transection, I cut the optic nerve of P18 transgenic and wild-type mice and examined the nerves after 4 days. I stained frozen sections with the RT97 monoclonal anti-neurofilament antibody (Wood and Anderton, 1981) to visualise the axon cytoskeleton. Very few intact axons were seen in either the wild-type or transgenic cut nerves, either by RT97 staining or by phase contrast microscopy (Figure 4.1). Thus *bcl-2* expression in RGCs does not protect their axons from Wallerian degeneration, even though it was able to protect the soma.

To confirm the presence of human Bcl-2 in transgenic RGC axons (suggested by the weak staining of axons in frozen sections of adult optic nerves), I cultured explants of retina from neonatal transgenic mice and stained the cultures with anti-human Bcl-2 antibody. As shown in Figure 4.2, RGC axons were strongly Bcl-2$^+$.

Figure 4.1 (Overleaf)

Immunofluoresence and corresponding phase contrast micrographs of frozen sections of normal (A-C) or cut (D-I) P18 optic nerves from wild-type (A-F) or transgenic (G-I) mice. The cut optic nerves were studied 4 days after transection. The sections were stained with either anti-neurofilament antibody (A, D, G) or PI (C, F, I). Arrows point to examples of dead cells. Scale bar = 100 μm.

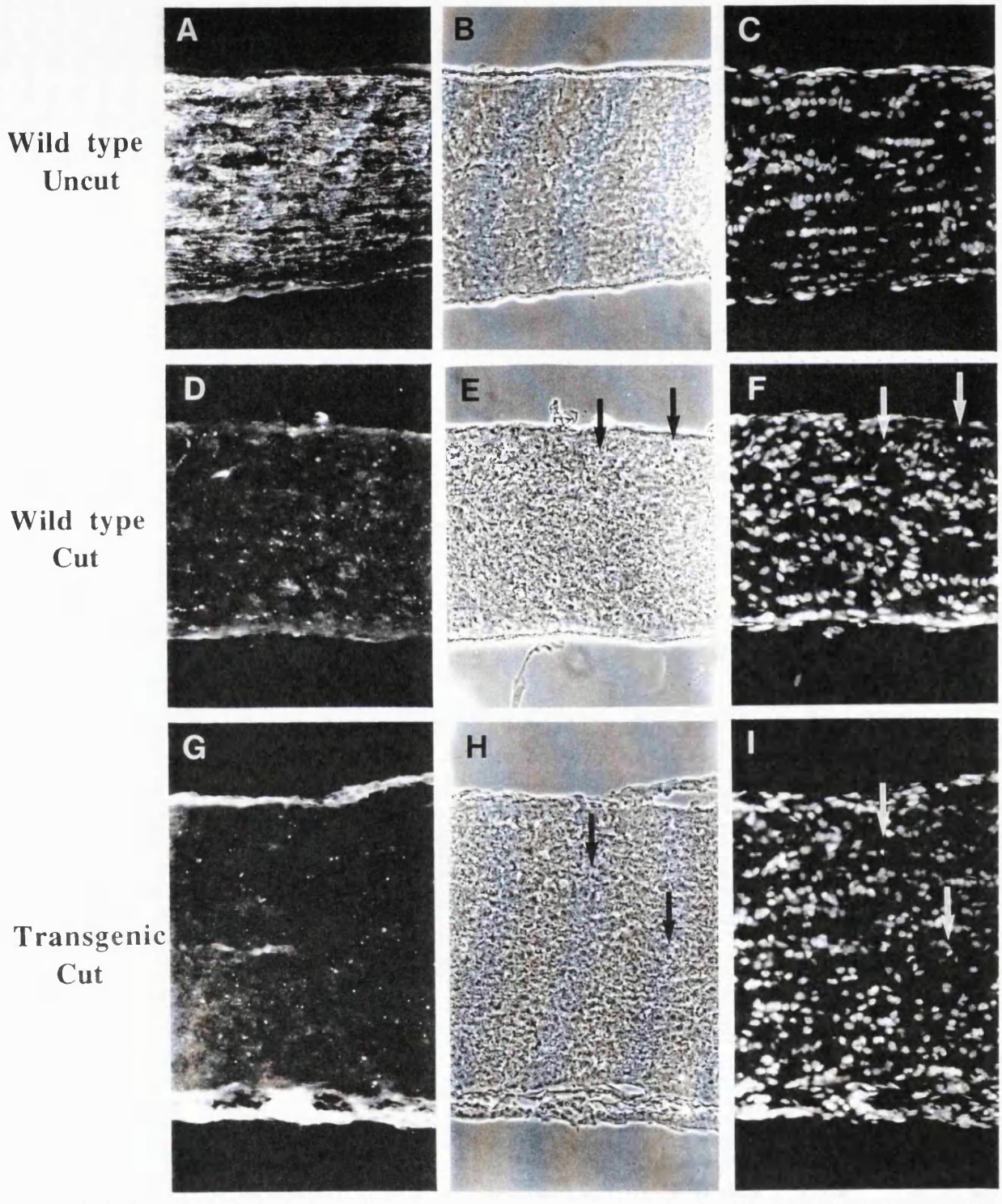
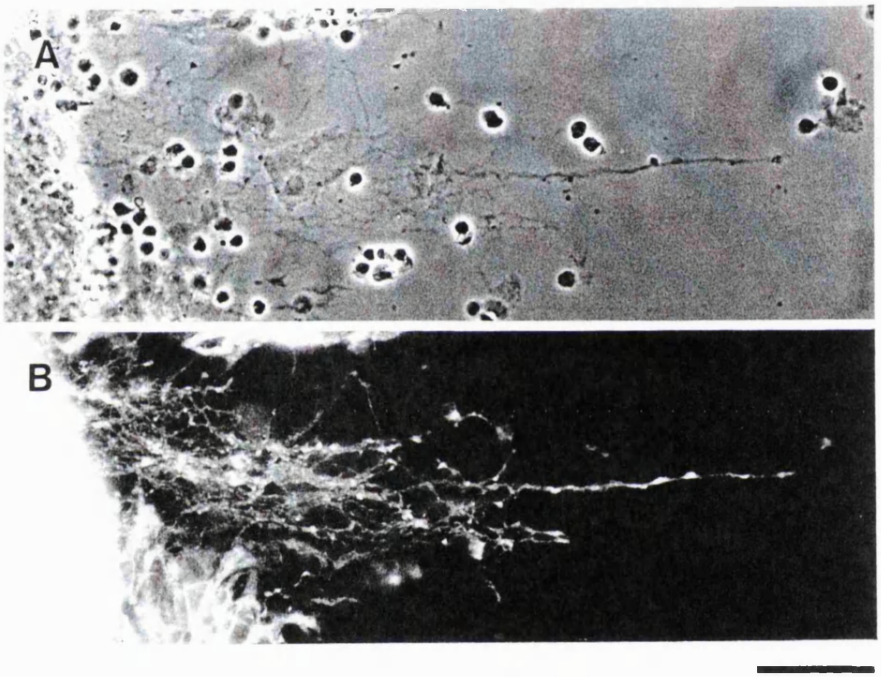

A
B
C
D
E
F
G
H
I
Wild type
Uncut
Wild type
Cut
Transgenic
Cut

Figure 4.2 (Overleaf)

Immunofluoresence (A) and corresponding phase contrast (B) micrograph of a retinal explant from a P1 transgenic (line 73) mouse after 2 days in culture. The culture was stained with an anti-human Bcl-2 antibody. Note that Bcl-2 protein is present in the axons. Scale bar = 50 μm.


A
B

Role of caspases in Wallerian degeneration

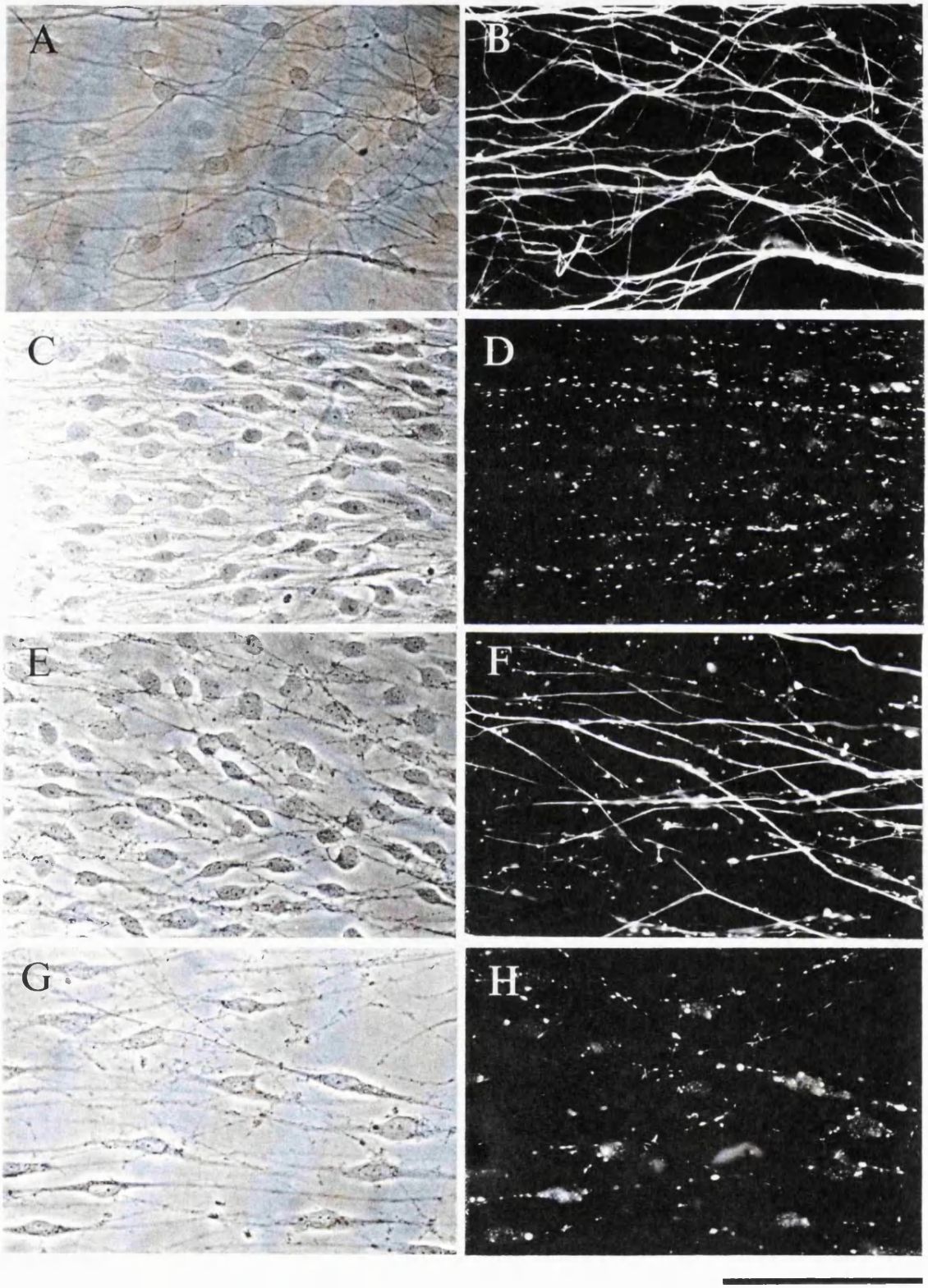
To study Wallerian degeneration in culture, I used mouse DRG neurons. Newborn mouse DRGs were cultured as explants for 7–10 days on laminin and in the presence of NGF. The explants cultured in this way produced extensive neurite outgrowth, far more than could be attained with mouse retinal explants. After 7-10 days *in-vitro*, I cut the axons with a blade, adjacent to the body of the explant, and removed the explant, leaving behind the isolated axons. In previous studies using DRG explants the anti-mitotic drug cytosine arabinoside (AraC) was added to the cultures to prevent Schwann cells from proliferating and migrating out along the axons (Buckmaster et al., 1995). However, I did not use AraC because I found that the Schwann cells associated with the axons helped to secure the axons to the substrate, particularly after the axons were cut.

As described by others, I found that DRG axons from wild-type mice degenerated within 24 hours after they were cut. Staining with RT97 antibody revealed that, although neurofilaments were still visible, most of them were fragmented (Figure 4.3). I tested whether the process of neurofilament degeneration could be prevented by treatment with two peptide caspase inhibitors, Z-Val-Ala-Asp(O-Me)-CH_2F (zVAD-fmk) (Shaw, 1990) and Boc-Asp(O-Me)-FMK (BAF) (Deshmukh et al., in press). zFA-fmk was used as a control because it has a similar structure but would not be expected to inhibit caspases, although it would inhibit capthepsin B proteases for which it was designed (Rasnick, 1985). The cultures were incubated in the presence of 200 μM of these inhibitors for 30 minutes before the axons were cut. Twenty-four hours later the axons were examined by phase contrast microscopy: in all conditions the axons appeared to have degenerated. However, when I fixed and stained them with the RT97 antibody, the untreated and zFA-fmk treated preparations

showed degenerated neurofilaments, while many neurofilaments were intact in the presence of zVAD and BAF (Figure 4.3). There was no difference when zVAD and BAF were added together or individually.

Figure 4.3 (Overleaf)

Phase contrast and corresponding immunofluoresence micrographs of axons in DRG explant cultures of uncut (A-B) or cut (C-D, E-F, G-H) axons from wild-type mice. Cultures of cut axons were treated with vehicle (C-D), zVAD (200 μM) + BAF (200 μM) (E-F), or zFA-fmk (200 μM) (G-H). The cut axons were studied 1 day after transection. The immunofluoresence staining was with anti-neurofilament antibody (B,D,F,H). Scale bar = 100 μm.


A
B
C
D
E
F
G
H

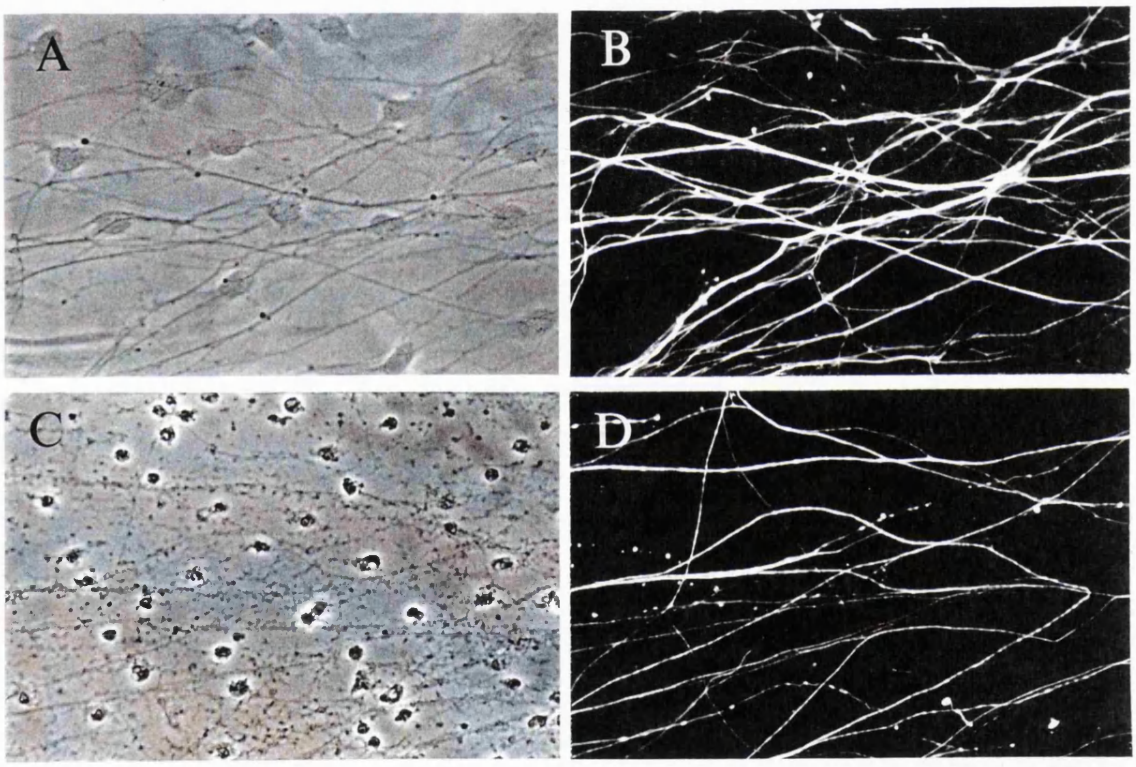
Effect of staurosporine on isolated axons

To test whether axons contain a death programme similar to PCD, I treated isolated axons with a general activator of PCD, staurosporine. To avoid Wallerian degeneration I prepared DRG explant cultures from newborn Wld^S mutant mice. I found, in agreement with other studies (Glass et al., 1993), that Wallerian degeneration of Wld^S axons was significantly delayed compared to wild-type axons: whereas wild-type axons all degenerated within 24 hours, mutant axons remained intact for many days after they were cut.

I found that 24 hours after the addition of a high concentration of staurosporine, with or without cycloheximide (CHX, which inhibits protein synthesis and enhances the PCD–inducing activity of staurosporine), most of the neurofilaments in the treated axons were apparently intact, although all the accompanying Schwann cells in the cultures had undergone typical apoptosis (Figure 4.4).

Figure 4.4 (Overleaf)

Phase contrast and corresponding immunofluoresence micrographs of cut axons in DRG explant cultures from Wld^S mice. Cultures of cut axons were treated with vehicle (A-B) or 5 μM staurosporine in the presence of 10 μg/ml CHX. The cut axons were studied 1 day after transection. The immunofluoresence staining was with anti-neurofilament antibody (B, D). Scale bar = 100 μm.


A
B
C
D

Discussion

Bcl-2 over-expression protects the cell body but not the axon after transection

The expression of the *bcl-2* transgene in RGCs protects the cell body from PCD when the axon is cut, but it does not protect the isolated axon from Wallerian degeneration, even though the transgene-encoded Bcl-2 protein is present in both the axon and the cell body. These results are consistent with other findings that suggest that the mechanisms that control PCD in the cell body and Wallerian degeneration in axons are different.

There are several findings of others that support this suggestion. Deckworth and Johnson (1994) showed that when sympathetic neurons from Wld^S mutant mice are cultured without NGF, their cell bodies undergo PCD, whereas their neurites remain intact. Moreover, when sympathetic (Garcia et al., 1992) and sensory (Gagliaridini et al., 1994) neurons protected by *bcl-2* or the anti-apoptotic viral gene *crm A*, respectively, are deprived of NGF, the cell bodies survive but the neurites usually do not. In addition, *bcl-2* over-expression in a mutant mouse with a progressive motor neuronopathy (the *pmn* mouse) prevents the loss of motor neuron cell bodies, but not of the motor neuron axons, and the progressive weakness and death of the mouse is unaffected (Sagot et al., 1995).

Role of caspases in Wallerian degeneration

There are a number of examples of PCD where Bcl-2 or CrmA do not protect the cell. Thus the failure of Bcl-2 or CrmA to block Wallerian degeneration does not necessarily mean that Wallerian degeneration is

not a form of PCD. To test the possibility that Wallerian degeneration is a form of PCD I tried inducing Wallerian degeneration in cut DRG axons in culture with peptide caspase inhibitors. Whereas the axons did not seem to be protected when assessed by phase contrast microscopy, when I stained them with antibodies against neurofilament protein after 24 hours, I found that the inhibitors prevented the break-up of many of the neurofilaments, suggesting that caspases may be involved in Wallerian degeneration. To my knowledge this is the first evidence to suggest a role for caspases in Wallerian degeneration.

Although calcium-activated proteases (calpains) are thought to be responsible for neurofilament degradation (Zimmerman and Schlaepfer, 1982), and the inhibitors used here do not affect the activity of this class of proteases, my preliminary findings suggest that caspases may act up-stream of calpains in Wallerian degeneration. There is evidence that calpains can also be involved in PCD. For example, calpain 1 is involved in apoptosis induced by protein kinase C inhibition in a neuroblastoma cell line (Behrens et al., 1995). It remains to be determined if the activation of caspases leads to the activation of calpains in PCD (Martin and Green, 1995).

The effect of Staurosporine on isolated axons

The mutation in the Wld^S mouse has been located on chromosome 3, but the product of the gene has not been identified. It seems likely that the normal gene product is involved in activating Wallerian degeneration, but it is not clear how it may act. Staurosporine, especially in the presence of CHX, induces PCD in all nucleated cell types that have been studied. It also induces PCD in enucleated cytoplasts, although it does not kill red blood cells, which do not have a nucleus or other organelles (Weil

et al., 1996). To test whether staurosporine induces PCD in isolated axons, I had to use Wld^S mice, whose axons undergo Wallerian degeneration only after a long delay, as wild-type axons rapidly undergo Wallerian degeneration spontaneously, whether or not they are treated with staurosporine. When cut Wld^S DRG axons are treated with staurosporine (1 or 5 μM) for 24 hr in the presence or absence of CHX, most of their neurofilaments remain intact. All the Schwann cells in the treated cultures, however, show the characteristic changes of apoptosis. Because of the cellular debris from dead Schwann cells, I was unable to determine if the axonal plasma membrane was preserved in these circumstances.

Staurosporine has previously been used in a study of the local control of neurite survival by NGF in neurons from the superior cervical ganglia of newborn rats (Campenot, 1982). In this study it was demonstrated that if NGF was withdrawn from only the most distal axons, only these distal segments degenerated, while the cell body and the rest of the axon was unaffected. When concentrations of up to 1 μM staurosporine were applied to the distal neurites in the presence of NGF, no axon degeneration was seen, in agreement with my findings.

It is unclear whether the failure of staurosporine to induce the degeneration of neurofilaments reflects the lack of a death programme in the axons or the lack of a staurosporine–sensitive activation pathway. My finding that caspase inhibitors blocked the breakdown of neurofilaments in some cut wild-type axons makes the second explanation more likely.

Material and Methods

Retinal explant cultures

Neural retinae were removed from adult mice in phosphate buffered saline (PBS). The whole retina was placed (pigment layer downwards) on a polycarbonate filter floating in 10 ml of DMEM in a Petri dish at 37°C in 5% CO_2. After 4 days the tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate, cryoprotected with 1 M sucrose, and 10 µm frozen sections were cut through the nerve head to the periphery of the retina; every third section was saved. The sections were post-fixed with methanol and stained with antiserum against protein gene product 9.5 (PGP 9.5), which is restricted to horizontal cells and RGCs in the mammalian retina (Bonfanti et al., 1992). The antiserum was diluted 1:1000 in TBLS, applied overnight at 4°C, and visualised with Sh anti-RIg TR. To count the number of RGC cell somata that remained after 4 days in culture, PGP^+ cells in the ganglion cell layer were counted in 40 x 0.25 mm lengths (measured with a graticule), sampled at random from the nerve head to the periphery of the retina.

To determine whether the human Bcl-2 protein is expressed in the axons of transgenic RGCs, as well as in their cell bodies, small fragments of P0 retina were cultured on glass cover-slips coated with PDL and laminin (5 µg/ml). Explants were grown in B-S medium containing 1% FCS and insulin (10µg/ml) at 37°C in 5% CO_2. After 3 days, when axons had grown out from the explants, the cultures were stained with anti-human Bcl-2 antibody as described.

Optic nerve transection

Optic nerve transection using P18 mice was as described in chapter 3. The preparation and PI staining of optic nerve sections from transected nerves was carried out as described in Chapter 2, except that the sections were also stained with the RT97 monoclonal anti-neurofilament antibody (Wood and Anderton, 1981), diluted 1:200 in TBLS, incubated overnight at 4°C and visualised with anti-MIg Fl.

DRG cultures

Whole dorsal root ganglia from either wild-type or Wld^S newborn mice were cultured on glass cover-slips that had previously been coated with PDL and laminin. Single DRG cultures were grown in 0.5 ml of Neurobasal medium supplemented with Sato components and 10 µg/ml NGF, (Boehringer-Mannheim). After 10 days in culture, extensive neurite outgrowth was observed, and axons were cut from the DRGs using a fine blade. Some cultures were treated with the caspase inhibitors zVAD (200 µM), BAF (200 µM) either together or alone, and the control peptide FA-fmk (200 µM), which were added to the medium 30 minutes before the axons were cut (zVAD, BAF and FA-fmk were from Enzyme Systems Products, Inc). In some cases staurosporine (1 or 5 µM) was added instead of the inhibitors, either with or without 10 µg/ml CHX; it was added at the same time as the axons were cut. The vehicle for these additives was DMSO, and an identical volume of this compound was added to control cultures. Twenty-four hours after the axons were cut the cultures were fixed with methanol and stained for neurofilaments with the RT97 monoclonal anti-neurofilament antibody, as described above for optic nerve sections, except that the incubation time with each antibody was reduced to 30 minutes.

CHAPTER 5

GENERAL DISCUSSION

The primary aim of this study was to establish whether RGC axons regulate glial cell numbers in the developing rodent optic nerve and, if so, to determine how they do it. In this final chapter I discuss the various forms of axon-glial signalling that are possible in the optic nerve. Finally, as a secondary issue, I discuss whether the pruning of axon branches, that occurs during normal development may be mediated by an intrinsic death programme in the axons.

Control of glial cell numbers in the optic nerve

The finding that the ratios of the axons to the three major glial cell types in the optic nerve are maintained in the enlarged *bcl-2* transgenic nerve is remarkable. It indicates that the different cell types can interact with one another and with axons to control their proliferation and survival in a co-ordinated manner to adjust their numbers when the number of axons is increased. This finding illustrates why it is advantageous to build animals such that their cells cannot survive or divide without signals from other cells. These social controls ensure that cells only survive and divide when it is appropriate for the animal as a whole. They enable organisms to control cell numbers so that each tissue and organ contains the right number of each cell type.

In this study I have found examples of the two main ways that animals control cell number—controls on cell survival and controls on cell proliferation. In the rodent optic nerve axons regulate astrocyte numbers solely by stimulating astrocyte proliferation. They regulate oligodendrocyte numbers mainly by controlling the survival of oligodendrocytes, and probably of their precursors.

Taken together with previous findings, my results suggest that RGC axons use at least three distinct mechanisms to regulate glial cell

proliferation and survival in the developing optic nerve. (1) They can promote the survival of newly-formed oligodendrocytes (Barres et al., 1992) by a mechanism that does not depend on either electrical activity (Barres and Raff, 1993) or axonal transport (Burne and Raff, 1997). (2) They can promote the proliferation and/or survival of oligodendrocyte precursor cells by a mechanism that depends on electrical activity (Barres and Raff, 1993) but not on fast axonal transport (Burne and Raff, 1997). (3) They can stimulate astrocyte proliferation by a mechanism that depends on axonal transport but not on electrical activity (Burne and Raff, 1997). Sadly, the molecular bases for these interactions are still unknown.

The molecular basis of axon-glial signalling

In principle, one of the ways that RGC axons could signal to glial cells in the optic nerve is via secreted signals. A number of growth factors and cytokines have been shown to promote the survival of newly-formed optic nerve oligodendrocytes and their progenitors in culture (Barres et al., 1993a), and specifically PDGF *in vivo* (Barres et al., 1992). It is not known if any of these cytokines or growth factors are normally involved in survival signalling from axons to oligodendrocyte lineage cells *in vivo*.

I have demonstrated that the axon signal(s) that stimulates astrocyte division must be transported down the axon. The signal seems to be short lived because it is rapidly lost from the Wld^S axons after axotomy, over the time that the oligodendrocyte survival signal remains (Barres et al., 1993b). I have shown that bFGF is a mitogen for optic nerve astrocytes in culture. bFGF does not have a classical signal for secretion (Abraham et al., 1986), and it remains a mystery how it is released by cells. Other members of the family do have conventional signal sequences, however. At the moment one or more members of the FGF family have to be strong candidates for

the astrocyte mitogen that is axonally transported. Unfortunately, it is not known which other FGF, if any, RGCs make, whether it is transported down the axon or released by them.

There is little evidence how mammalian axons might release cytokines or growth factors along their lengths and none that they communicate via gap junctions. During the development of the rat optic nerve it is however, possible with an electron microscope, to observe vesicular elements that appear to fuse with the axolemma, but it is not clear whether these transport vesicles are carrying material into the axon or releasing material to the extracellular space (Hildebrand and Waxman, 1984). Until a secretary pathway for cytokines is demonstrated in mammalian axons, it seems sensible to assume that the signalling between axons and glial cells in the optic nerve is mediated by cell–cell contact.

RGC axons probably signal to glial cells in the optic nerve via cell surface bound signals. The signal(s) from axons that is responsible for the promotion of oligodendrocytes survival is independent of both activity and axon transport and therefore presumably involves membrane to membrane interactions or short range soluble factors. A similar situation seems to exist in peripheral nervous system. Schwann cell precursors seem to be over-produced during development in a way that is analogous to the oligodendrocytes lineage cells in the central nervous system (Jessen et al., 1994). Schwann cell and oligodendrocyte development are also alike in that in both cases an axon–derived survival signal(s) is involved. Members of the neuregulin family (reviewed in Mudge, 1993) have been implicated in the survival, maturation and proliferation of Schwann cell precursors (Dong et al., 1995). They have also been shown to promote the survival of oligodendrocyte lineage cells in culture (Canoll et al., 1996). It may be therefore that neuregulins will turn out to be involved in the axon

surface signal that promotes the survival of oligodendrocyte lineage cell survival *in vivo.*

Another molecule that may be involved in axon-glial signalling is the Shh expressed by RGCs as it probably diffuses down the axon (Jensen and Wallace, 1997), but whether Shh plays any role in glial cell development is currently unknown.

Axon-glial signalling in the optic nerve may be indirect. One such pathway would be from axons to oligodendrocytes and precursors via astrocytes because they are known to make a number of the growth factors and cytokines shown to promote the survival of newly-formed optic nerve oligodendrocytes in culture. The decrease in the number of dividing oligodendrocyte precursor cells seen after an intraocular TTX injection can be prevented by the administration of PDGF (Barres and Raff, 1993), but it is still not known whether electrical activity in axons is required for the production or release of mitogens such as PDGF, or whether the axons or astrocytes are the source of the mitogens. Activity dependent axoglial signalling in the optic nerve is probably mediated via the extracellular space because there is no evidence that axons and glial cells in the optic nerve make either synaptic or gap-junction contact with one another. There is however, evidence that glutamate is released along optic nerve axons via the reversal of the glutamate transporter, which is dependent on sodium and potassium gradients (Kriegler and Chiu, 1993). Astrocytes have the capacity to respond to glutamate released from axons since they express receptors for glutamate, as well as for other neurotransmitters (Barres, 1991). If astrocytes are the main source of mitogens such as PDGF, mitogen release could be mediated by activity–dependent axon-glial signalling of this type.

Is there local control of axon degeneration?

During the development of the mammalian nervous system widespread and extensive remodelling of axon branches and terminals takes place. For example, synapse elimination (Purves and Lichtman, 1980) and axon branch loss (O'Leary et al., 1981) are important processes in neural development. This could involve the rapid degeneration of branches and terminals that are eliminated or it could be that the branches and terminals simply withdraw into the nearest axon branch or axon trunk.

The question of whether axon branches are locally withdrawn, or degenerate locally, could be addressed by examining the nervous system of the Wld^S mouse, as one would predict that if local axonal degeneration does occur, it would be delayed in these animals whereas axon withdrawals should occur normally (Deckwerth and Johnson, 1994).

There are two lines of evidence that favour the possibility that axon branches could degenerate during withdrawal. First, the local control of neurite survival by NGF has been demonstrated in compartmentalised cultures (Campenot, 1982). In this system it is possible to selectively withdraw NGF from peripheral neurites, while leaving the cell body and proximal neurites exposed to NGF: in these circumstances only the neurites deprived of NGF degenerate. Campenot suggests that this is a possible explanation for the eliminating of axon collaterals during development, where it is though that there is a competition for limiting amounts of neurotrophins. Second, axon branch degeneration has been demonstrated *in vivo* in dye labelled chick retinotectal axons during course corrections and axon remodelling. Aberrant projections were seen

to become dye-labelled debris without ganglion cell death (Nakamura and O'Leary, 1989).

By analogy the disappearance of apoptotic cells during development, the removal of axon fragments would presumably be very rapid. If so, it would be hard to detect microscopically. This may explain why there is a widespread belief amongst neuroscientists that axon remodelling in the nervous system occurs via terminal and branch withdrawal rather than by local degeneration.

My findings suggest that caspases may operate during Wallerian degeneration, which in turn suggests that axons and their branches may well contain the protein components required for self–destruction. If so, it seems likely that these components are used in axonal remodelling that occurs during development, and perhaps during the plasticity now known to occur during learning and memory. This possibility deserves further study.

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