RETINAL GANGLION CELL LOSS AFTER DIFFERENT TYPES OF AXOTOMY IN THE OPTIC NERVE

by

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RETINAL GANGLION CELL LOSS AFTER AXOTOMY
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ABSTRACT

To investigate differences in neuronal responses to axotomy in the mammalian central nervous system, I compared the patterns of retinal ganglion cell (RGC) survival after intracranial optic nerve (ON) cut or crush in adult rats. After ON cut, approximately 40% of the RGC's were lost in the second week, followed by a more protracted cell loss, while ON crush gave rise to a slow RGC loss beginning at one week. Retrograde axonal degeneration and macrophage invasion into the ON stump was more marked after ON cut than crush, but progressive retrograde degeneration of axons into the retina did not precede the onset of RGC loss. In parallel experiments, I found evidence that brain derived neurotrophic factor mRNA is expressed in non-neuronal cells of the uninjured ON. These observations suggest that events other than neuron-target separation play a role in the timing and severity of neuronal loss after axotomy.

RESUME

Dans le but d'étudier les réponse des neurones du système nerveux central des mammaliens à différents types d'axotomie, j'ai comparé les modes de survie des cellules ganglionnaires de la rétine, soit après une section totale, soit après un écrasement du nerf optique dans sa portion intracrânienne. Une section du nerf entraîne, sur une période de deux semaines, la mort de près de 40% de la population totale des cellules ganglionnaires, une mort qui se prolonge ultérieurement. L'écrasement du nerf optique entraîne, quant à lui, une phase de mort neuronale nettement plus lente et qui ne débute qu'une semaine après l'axotomie. Il me faut souligner que la dégénérescence, rétrograde et progressive des axones du nerf optique est un phénomène qui ne précède pas la mort des cellules ganglionnaires. Cette dégénérescence ainsi que l'invasion de cellules macrophages dans la rétine sont plus marquées après une section qu'après un écrasement. Pour explorer les mécanismes moléculaires impliqués dans ces différences de réponses à l'axotomie, j'ai, dans des expériences parallèles, montré que l'expression de l'ARN messager du BDNF (Brain Derived Nerve growth Factor), existait au niveau des cellules non-neuronales du nerf optique intact. Toutes ces observations suggèrent qu'après une axotomie, des évènements, autres que la séparation neurone-cible, ont une incidence sur la sévérité et l'initiation temporelle de la mort neuronale.

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LIST OF ABBREVIATIONS

BDNF - brain derived neurotrophic factor
bFGF - basic fibroblast growth factor
BSA - bovine serum albumin
cAMP - cyclic adenosine monophosphate
CNS - central nervous system
CNTF - ciliary neurotrophic factor
diI - 1,1 dioctadecyl 3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMSO - dimethylsulfoxide
EDTA - disodium ethylenediamine tetraacetate
FITC - fluorescene isothiocyanate
GAP43 - growth associated protein 43
GCL - ganglion cell layer
GFAP - glial fibrillary acidic protein
HRP - horseradish peroxidase
IgG - immunoglobin G
IL-1 - interleukin-1
IL-2 - interleukin 2
MAP2 - microtubule associated protein 2
MF - myelinated fiber
mRNA - messenger ribonucleic acid
NGF - nerve growth factor
NT-3 - neurotrophin 3
NT-4 - neurotrophin 4
NT-5 - neurotrophin 5
NMDA - N-methyl-D-aspartate
ON - optic nerve
PBS - phosphate buffered saline
PNS - peripheral nervous system
RGC - retinal ganglion cell
SDS - sodium dodecyl sulfate

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CLAIMS FOR ORIGINALITY

I have documented, in adult Sprague-Dawley rats, with intracranial optic nerve interruption by cut or crush, that:

1) Retinal ganglion cell loss is delayed for approximately one week after intracranial optic nerve interruption by cut or crush.

2) The pattern of retinal ganglion cell loss is dependent on the type of lesion: there is an early, rapid cell loss and a later, protracted cell loss after optic nerve cut, while retinal ganglion cells are lost at a single, slow rate after optic nerve crush.

3) Retrograde degeneration of retinal ganglion cell axons in the optic nerve and retina occurs more rapidly after optic nerve cut than crush.

In normal Sprague-Dawley rats, I have presented preliminary evidence that,

1) Brain derived neurotrophic factor mRNA is expressed in the optic nerve.

CHAPTER 1: INTRODUCTION

The aim of this chapter is to provide a review of the current literature pertaining to the events which follow axon injury as an introduction to the experiments presented in this thesis. Firstly, I outline the events which are known to occur in neuronal and non-neuronal cells after axotomy of cells in the peripheral nervous system (PNS) and central nervous system (CNS) in general and retinal ganglion cells (RGCs) in particular. Secondly, I present the mechanisms that have been proposed for developmental and injury-induced cell death in the nervous system. Thirdly, I examine the role of trophic factors in the survival of neurons after injury and fourthly, I describe the anatomical features of the paradigm used in the present study, the rat retinotectal system. Following this review, I present the rationale for undertaking the experiments described in this thesis.

I. RESPONSES TO NEURONAL AXOTOMY

A. Neuronal Responses to Axonal Injury

The neuronal responses that accompany axotomy involve the entire neuron, both structurally and functionally. These events include morphological, electrophysiological and molecular changes and in the most severe case, neuronal death. Despite the lack of a known mechanism for the signaling of these cellular events after injury, they have been extensively documented in many systems. In this section, I outline the events which occur after injury in the CNS and PNS, presenting examples from the literature. The responses documented for axotomy

of RGCs, the paradigm used in the present study, are given in detail in a later section (see Section D).

Axotomy results in the separation of the axon into a proximal (nearest the soma) and a distal (nearest the terminal) segment. The morphological changes resulting from axotomy, therefore, can occur in two parts of the neuron in relation to the site of injury. Morphological events occurring in the distal portion of the nerve are termed orthograde or Wallerian degeneration. Wallerian degeneration is characterized by a breakdown of axons, followed by their phagocytosis by invading macrophages (reviewed by Fawcet and Keynes, 1990). It is reported to proceed more rapidly in the PNS compared to the CNS (Perry, 1987, Stoll, 1989) and after PNS cut compared to crush (Lunn et al., 1990).

Several morphological events that occur in the proximal axon stump and the attached cell soma are termed retrograde degeneration. Within the proximal portion, as in the distal stump, the axons are lost and subsequently phagocytosed. This process occurs rapidly after transection of the ON (Richardson et al., 1982) and peripheral nerves (Hendry and Campbell, 1976; Aldskogius et al., 1980). In the soma, the retrograde response involves chromatolysis, nuclear eccentricity, nucleolar enlargement and hypertrophy. Chromatolysis is observed as a breakdown of the Nissl bodies (aggregates of granular endoplasmic reticulum and ribosomes). Chromatolysis is accompanied by an enlargement of the nucleolus and often increases in nucleolar and perikaryal volume that result in cell swelling (review: Lieberman, 1974; Barron, 1983 a,b, 1989). However, not all cells react to axotomy by cell swelling; some dorsal root ganglion neurons, for example, shrink after axotomy (Verge et al., 1989).

Neuronal surface changes, including the formation of new processes from the soma or dendrites is another morphological response to injury observed in

some systems. For example, Engel and Kreutzberg (1988) reported chromatolysis, cell surface invaginations and the formation of flap-like processes after axonal transection of guinea pig dorsal vagal motor neurons.

In addition to morphological changes, the electrophysiological properties of neurons are also altered after axotomy. Electrophysiological changes include variations in axon conduction velocity, membrane potentials and synaptic potentials (reviewed by Mendell, 1984; Purves, 1976; Titmus and Farber, 1990). In general, conduction velocity in large myelinated neurons decrease accompanied by a decrease in axon diameter, action potential waveforms are altered, the initial segment-axon hillock region decreases in excitability and synaptic transmission decreases

Axotomy can also affect neurotransmitter production by the affected neurons. For RGCs, the putative neurotransmitter is N-acetylaspartylglutamate (NAAG) Although the expression of this molecule decreases in RGC targets after ON lesion (Moffet et al.,1991), it has not been determined if NAAG synthesis decreases in the RGC cell bodies.

Axotomy induces changes in the molecular functioning of the neuron as well, including protein levels, axonal transport and gene expression (reviewed by Purves, 1976; Barron 1983 a,b, 1989; Grafstein 1983, 1986). Protein levels can either rise or fall after axotomy For example, axotomy of dorsal root ganglion axons resulted in increased levels of growth associated protein, GAP43 (Verge et al., 1990a), and a 57 kDa intermediate filament protein (Oblinger et al., 1989), while causing a decrease in the levels of medium neurofilament subunit (Verge et al., 1990b) and the low affinity nerve growth factor (NGF) receptor (Verge et al., 1989). Protein levels may also change in tissues near the injured neuron; for

example, increased 5' nucleotidase was observed in rat superior cervical ganglion after pre- and post-ganglionic axotomy (Nacimiento and Kreutzberg, 1990)

Anterograde axonal transport, which moves organelles and materials from the soma to the terminals is divided into two classes: fast and slow axonal transport. The pattern of and levels of protein transported can be altered following axotomy. For example, neurofilaments, which are transported into the axon and influence the caliber of the axon, show decreased expression after sciatic nerve transection (Verge et al., 1990). Retrograde transport of molecules may also be altered after injury. Singer and Meher (1980) reported that motor neurons increase their uptake of 2-deoxyglucose, a metabolic marker, immediately after injury. The increased retrograde transport of certain molecules may be an important signal for degeneration because blockage of axonal transport at the time of the increased uptake delayed the onset of chromatolysis (Singer et al., 1982)

Gene expression is also changed following axon injury, both in the messenger RNAs (mRNAs) produced and the level of their production. For example, the expression of high molecular weight microtubule associated protein, MAP2, is decreased after axotomy of the hypoglossal nucleus of the adult rat (Svensson and Aldskogius, 1992). Armstrong et al. (1991) used *in situ* hybridization to show that transection or crushing of the hypoglossal nerve causes a decreased expression of choline acetyl transferase mRNA and an increased expression of nerve growth factor (NGF) receptor mRNA.

The most drastic consequence of axotomy, neuronal death, has been documented in many systems. In the peripheral nervous system, neuronal loss is not severe: Devor et al. (1985) calculated the rate of primary sensory neuron loss after sciatic nerve lesion as 8% per 100 post-operative days. In the CNS, cell death after axotomy is more pronounced. Neurons in the rat medial septum and

vertical limb of the diagonal band of Broca undergo cell death after the fimbra-fornix is transected, such that 2 weeks after injury, 50% of the total neurons (70% of the cholinergic neurons) are lost in the medial septum and 30% of the total neurons (40% of the cholinergic neurons) are lost in the vertical limb (Williams et al., 1986) and 4 weeks after injury 50% of the cholinergic neurons are lost from both structures (Hefti, 1986)

These responses to axotomy are not uniform after every injury, but are influenced by many variables. These influences will be discussed in Section C and will be addressed in this thesis.

B. Responses of Non-neuronal Cells to Neuronal Injury

The effects of neuronal damage are not restricted to the neurons themselves. The glial elements of the nervous system, both macro- and microglia, as well as the immune system elements, especially macrophages, are active components of the events that follow axon injury In this section, I examine some of the responses of macroglia, microglia and macrophages to PNS and CNS injury.

1. Macroglial Response

The morphological response of macroglia, including Schwann cells, astrocytes and oligodendrocytes, to neuronal injury has been extensively examined (Thomas, 1970; Raff et al., 1987; Reier, 1989). However, more recent investigations, focusing on the molecular glial response, have indicated that glial cells are capable of supplying growth factors to injured neurons.

Axon injury results in hypertrophy of astrocytes and an increase in their content of glial fibrillary acidic protein (GFAP). This phenomenon has been described in many systems, including motoneuron transection (Kreutzberg et al.,

1989) and the astrocytes and Müller cells of the retina after ON transection (Miller and Oberdorfer, 1981). In motoneurons, capable of regeneration after interruption of their axons in the PNS, this initial astrocyte response is known as the early response, occurring approximately two days after injury and is followed by a delayed astrocyte reaction that is characterized by the reshaping of cell processes to form long extensions (Graeber and Kreutzberg, 1990) In the CNS, astrocytic hypertrophy and proliferation creates a mass of astrocytes at the site of injury called an astrocytic scar. It has been suggested that this astrocytic gliosis is inhibitory to axonal growth (Reier, 1983, 1989) However, the role of astrocytic gliosis after injury is not clearly defined.

In the PNS, Schwann cells have been proposed as an essential component in the supply of NGF. Sciatic nerve injury results in the expression of NGF and the NGF low affinity receptor by Schwann cells (Taniuchi et al., 1986, Heumann et al., 1987b). The target of this NGF was assumed to be the injured neuron However, the sensory neurons decreased their production of NGF receptors after axotomy until regeneration was completed (Raivich and Kreutzberg, 1987) This reduction in receptor expression is accompanied by a decrease in the axonal uptake and retrograde transport of NGF after axotomy (Raivich et al., 1991) These authors suggest that the denervated Schwann cells may become the target for the large amounts of NGF produced after injury.

In the CNS, astrocytes are also reported to produce growth factors *in vitro* and *in vivo*, indicating that gliosis after injury may not be inhibitory Furukawa et al. (1987) reported the production of NGF associated with cell growth in cultured mouse astroglial cells. Vigé and colleagues (Vigé et al., 1991, Carmen-Krzan et al., 1991) showed that NGF secretion and NGF mRNA expression by neonatal rat cortical astrocytes was upregulated by the application of interleukin-1 (IL-1) or

basic fibroblast growth factor (bFGF) to the cultures. Rudge et al. (1992), showed the expression of NGF, ciliary neurotrophic factor (CNTF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) mRNA in cultured rat hippocampal astrocytes. Bakhit et al., (1991) reported that *in vivo* NGF immunoreactivity of astrocytes was increased after destruction of hippocampal neurons. Basic fibroblast growth factor (bFGF) was also found to be produced by astroglial cells *in vitro* (Araujo and Cotman, 1992) and immunohistochemically identified in astrocytes *in vivo* (Ferrara et al., 1988; Hatten et al., 1988). In the rat ON, Lu et al. (1991) found an 8-fold increase in NGF expression after enucleation. This response must be non-neuronal since there are no neuronal cell bodies in the ON.

Thus, in both the PNS and CNS, glial cells respond to axonal injury by morphological and molecular changes and play an important role in determining the availability of growth factors although the target of these growth factors has not been determined.

2 Macrophages and Microglia

Injury to the nervous system, as in any other tissue, may result in an immunological response such as inflammation. The CNS is characterized as an immunologically privileged site, but this term refers more to the intrinsic level of immune components rather than the ability to activate an immune response. Recently, macrophages have been recognized as important components of the events that follow axotomy in the PNS and the role of invading macrophages and microglia, the resident brain macrophages, in CNS degenerative events is beginning to be elucidated.

Macrophages and microglia are two components of the mononuclear phagocytic system and both have been reviewed extensively (Nathan, 1987, Johnston, 1988; Perry and Gordon, 1988; Papadimitriou and Ashman, 1989; Streit et al., 1988; Thomas, 1992). Their functions include many aspects of lipid metabolism, regulation of granulocyte and erythrocyte pools, host defense against microorganisms, wound healing, host defense against neoplasm and scavenging (Papadimitriou and Ashman, 1989) They secrete over one hundred different substances, including hormones, growth factors, complement components, coagulation factors, enzymes, cell matrix or cell adhesion proteins, lipid, reactive oxygen and nitrogen intermediates (Nathan, 1987). These partial lists of functions and secreted factors indicate the complexity of these cells Their role in the nervous system, especially after injury, has recently been the focus of many neurobiologists and the accumulating data suggests that they are important determinants of the neuronal response to injury

In the PNS, compared to the CNS, the removal of debris in the distal axon stump is completed very quickly (Perry et al., 1987). In the sciatic nerve, after transection, macrophages are recruited into the distal stump within the first 3 to 5 days, phagocytosing myelin debris Heumann and colleagues (1987) reported that sciatic nerve transection resulted in increased NGF mRNA in Schwann cells and fibroblast-like cells of the distal stump This increase occurred in two peaks, the first was observed within 6 hours of injury and is reported to be induced by c-fos activation (Heumann, 1990) The second peak occurred between the second and seventh day after transection. This second peak is induced by macrophages that secrete IL-1 (Heumann et al., 1987b; Lindholm et al., 1987, Heumann et al., 1990), prompting Schwann cells to upregulate the expression of NGF and NGF low affinity receptor (Heumann, 1987b). Following the reinnervation of the

stump, the level of NGF receptor mRNA decreases in the Schwann cells (Taniuchi et al., 1988) and when the macrophages leave the nerve stump, the levels of NGF mRNA return to normal (Heumann, 1987b; Johnson et al., 1988).

The relationship between macrophages and NGF production in the PNS is substantiated by observations in the C57BL/Ola mouse by Brown et al. (1991). These mice display very slow Wallerian degeneration (Lunn et al., 1989). Brown et al. (1991) reported that cutting the saphenous nerve in the C57BL/Ola mouse resulted in reduced macrophage recruitment and near normal mRNA levels for NGF and its receptor. In addition, sensory axons, which depend on NGF, showed impaired regeneration while motor axons, independent of NGF, showed normal regeneration. Thus, in the PNS, macrophage invasion is an important determinant of axonal regeneration.

In contrast to the PNS, the role of macrophages and microglia in the CNS is less defined. Microglia are the third most abundant cell type in the CNS, after neurons and macroglia (Vrabac, 1970). Within the rabbit retina, microglia are reported to respond to ON transection by swelling, increasing NADPase activity and transiently increasing in density (Schnitzer and Scherer, 1990). Retinal microglia, as identified by OX-42 immunoreactivity, were found to phagocytose RGCs and retain the retrograde neuronal tracer dil after ON interruption in the adult rat (Villegas-Pérez et al., 1992). Differences between microglial responses in the rat neonate and adult were examined by Thanos (1991). The retinal microglial cells that phagocytosed RGCs during developmental cell death were also present in the adult and were responsible for RGC phagocytosis following axotomy-induced cell death. After transection of motoneurons, microglia leave their perineurial positions and migrate to the neuropil (Kreutzberg et al., 1989). They become activated microglia (proliferating but non-phagocytic), increase in

CR3 complement receptors and express vimentin and major histocompatability class I antigens. If degeneration results, for example, from application of the toxic lectin from *Ricinus communis*, the microglia become brain macrophages and are capable of phagocytosis (Kreutzberg et al., 1989) Microglia, as illustrated by these examples, are capable of undergoing morphological, molecular and functional changes after neuronal injury.

In tissues near the site of injury, microglia are difficult to distinguish from invading macrophages Using recently developed microglial and macrophage markers, Milligan et al., (1991) found that in the developing brain, visual cortex lesions resulted in faster debris clearing by invading macrophages and less scarring compared to lesions in the adult, where primarily the microglia respond Ludwin (1990), however, reported that in the rat ON, Wallerian degeneration after enucleation was characterized by debris clearance achieved by macrophages, microglia and to a small degree, oligodendrocytes

David et al (1990) suggest that macrophages may improve the ON's permissiveness to axon growth after injury. They determined *in vitro* that the permissiveness of the area near transection was associated with an abundance of macrophages. In addition, normal ONs treated with activated macrophages became permissive to neurite growth Macrophages are capable of stimulating astrocytic gliosis (Giulian et al , 1989) and astrocytes are known to secrete neurotrophic factors (Rudge et al , (1992)

In contrast, in the C57BL/Ola mouse, a strain of mice characterized by impaired macrophage recruitment, the rate of RGC loss after ON transection is slowed compared to rats with normal macrophage invasion after injury (Lunn et al., 1991). Although the link between the lack of macrophages and greater cell

survival has not been discovered, this observation supports the suggestion that macrophages are deleterious to RGC survival.

The role of microglia and macrophages in the CNS after injury is an area of active research. At present, no definitive role has been elucidated but the data suggests that they are potentially important factors in determining the response of CNS neurons to axon interruption. In the present study, the response of non-neuronal cells to optic nerve transection will be examined to further define their role in the CNS response to injury.

C. Variables that Influence Responses to Axonal Injury

The response, neuronal and non-neuronal, to axotomy is influenced by a number of variables, including the animal species and age, type of neuron, location and type of injury (reviewed by Lieberman, 1974). The species of animal is an important determinant of the type of neuronal response, especially in the CNS. CNS neurons of mammals show some evidence of abortive sprouting, but are unable to regenerate after injury. In contrast, CNS neurons in fish are able to regenerate and restore function after axotomy (reviewed by Grafstein, 1986).

The age of the animal also influences the response to neuronal injury. For example, intraorbital ON crush in mice results in more marked axonal degeneration in neonates when compared to adults; 0 to 20% of RGCs (75-155 μm^2) survived 10 days after neonatal injury while 20 to 40 % of RGCs survived 20 days after lesion in the adult (Allcutt et al., 1984). This response, known as the Gudden effect, occurs in most systems (reviewed by Lieberman, 1974).

The type of neuron is a third factor influencing the response to neuronal injury. Neurons of the mammalian CNS are unable to regenerate while PNS axons in the same animal are capable of regeneration and functional recovery (Ramon y

Cajal, 1914). A study comparing cell death after axotomy of dorsal motor vagal neurons and hypoglossal neurons revealed that despite the use of similar protocols, cell death was greater in the dorsal motor vagal neuron; 70% and 25% of cells were lost 164 days after axotomy of the dorsal motor vagal and hypoglossal neurons respectively (Aldskogius, 1980)

The location of the injury along the length of the axon also determines the nature and extent of the response to injury In the CNS, the pattern of RGC loss after ON interruption at four different distances from the eye has been investigated by Villegas-Perez et al (1992) The loss of RGCs was more severe after lesions closer to the eye and the pattern of cell loss was also altered by the level of the injury. This observation is supported by earlier quantitative studies of RGC loss (Grafstein and Ingoglia, 1982, Allcutt et al , 1984, Misantone et al , 1984, Barron et al., 1986) A comparison of these reports (see section D) suggests that RGC loss is greater after lesions close to the eye Sofroniew and Isaeson (1988) correlated the loss of cholinergic cells in the septum with the proximity of the neuronal somata to the lesion. lesions within 2500μm caused pronounced cell death (<30% survival), while lesions over 4000μm away did not cause severe cell loss (>80% survival).

The type of injury was described by Lieberman (1974) as "perhaps the most significant variable determining the character of the retrograde response" This statement was based primarily on the response of PNS neurons in mammals and PNS and CNS neurons in frogs and fish because in these neurons, the type of injury determines the success of regeneration the more severe injuries, such as capping or ablation of the distal nerve stump, which do not provide a pathway for the regenerating axon generally do not permit regeneration while brief crushes or freezing allow regeneration.

The rate of Wallerian degeneration in the PNS has been determined following cut, crush and ligation of the sciatic nerve in the adult mouse (Lunn et al., 1990). Degeneration proceeded more rapidly after transection than crushing. Freezing of a large length of nerve or prolonged ligature resulted in rapid degeneration, comparable to cut lesions. Crush lesions where the perineurium was also cut open and the blood supply damaged, and nerve ligature followed by immediate untying resulted in slow degeneration, similar to that seen after crush lesion. The authors suggested that the continuing contact of the proximal and distal stumps promoted slower degeneration.

The influence of the type of injury on responses to axotomy has not been extensively studied in the CNS. In lower vertebrates, Humphrey and colleagues found similar proportions of cell death after ON cut or crush in *Rana pipiens* (Humphrey, 1987) and reported that capping or ligation of the ON in the frog *Hyla moorei* prevented regeneration, caused a delay in RGC death and eventually resulted in greater cell death compared to ON crush. In mammals, there have been several quantitative reports which examined RGC loss after ON cut or crush (Grafstein et al., 1982, Allcutt et al., 1984, Misantone et al., 1984, Barron et al., 1986), but no single report has compared the two types of injury. These reports are described in detail in Section D. A comparison of the data reveals a trend toward greater RGC loss following ON cut compared to crush but the reports cannot be accurately compared due to the differences in the species, methods of producing the lesions and methods of determining RGC counts. Mantz and Klein (1951) qualitatively examined RGC loss after intraorbital ligation and transection and described greater cell loss after transection.

D. Responses of RGCs to Axotomy

The experimental system examined in this thesis is the rat visual system. The RGC has been utilized extensively for the investigation of neuronal responses to CNS injury. This section describes previous experiments performed in the rodent visual system and highlights the major results to provide a background for the present study.

Several early papers reported that in mammals, RGCs were lost after ON injury (Leinfelder (1938); Mantz and Klein, 1951, Hayrs, 1952, Polyak, 1957; Stone, 1965; Lin and Ingram, 1973, 1974, Quigley, 1977; Radius and Anderson, 1978; Cowey and Perry, 1979). Mantz and Klein (1951), in a qualitative study of the retinal ganglion cell morphology found that RGCs were more sensitive to intraorbital ON section than to ON ligature. Leinfelder (1938) and Mantz and Klein (1951) reported that the distance from the soma to the injury influenced the amount of cell death while Radius and Anderson (1978), Quigley (1977) and Lin and Ingram (1973, 1974) claimed that the location of the lesion did not influence survival.

In the past decade, several publications have examined quantitatively the loss of RGCs in the rodent visual system after ON injury (Grafstein and Ingoglia, 1982, Allcutt et al., 1984, Misantone et al., 1984 and Barron et al , 1986, Villegas-Pérez et al., 1992). Grafstein and Ingoglia (1982) determined the number and size of adult mouse RGCs after intracranial ON cut. Three days and 65 days after injury, survival of RGCs was 80% and 50% respectively. The average soma size decreased by 25% of control 3 days after transection, but returned to control values by 90 days.

Allcutt and colleagues (Allcutt et al., 1984) compared the RGC loss and cell size after intraorbital crush in neonatal and adult mice. The cell atrophy and death

was greater after injury in the neonate. In the adult, there was an initial sharp decline until 20 days when 20 to 40% of the cells (75 - 155 μm^2) were present. This was followed by a more gradual decline until 80 days when 0 to 20% of the RGCs remained

Misantone and colleagues (Misantone et al., 1984) examined RGC density following intracranial ON crush in the adult rat. They reported normal RGC densities in the first three months after injury but by 230 days after injury 60% of the RGCs remained. Cell size had declined to 50% of normal 1 month after ON crush. Changes in RGC density and size following intraorbital ON crush in the adult rat were investigated by Barron et al. (1986) They reported a cell survival of 64% at 7 days, followed by a slow cell loss such that 32% of the RGCs remained at 6 months after injury. The cel' size decreased between 28 and 90 days after injury.

A comparison of the loss of RGCs reported in these four publications would suggest that cut injury results in greater RGC loss than crush injury. However, it is difficult to ascertain the accuracy of such a comparison since the species are not consistent and the methods for producing the injury (ie. type of forceps, number and length of time of crushes) are not identical in each report. In addition, the methods used for identification of RGCs within the ganglion cell layer are not definitive and may have resulted in the inclusion of displaced amacrine cells that are also located in this retinal layer (Perry, 1981). In all cases, morphological characteristics (cell size, cell shape, ultrastructural observations) were used to identify RGCs These criteria may not be applicable following injury to the cell when atrophy and chromatolysis occur

A recent investigation in this laboratory utilized a retrograde, fluorescent tracer, diI, to enable measurement of RGC densities without the error of including

displaced amacrine cells (Villegas-Pérez et al., 1992) They quantitated the pattern of RGC loss after ON lesions at different distances from the eye in adult rats In addition to using an improved method for RGC identification, RGC densities were measured at many times after injury, from 2 weeks to 20 months, providing a comprehensive report of the time course of RGC loss.

RGC loss occurred in two phases: an early rapid cell loss and a later, more protracted cell loss. The initial period of rapid RGC loss was more severe after lesions close to the eye than distal lesions. Two weeks after injury RGC densities were 24.7 to 38.5% of control for proximal (0.5mm and 3 mm from the eye) and 57.4 to 65.6% of control for distal (8 mm and 10 mm from the eye) lesions.

RGC loss in the second phase, from 2 weeks to 20 months, was slow and protracted, such that at 12 months after injury, RGC densities were 3.2 to 5.7% for proximal lesions and 20.4 to 20.0% for distal lesions. The one-half survival times after intracranial lesions were approximately 1 month and 6 months for intraorbital and intracranial lesions respectively. A small population (approximately 5%) of the RGCs survived for as long as 20 months after intraorbital axotomy This report established that the location of the injury influenced both the severity and time course of cell death in the RGCs of the adult rat.

The morphological changes which occur in the soma after axotomy (ie chromatolysis) have also been examined in RGCs after injury Barron et al , (1986) describe disaggregation of ribosomal rosettes 3 to 14 days after intraorbital ON crush, a decrease in the number of mitochondria and slight decreases in nuclear size. Misantone et al (1984) also reported chromatolysis in RGCs following ON interruption The atrophy of RGCs after injury is described above in relation to cell death (Grafstein and Ingoglia, 1982, Allcutt et al , 1984, Misantone, 1984 and Barron et al., 1986).

Morphological responses that occur in the axon of RGCs after interruption have also been investigated. Allcutt et al. (1984) examined axons within the retina and ON stump after intraorbital crush. Ten days after injury, fascicles of axons in the nerve fiber layer of the retina were thinner than control retinas, and some axons were beaded and fragmented Axons were also observed growing aberrantly across fiber bundles and in other retinal layers. In the proximal stump, 10 days after injury, fascicles of axons either terminated at the lesion site or looped back into the retina Thirty days after injury the majority of axons in the retinal and proximal stump had degenerated, but some of the remaining axons continued to display aberrant growth. The distal nerve stump contained degrading axons 10 days after injury and by 85 days after injury it was filled with myelin debris.

Richardson et al. (1982) described retrograde degenerative changes in the ON after intracranial ON cut in the adult rat. One week after transection, they observed an area of central necrosis, containing myelin debris and macrophages, that extended 3-6 mm from the cut toward the eye. The peripheral area of the nerve and the portion of the nerve near the eye contained intact fibers. The retrograde degenerative process occurred rapidly: 1 month after cut the number of myelinated axons measured 1 mm from the eye decreased to 10% of normal. After 1 month, axon loss proceeded more slowly and by 4 months the nerve contained mainly astrocytic processes. Kiernan (1984) described the axonal changes after intraorbital ON crush. In the proximal stump, qualitative decreases in axon number were observed from the second week to the end of the study, 34 weeks, when some axons remained

Some of the molecular changes that result from axotomy, including alterations in RNA expression, protein levels and axonal transport, have also been reported in the RGCs of adult rodents. A monoclonal antibody, RT97, recognizing

the phosphorylated form of the heavy neurofilament protein is often used as a tool for visualizing the RGC fibers that traverse the retina. In control wholemounts, the RT97 immunoreactivity is restricted to the axons After axotomy, RT97 immunoreactivity is also observed in the somata of a small population of RGC's (Dräger and Hofbauer, 1984, Vidal-Sanz, PhD Thesis, McGill University, 1990). GAP43, a growth associated protein, is expressed in RGC's during the period of developmental axon growth Following ON transection close to the eye of adult rats, GAP43 immunoreactivity is observed in a subpopulation of RGC's. This immunoreactivity is not observed after intracranial transections (Lozano, 1988, Lozano et al., 1987, Doster et al., 1988).

McKerracher (1990b) investigated the rate of transport of cytoskeletal proteins after ON lesion in the adult rat. One week after crush the anterograde transport of neurofilaments and tubulin were slowed while other proteins were transported at normal rates Thus, selective alterations in the axonal transport of cytoskeletal proteins occur after ON injury.

The investigation of changes in RNA expression after axotomy is an area of active research because the molecular responses to injury have not yet been fully characterized. The expression of growth factors after RGC injury may be an important determinant of cell survival. Lu et al (1990) showed an eight-fold increase in NGF expression in the ON of adult rats one day after enucleation, followed by a decline to normal levels by one week There is no evidence, however, that NGF is a growth factor for RGC's.

E. Modification of Responses to Neuronal Injury

The first requirement for regeneration of neurons after injury is the survival of the neuron. In the CNS, this requirement is often not met and cell death occurs after axon interruption in many systems. Recent experiments which interfere with the normal CNS state, such as the insertion of fetal or PNS grafts or the application of growth factors, have illustrated the capacity of the CNS to alter its response to injury, resulting in a reduction of cell death or the regeneration of the severed axons.

One method successfully used to enhance regeneration of many CNS neurons and decrease the extent of cell death after injury is the attachment of peripheral nerve grafts to a CNS tract (David and Aguayo, 1981; Benfey and Aguayo, 1982; So and Aguayo, 1985). The apposition of a segment of peroneal nerve to the ON at the back of the eye results in the regeneration of up to 10% of the RGC population (Vidal-Sanz et al., 1985). When the PN graft is placed in the main RGC target, the superior colliculus, some of the regenerating axons formed terminal arborizations with pre- and post-synaptic membrane specializations (Vidal-Sanz et al., 1987, Carter et al., 1988, 1989). Electrophysiological testing indicated that some of the regenerated RGC axons responded normally to retinal stimulation with light (Kierstead et al., 1985) and transynaptically activated superior colliculus neurons (Kierstead et al., 1989).

Other CNS tissues capable of regenerating axons into PNS grafts include somatosensory (Benfey and Aguayo, 1981; Vidal-Sanz et al., 1984), motor cortex (Horvat and Aguayo, 1985), olfactory bulb (Friedman and Aguayo, 1985), basal ganglia (Benfey and Aguayo, 1982), thalamus and hypothalamus (Benfey and Aguayo, 1985), hippocampus and amygdala (Aguayo et al., 1983), brainstem

nuclei (Aguayo et al, 1983; Muntz et al., 1985) and spinal cord (Richardson et al , 1980,1982, 1984, Aguayo and David, 1981).

Another approach to modification of CNS responses to injury is the use of fetal brain grafts at the site of injury Bregman and Reier (1986) reported that most axotomized rubrospinal cells could be rescued by inserting fetal spinal cord tissue (E12-14) into midthoracic spinal cord lesions Adult rat RGC's regenerated after ON transection into E16 thalamus and tectum fetal grafts, forming arborizations and terminal varicosities. These results could not be replicated with laminin absorbed to nitrocellulose or an artificial basement membrane (Hausemann et al., 1989). The effectiveness of peripheral nerve and fetal grafts may be attributed to the availability of growth factors and good substrates for growth, including the non-neuronal cells and extracellular matrix

Growth factor application is another method used to reduce neuronal death and promote regeneration after CNS injury NGF causes a reduction in the death of sympathetic neurons (Korsching and Thoenen, 1983) Central cholinergic neurons can be rescued by intraventricular NGF (Hefti, 1986, Kromer, 1987; Montero and Hefti, 1988; Williams et al., 1986) or by secretion of NGF from non-neuronal cells genetically engineered to produce NGF (Rosenberg et al., 1988) Intracerebral infusion of NGF improves retention of spatial memory tasks and partly reverses atrophy of cholinergic cells in aged rats (Fischer, 1987) A combination of NGF infusions and the insertion of fetal hippocampal grafts resulted in more extensive reinnervation of the lesioned septo-hippocampal projection than either treatment performed separately (Tuszynski et al, 1990)

Fibroblast growth factor (FGF) is capable of partially rescuing RGC's after ON cut by application of the growth factor to the ON stump (Sievers et al , 1987). Cholinergic neurons, in addition to being rescued by NGF, are rescued by FGF

after fimbria fornix lesion in the adult rat (Anderson et al., 1988). CNTF has also been reported to influence neuronal responses to injury by preventing motor neuron degeneration in the newborn rat (Sendtner et al., 1991).

These examples of growth factors rescuing axotomized neurons suggest that neuronal survival is intimately linked to the availability of growth factors. It has been suggested that neuronal death is caused by growth factor deprivation in some cases (reviewed by Oppenheim, 1990)

Another means of modifying neuronal responses to injury was reported by Richardson and Issa (1984) One hu ed-fold greater numbers of rat dorsal root ganglion central axons regenerated at a faster rate when peripheral branches were cut. This increase in regeneration was influenced by the distance between the soma and the site of peripheral cut. increased distances resulted in a smaller regenerative response (Richardson and Verge, 1986). The application of colchicine or nerve crush could not produce a similar increase in central regeneration (Richardson and Verge, 1986). However, the injection of *Corynebacteria parvum* to provoke an inflammatory response did enhance regeneration (Lu and Richardson, 1991). Although deterring the glial proliferation induced by axotomy did not reduce the regeneration, the authors suggest that the non-neuronal cells in the dorsal root ganglion may influence the regenerative responses of these neurons after injury.

The successful use of peripheral and fetal grafts, growth factors and conditioning lesions suggests that neurons, if given the correct stimulation, can survive axotomy However, these techniques are unable to rescue the entire population of axotomized neurons This limitation may result from an insufficient supply of the necessary factor(s), the inability of all of the neurons to receive the

aid (ie. proximity to the graft) of the existence of sub-populations of neurons that have different requirements for survival.

II. MECHANISMS OF NEURONAL DEATH

The cellular and molecular mechanisms of cell death are, at present, not fully understood. Some information has been gained from studies of developmental and injury-induced cell death in the nervous system. These studies suggest that the morphology of the dying neuron is not uniform, but depends, in part, on differences in mechanisms that lead to cell death. The two main forms of cell death based on the morphological aspects of the dying process are apoptosis and necrosis (Wyllie, 1981). Apoptosis is characterized by a progressive loss of cell volume and chromatic condensation while necrosis involves cellular edema, plasma membrane rupture and leakage of the cell contents. It is not known, however, if different morphological "forms" of cell death, such as apoptosis and necrosis, share some of the same biochemical and molecular events.

A. Developmental and Injury-Induced Neuronal Death

The neuronal death that occurs after injury and during development has been extensively studied and our present understanding of the mechanisms of cell death has stemmed predominantly from this research. The factors which influence and modify injury-induced cell death have been described in previous sections (Section I. C and E). Developmental cell death is linked to factors such as target tissues, afferents, hormones and interaction with non-neuronal cells and extracellular matrix components (reviewed by Oppenheim, 1991).

Oppenheim (1991) argues that developmental cell death in vertebrates, unlike invertebrates, is not likely to be entirely genetically programmed, but is influenced by many external factors. Due to the coincidence of cell death with the initiation of synaptic connections, cell death in the vertebrate is often attributed to interactions with the target tissue or with afferent inputs. The role of the target tissue in influencing cell death was proposed at the time of the discovery of NGF. Early experiments showed that the size of the NGF-producing target determined the extent of developmental cell death (Oppenheim, 1981, 1985; Hamburger and Oppenheim, 1982; Lamb, 1984). However, the amount of size increase or decrease of the target did not always directly correlate with the effect on cell death (Farel, 1989; Lamb, 1980), suggesting that the control of cell death was not restricted to one factor.

The afferent input is a second factor that is an important determinant of cell death. In the cochlear nuclei of the rat, a reduction in the afferent input before or during cell death results in increased neuronal death (Trune, 1982). This relation to afferent input is also evident in many other systems including the rat retina (Linden and Perry, 1982) and in the marsupial cat superior colliculus (Crewther et al., 1988). Dissociated neonatal RGCs *in vitro* are also dependent on afferent input; blocking synaptic activity by applying tetrodotoxin or low-Ca^{2+}/high-Mg^{2+} medium resulted in cell death (Lipton, 1986).

Neuronal activity may also influence neuron survival. The blockade of neuromuscular activity in the chick embryo during the period of normal motoneuron death resulted in increased neuron survival (Oppenheim, 1987) which does not appear to be related to a retardation of motoneuron differentiation (Oppenheim et al., 1989). This phenomenon has been observed in other systems (reviewed by Oppenheim, 1991).

Hormones have also been implicated as determinants of developmental cell death. Thyroxin regulates the onset and rate of naturally occurring motoneuron death in the frog spinal cord (Kollros, 1981) where lower levels decrease cell loss. In the moth, *Manduca sexta*, ecdysteroid hormones control the timing and initiation of the degeneration of a set of motoneurons and their target muscle (Trueman and Schwartz, 1984). In the rat, sexual dimorphism in the spinal nuclei is controlled by androgens (Nordeen et al., 1985), which are also responsible for sexual dimorphism in other neuronal types (reviewed by Oppenheim, 1991).

In invertebrates, neuronal death is generally genetically programmed. In the nematode, *C. elegans*, cell lineages are strictly controlled such that certain cells always undergo degeneration and cell death (Horvitz et al., 1982). The genetic control of this cell death is demonstrated by the existence of mutants, like ced-3 and ced-4, in which all normal cell death is prevented (reviewed by Oppenheim, 1991).

Glia and the extracellular matrix may also be involved in the determination of developmental cell death. Recent reports revealed that trophic factors and trophic-like effects can be derived from glia and the extracellular matrix (Lipton, 1986; Walicke, 1989; Johnson et al., 1988, Rudge, 1992). If trophic factor deprivation is one cause of developmental neuronal death, the glial and matrix elements, in addition to the target tissue, may be involved in mediating this death (reviewed by Oppenheim, 1991).

This overview of the factors that influence developmental cell death suggests that, like injury-induced cell death, it is a complex phenomenon. The many factors that influence cell death may indicate that there are multiple mechanisms of cell death. In contrast, there may be only a single mechanism, which is controlled and influenced by a variety of factors. Some of the

biochemical and molecular events leading to cell death during development and after injury are examined in the following Section.

B. Biochemical and Molecular Mechanisms

The molecular and biochemical basis for injury-induced and developmental cell death has also been investigated. At present, the mechanisms are not fully understood, however, certain events and molecules are known to be involved in the process of cell death

Several ions have been implicated in the process of cell death, including sodium, potassium and predominantly, calcium. Extracellular sodium and potassium are known to be necessary in the first phase of neuronal damage after ischemia, when the neuron swells immediately after the injury (reviewed by Choi, 1988a; Choi and Rothman, 1990). The second phase of neuronal damage after ischemia, delayed until 48-72 hours after the injury, involves the disintegration of the cell and depends on the presence of extracellular calcium (Choi, 1988a).

The importance of calcium is not restricted to ischemic damage. Orrenius et al. (1990) describe the role of calcium in toxic and programmed cell death and examine the possible methods by which calcium could determine cell death. In the normal cell cytoplasm, calcium levels are maintained at a very low concentration (10^{-7}M) compared to external levels (10^{-3}M). Calcium is also stored intracellularly and the concentrations are maintained by pumps, most importantly the Ca^{2+}, Mg^{2+}-ATPases in the endoplasmic reticulum, mitochondria and the plasma membrane. Toxic chemicals and their reactive metabolites inhibit calcium transport systems in the endoplasmic reticulum and mitochondria, causing calcium to be released from intracellular stores (Orrenius and Bellomo, 1986). During

apoptosis in the immune system, sustained, elevated Ca^{2+}, caused by an influx of extracellular calcium, is a critical event.

The mechanism of cell destruction after calcium levels have been elevated, is related to disruption of the cytoskeletal network and the uncontrolled activation of calcium dependent catabolic enzymes. Increased levels of calcium cause cytoskeletal disruption by the dissociation of microfilaments from alpha-actin, the activation of proteases that cleave actin-binding proteins, thereby removing the anchor between the plasma membrane and the cytoskeleton (oxidative stress in platelets, Mirabelli et al., 1989), and the activation of transglutaminase, causing cytoskeletal alterations (e.g., liver cells undergoing programmed cell death Fesus, 1989).

The activation of catabolic enzymes by elevated calcium involves phospholipases, resulting in membrane lipid breakdown and the generation of toxic metabolites, neutral proteases (eg. toxic injury in hepatocytes, Nicotera et al., 1986), and endonucleases that cleave DNA into oligonucleosome-length fragments (thymocytes, Wyllie, 1980; McConkey et al., 1988, liver cells, Hibino et al., 1989)

Thus, the consequences of elevated calcium are severe, and may be involved in developmental and toxin-induced cell death in non-neuronal cells. Recent reports also implicate calcium in neuronal cell death. Koike and Tanake (1990) showed that there is a correlation between *in vitro* sympathetic ganglion cell survival and concentrations of intracellular calcium during NGF deprivation. In addition, Rich and Hollowell (1990) reported that flunarizine, a calcium channel blocking agent protects embryonic sensory and sympathetic neurons from cell death by NGF deprivation. However, the high concentration required for this effect suggests that the flunarizine is not acting as a calcium channel blocker. At high concentrations, it is able to perform other functions such as the inhibition of

calmodulin (Rich and Hollowell, 1990). These reports indicate that, at least *in vitro*, growth factor deprivation-induced cell death may be related to calcium levels.

Axotomy-induced cell death may also be related to calcium levels. Strautman et al (1990) reported that calcium levels rise in the axons of transected spinal axons. Tecoma et al (1989) investigated the effect of N-methyl-D-aspartate (NMDA) antagonists on the neuronal degeneration that follows mechanical damage in vitro. The NMDA antagonists, dextrorphan and D-2-amino-5 phosphonovalerate, protected fetal neocortical cells from degenerative changes, suggesting that excitatory amino acids, acting on NMDA receptors, resulting in increased intracellular calcium, may participate in injury-induced cell death.

The relationship between excitotoxin-induced cell death and calcium levels is less clear. Many reports suggest that excitotoxic cell death is mediated by calcium entry through NMDA-receptors (MacDermott et al., 1986; Siesjö et al., 1989, Choi, 1990; Pauwels et al., 1991). Michaels and Rothman (1990) found that the excitatory amino acid, glutamate, mediates cell toxicity by receptors such that an NMDA antagonist (MK-801) blocks the neurotoxic effect. However, only a poor correlation was found between the calcium concentration and cell death. The role of calcium in excitotoxic cell death is also supported by the observation that the presence of calcium-binding proteins, such as calbindin and parvalbumin, decrease the susceptibility of these neurons to excitotoxic amino acids (Weiss et al., 1990, Mattson et al., 1991).

In addition to ions such as calcium, gene expression and protein synthesis have also been determined to be required for some instances of cell death. Oppenheim (1990) found that inhibition of protein and RNA synthesis resulted in

decreased 1) developmental cell death, 2) cell death caused by limb bud removal and 3) cell death due to peripheral axotomy in chick motoneurons. Thus, developmental, target derived growth factor deprivation- induced and axotomy-induced neuronal death appear to require active gene expression and protein synthesis (Oppenheim, 1990). Scott and Davies (1990) reported a similar requirement for protein synthesis during the cell death of sensory and parasympathetic neurons after NGF deprivation. There may be a connection between the induction of gene expression and calcium levels. Sheng et al. (1990) reported that c-fos expression is calcium dependent.

The expression or regulation of certain proteins has also been associated with cell death in some systems. Polyubiquitin expression increases at the commencement of intersegmental muscle degeneration in *Manduca sexta* (Schwartz et al., 1990). This expression is delayed when the degeneration is chemically delayed. Polyubiquitin is involved in targeting intracellular proteins for degradation (Schwartz et al., 1990). Naegle et al. (1991) reported that the commencement of developmental cell death in the subplate neurons of the cerebral cortex is associated with increased levels of a 56 kDa protein, as assessed by immunohistochemistry. In regard to neuronal death following the removal of survival factors, in addition to the role of gene expression, the regulation of several enzymes have been associated with these processes. Rukenstein et al. (1991) reported that the death of PC12 cells following serum removal can be attenuated by adding a variety of agents, including forskolin, permeant cyclic adenosine monophosphate (cAMP) analogs or insulin. The action of these agents appears to involve the regulation of protein kinase activity, suggesting that protein kinases may play a role in the prevention of cell death. The loss of sympathetic neurons by the process of apoptosis *in vitro* due to NGF deprivation can be prevented by

adding NGF very late after the initiation of cell death, at a time when protein-synthesis inhibitors are no longer effective (Edwards et al., 1991). This rescue is mimicked by the addition of aurintricarboxylic acid, a suppressor of endonuclease activity (Batistatou and Greene, 1991). This observation suggests that endonuclease, an enzyme that cuts DNA into fragments whose sizes are multiples of 185 kDa, may be an essential component of the later events in growth factor-deprivation mediated neuronal death. These reports indicate that the expression of certain proteins may be crucial in the process of cell death.

C. Morphological Correlates of Cell Death

In addition to determining the biochemical and molecular events that lead to cell death, researchers have attempted to classify neuronal death according to the morphology of the cells as they are lost Morphological classification may reflect the existence of different basic mechanisms of cell death as the molecular and morphological changes in the cell occur in tandem.

Kerr and Wyllie (Wyllie et al., 1980, Wyllie, 1981; Kerr et al., 1987), distinguish two general morphological classes of cell death; necrosis and apoptosis. Necrosis is characterized by cellular edema, rupture of plasma and nuclear membranes and leakage of cellular contents. This type of cell death is generally associated with traumatic injury. The cellular events which may be involved include interference with mitochondrial oxidative phosphorylation, cell membrane damage and disruption of transmembrane transport (reviewed by Trump et al., 1981, Dean, 1987).

Apoptosis is characterized by a progressive loss of cell volume, and chromatic condensation (Bursch et al., 1990). Cells undergoing apoptosis separate into membrane - bound fragments that are rapidly phagocytosed. Bursch et al.

(1990) describe apoptosis in distinct steps. After stimulation, there is a period where no histological changes are evident. The first visible stage is characterized by the chromatin condensation and the isolation of the affected cell from its neighbors. This condensation requires the activation of an endogenous endonuclease that degrades the DNA. The second step, cytoplasmic condensation occurs when the cytoskeleton is disrupted, the intermediate filaments condense and the cell surface becomes distorted. In the final stage the cell is fragmented into "apoptotic bodies" which are phagocytosed. The apoptotic process is completed in about 3 hours. Increased transglutaminase activity has been associated with apoptosis (Fesus et al., 1987) and may be responsible for cross-linking of the cytoskeletal components. Apoptosis is viewed as the main type of cell death during development (reviewed by Kerr et al., 1987) and in cell death after hormonal or growth factor withdrawal, such as in T-lymphocytes in culture, after interleukin-2 (IL-2) deprivation (Kerr et al., 1987).

Schweichel and Merker (1973) have defined three types of cell death based on the involvement of lysosomes. Clarke (1990) expanded on these three types and divided the third type into two subtypes. The first type of cell death is apoptosis as described by Kerr and Wyllie (Wyllie et al., 1980, Wyllie, 1981, Kerr et al., 1987). It is characterized by the eventual destruction of fragments in the secondary lysosomes of other cells (heterophagocytosis). Clarke (1990) noted that this type of cell death occurred primarily in isolated cells, rather than areas of mass destruction.

The second type of cell death defined by Schweichel and Merker (1973) is autophagocytosis, where the cell is destroyed in its own lysosomes. The cell forms numerous autophagic vacuoles, the organelles often dilate and the plasma membrane is often altered. Clarke (1990) noted that this type of cell death

generally occurred in regions where all the cells were degenerating simultaneously.

The third type of cell death described by Scweichel and Merker (1973) did not involve lysosomes Clarke (1990) called this type non-lysosomal vesiculate degradation, and divided it into non-lysosomal disintegration where the cell disintegrates into smaller and smaller pieces and the "cytoplasmic" type of degeneration, where the organelles dilate and break into vesicles, followed by the rounding of the cell and eventual phagocytosis. This latter description is similar to necrosis.

In regard to the distinction between developmental and injury-induced cell death, Clarke (1990) suggests that these three classes are represented in both circumstances Thus, the different morphological descriptions of cell death may reflect overlapping mechanisms for cell death during development and after injury.

III. ROLE OF TROPHIC FACTORS IN NEURONAL SURVIVAL

A. Neurotrophic Factors

The main neurotrophic factors that have been identified are: the neurotrophins, ciliary neurotrophic factor (CNTF) and fibroblast growth factor (FGF). These molecules are involved in the survival and growth of neurons during development, the maintenance of neurons in the adult, and in the survival of neurons after injury Despite this common function, these factors display both structural and target diversity

The neurotrophins - nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) and neurotrophin-5 (NT-5) - are a structurally related family of factors which promote the survival of

neurons. NGF was the first factor discovered, purified from the mouse salivary gland (Cohen, 1960) NGF is a dimer of two polypeptides, 118 amino acids each, that has a thin elongate shape (McDonald, 1991). The dimer is formed by strong hydrophobic interactions.

BDNF was isolated by Barde et al. (1982) and later cloned by Liebrock et al. (1989). This discovery led to the identification of the three other neurotrophins: NT-3 (Maissonpierre et al, 1990; Hohn et al., 1990, Rosenthal et al , 1990, Jones and Reichardt, 1990), NT-4 (Hallböök et al., 1991) and NT-5 (Berkemeier et al., 1991). All five neurotrophins are highly homologous and have specific as well as overlapping target neuronal populations (reviewed by Bailey et al., 1991; Neuroscience Facts, Fidia Research Foundation, 1992).

Basic and acidic fibroblast growth factors (bFGF and aFGF), are structurally related proteins of about 16 000 daltons that are mitogenic and can promote the survival of some neurons *in vitro* and *in vivo* They are not members of the neurotrophin family, but rather, belong to the FGF family, which includes other structurally related mitogens. They are present in the embryonic brain (Risau et al., 1988) but are also abundant in the adult brain (Gospodarowicz, 1986).

Ciliary neurotrophic factor (CNTF), also a potent neurotrophic factor, is not included in the neurotrophin family. It is a 200 amino acid protein originally identified in avian ovular tissue (Alder et al., 1979) and later purified from rodent sciatic nerve (Manthorpe et al., 1986; Lin et al , 1990). Curiously, CNTF lacks a classical peptide signal sequence for secretion, obscuring the mechanism for its biological activity.

The tissue and cellular localization, targets, and functions of these neurotrophic factors has not been completely elucidated. The present data

suggests that they have a critical role in the development, maintenance and injury-response of the nervous system.

B. Neurotrophic Factors and Developmental Cell Death

The naturally occurring cell death observed in most neuronal populations has been linked to the supply of neurotrophic factors in the target tissue. As discussed earlier (Section IIA), the timing of developmental death coincides with the arrival of the neurons at their target, suggesting that the target tissue may be involved in this death. In many vertebrate neuronal systems, the target tissue produces a limited amount of specific molecules required for neuronal survival, creating a competition for the available trophic factors (reviewed by Barde, 1989).

NGF was the first neurotrophin discovered and the first to be implicated as a target derived factor involved in developmental cell death. Cohen (1960), observed that anti-NGF antibodies injected into newborn rodents resulted in destruction of the peripheral sympathetic nervous system. To substantiate the role of NGF in the cell death of these neurons, NGF administration was found to decrease the number of pyknotic neurons (Oppenheim, 1982) and NGF (Korsching and Thoenen, 1983) and its mRNA (Heumann et al , 1984; Shelton and Reichardt, 1984) were shown to be located in the target tissues of the sympathetic neurons. In addition, the interruption of retrograde axonal transport resulted in a similar cell loss as anti-NGF treatment (Hendry, 1975; Johnson, 1978)

NGF has also been proposed as a target derived factor for sensory neurons (Hamburger et al., 1981, Johnson et al., 1986). In the sensory system, anti-NGF differentially affects sub-populations of sensory neurons; neurons derived from ectodermal placodes (Pearson et al., 1983) and large myelinated sensory axons (Goedert et al , 1984) were not affected. A tight correlation between the time of

target innervation and the detection of NGF mRNA has been determined (Davies et al., 1987).

Central cholinergic neurons are a third neuronal population influenced by NGF. NGF and its mRNA are located in the target tissue, the pyramidal cells of the hippocampal neurons of the dentate gyrus (Korsching et al., 1985, Shelton and Reichardt, 1986; Whittemore et al., 1986)

In addition to NGF, other neurotrophic factors have been proposed as target derived survival factors during development. BDNF supports the survival of sub-populations of embryonic primary sensory neurons, both *in vitro* and *in vivo* (reviewed by Barde, 1989). The ability of BDNF to support cell survival *in vitro* has also been reported for embryonic rat RGCs (Johnson et al., 1986), basal forebrain cholinergic neurons (Alderson et al., 1990) and dopaminergic neurons in the substantia nigra (Hyman et al., 1991).

Both acidic and basic FGF have been reported to promote *in vitro* the survival of embryonic neurons from the hippocampus (Walicke et al , 1986), the cerebral cortex (Morrison et al., 1986), the striatum, septum and thalamus of E18 rats (Walicke, 1989), early postnatal mouse cerebellum (Hatten et al , 1988), and chick ciliary ganglia and spinal cord (Unsicker et al , 1987).

The *in vivo* functions of the most recently discovered neurotrophins, NT-3, NT-4 and NT-5 have not yet been fully investigated However, *in vitro*, NT-3 has been reported to increase survival of primary sensory neurons (Hohn et al , 1990, Rosenthal et al., 1990) and NT-4 was shown to be capable of promoting neurite outgrowth from embryonic chick nodose and dorsal root ganglia (Hallbook et al , 1991).

This overview of the involvement of neurotrophic factors in developmental cell death suggests that, although a deficit in the amounts of target derived factors

have not been established as the cause of developmental cell death in all systems, such a mechanism appears to function in some neuronal systems.

C. Injury-Induced Neuronal Death and Neurotrophic Factors

Due to the dependence of many neuronal types on target derived trophic factors during development (reviewed in Section A), it has been postulated that adult neurons still require trophic support. Thus, axotomy-induced neuronal death in the adult may result from the separation of the neuron from the target-derived factors (reviewed by Oppenheim, 1991). The involvement of several trophic factors in injury-induced cell death has been investigated.

Application of NGF was shown to increase the survival of basal forebrain cholinergic neurons and dorsal root ganglion neurons after axotomy (Hefti, 1986; Williams, 1986, Kromer, 1987; Otto et al., 1987; Rich et al., 1987; Koliatsos et al., 1990, Tuszynski et al., 1990). Anti-NGF antibodies given to adult mice for one month resulted in the death of 25% of the sympathetic neurons in the superior cervical ganglia (Ruit et al., (1990) suggesting that in this system, adult neurons require NGF. However, some neurons that are dependent on NGF in the embryo are no longer NGF-responsive in adulthood, for example, embryonic rat dorsal root ganglion neurons (Lindsay, 1988; reviewed by Barde, 1989). In addition, the removal of the target tissue of some adult neurons does not cause cell death: for example, adult basal forebrain cholinergic neurons survive after excitotoxic ablation of their target (Sofroniew et al., 1990).

The ability of FGF to rescue adult cholinergic neurons and RGCs after axotomy has also been examined. Cholinergic neurons are rescued by FGF after fimbria fornix lesions (Anderson et al., 1988). Application of FGF to the cut stump of the ON resulted in a 3-fold (basic FGF) to 4-fold (acidic FGF) increase in

RGC survival (Sievers et al., 1987). Adult RGCs may also be responsive to BDNF: in retinal explants, RGCs from adult rats were supported and their rate of axon elongation increased in the presence of BDNF (Thanos et al., 1989).

These observations do not directly implicate target deprivation as the cause of axotomy-induced cell death, but suggest that some adult neurons are responsive to neurotrophic factors before or after injury. The discovery that trophic factors and trophic-like effects are also derived from glia and the extracellular matrix (Walicke, 1989; Johnson et al., 1988, Lipton, 1986, Rudge et al., 1992) indicates that if neurons are dependent on trophic support, these factors may be released from the glia.

D. Neurotrophin Receptors

The neurotrophins (NGF, BDNF, NT-3, NT-4 and NT-5) are capable of initiating biological responses at very low concentrations. This is accomplished by binding to high affinity receptors on the target cell. NGF has two structurally unrelated receptors, a low affinity receptor, $p75^{NGFR}$, which also serves as a receptor for all the known neurotrophins and a second, high-affinity receptor, $p140^{trkA}$. The second NGF receptor is a member of the trk family of tyrosine kinase receptors, including $p145^{trkB}$, the receptor for BDNF and NT-4, and $p145^{trkC}$, the receptor for NT-3.

Nerve growth factor binds its receptors on the surface of the nerve terminal, followed by clustering of the receptors and internalization in membrane-bound vesicles. The vesicles travel along microtubules to deliver intact, biologically active NGF to the neuronal cell body (Thoenen and Barde, 1980). Signal transduction occurs during receptor binding but there is also a possibility that additional signalling takes place during transport (Meakin and Shooter, 1991).

The low affinity NGF receptor is a single peptide chain of approximately 400 amino acid residues, containing a single membrane spanning domain, an intracellular domain and an extracellular domain. Its distribution is widespread, both in the CNS and other tissues (Ernfors et al, 1990a).

The $p140^{trkA}$ NGF receptor is a 790 amino acid chain, containing a single membrane spanning domain. Its tyrosine kinase activity is in the cytoplasmic domain. The BDNF receptor, $p145^{trkB}$, has a number of differentially expressed transcripts, one encoding a truncated form, $p95^{trkB}$, which lacks the cytoplasmic domain (Klein et al., 1990). The three trk receptors, A,B and C, have similar sequences, especially in the tyrosine kinase domains (Meakin and Shooter, 1991). Binding studies suggest that $p140^{trkA}$ and $p145^{trkB}$, but not $p145^{trkC}$ have overlapping specificities to the neurotrophins (Meakin and Shooter, 1991)

All three trks are able to mediate biological function without $p75^{NGFR}$ although the low affinity receptor may be involved in modulating the binding and signalling of the trks (Meakin and Shooter, 1991).

E. BDNF as the Neurotrophin for RGCs

Several observations support the suggestion that RGCs are responsive to BDNF, one of the neurotrophins. Johnson et al., (1986) reported that BDNF supported the survival of RGCs in E17 retinal cultures. RGCs were identified by Thy-1 immunohistochemistry and by retrograde HRP labelling. This observation reveals that embryonic RGCs, at least *in vitro*, are responsive to BDNF. Thanos et al., (1989), showed that RGCs in an adult retinal explant, display increased survival (51% survival at 6 days, compared to control survival of 20-30%) and substantial axonal elongation (> 500 μm). The RGCs were identified by the retrograde label, rhodamine isothiocyanate. Hofer et al., (1990) examined the

regional distribution of BDNF mRNA and reported that the superior colliculus of the adult mouse expresses low levels of BDNF. These observations support the idea that BDNF is the RGC neurotrophin. However, FGF has also been shown to support adult RGC axon elongation *in vitro* (Thanos et al, 1989) and to decrease RGC loss after ON cut *in vivo* when applied to the nerve stump (Sievers et al, 1987). There may also be subpopulations of RGCs which respond to different neurotrophins. Thus, although BDNF is a plausible candidate, the precise neurotrophin(s) that support RGCs in the developing and adult retina may not yet have been identified.

IV. RETINOTECTAL SYSTEM

A. Retina

1. Retinal structure:

The rat retina is a complex structure, containing nine separate layers. Beginning at the vitreal surface, these layers are: 1) the inner limiting membrane which forms the edge of the retina, 2) the fiber layer, consisting of RGC axons traversing in large bundles from the soma to the optic disk, where they join to form the optic disk, 3) the ganglion cell layer, containing the RGC somas as well as displaced amacrine cells, 4) the inner plexiform layer, containing amacrine cell processes, RGC dendrites, and bipolar cell axons, 5) the inner nuclear layer, containing amacrine cells, bipolar cell somata and horizontal cell somata, 6) the outer plexiform layer, containing the horizontal cell processes, the bipolar cell processes and the base of the photoreceptor cells, 7) the outer nuclear layer, containing the receptor cell somata, 8) the outer limiting membrane, which separates the receptor cell somata from their outer segment, 9) the receptor

segment layer, containing the outer segments of the receptor cells that receive the visual input. Directly beside the receptors cells is the pigmented epithelial layer, which gives color to the eye and lacks pigment in the albino rat.

The visual input passes through the retinal layers and is received by the photoreceptors. The input is transmitted to the bipolar cells and then the ganglion cells, while the amacrine and horizontal cells are responsible for directing signals transversely in the retina.

The glial components of the retina are the astrocytes, microglia, Müller cells and oligodendrocytes. The astrocytes reside next to blood vessels in the outer plexiform layers and their processes are located deeper retinal layers. In the normal retina, microglia are regularly arrayed and have long, branched, fine processes (Perry and Gordon, 1988). Müller cell somata are located in the inner nuclear layer, but their processes extend from the outer limiting membrane to the inner limiting membrane where they spread along this membrane in structures called endfeet (Hughes, 1985). A small number of oligodendrocytes can also be identified in the RGC layer of the retina (reviewed by Stone, 1981).

2. Retinal Ganglion Cells:

The RGCs comprise only 50% of the cells within the ganglion cell layer (GCL) of the rat retina. Ramon y Cajal (1893) described displaced amacrine cells in the GCL and Perry (1981) discovered that only 50% of the cells in the GCL are labelled by application of horseradish peroxidase (HRP) at the target tissue, and that the unlabelled population, consisting of displaced amacrine cells, survives ON axotomy.

Some RGCs, called displaced RGCs are located in the inner nuclear layer. Linden (1987) reported that in the rat, these RGCs have a normal size range and

represent 1% of the total RGC population. In addition, RGCs that do not project outside the retina, called association RGCs, have been observed in the human, dog, mouse and rat retina (Gallego and Cruz, 1965, Drager et al., 1984)

The density of RGCs is not uniform along the area of the retina. The rat, in contrast to other mammals, does not have a fovea centralis but does contain a region of high RGC density in the superotemporal quadrant, 1-2 mm from the optic disc, called the area centralis (Sefton and Dreher, 1985). The total number of RGCs has been calculated to be from 110 000 to 115 000 (Schober and Gruschka, 1977; Fukuda, 1977; Perry, 1981, Dreher et al., 1984). RGC density in the central region, approximately 2500/mm^2 (Perry, 1981; Villegas-Pérez et al., 1992) is greater than in the periphery, approximately 1600/mm^2 (Perry, 1981).

Rat RGCs have been classified into three groups based on their sizes (Fukuda, 1977). Small RGCs, ranging in diameter from 6 to 11.5 μm, comprise 67% of the RGC population. Medium sized RGCs vary in diameter from 11.5 to 14.5 μm and represent 28% of the population. Large RGCs with diameters greater than 14.5 μm account for only 5% of the total. Small and large RGCs are evenly distributed across the retina while medium sized RGCs are preferentially located near the optic disc. Other means of classifying RGCs depend on either soma size, dendritic morphology, or a combination of the two: diffuse and stratified (Bunt, 1976), I, II and II, similar to the large, medium and small classification described above (Perry, 1979) and I, IIa, IIb, and III (Dreher et al., 1985).

B. Optic nerve

The optic nerve begins at the optic disc, where the axons of the RGCs leave the retina and join together, and extends approximately 10 mm to the optic chiasm. RGC axons traversing the retina are not myelinated but the majority of axons

(Perry and Lund, 1990) become myelinated as they pass through the lamina cribosa The number of axons in the ON of adult rats has been estimated as 100 000 in the albino rat (Fukuda et al., 1982; Sugimoto et al., 1984) and 117 000 (Forrester and Peters, 1976) to 120 000 (Hughs, 1977; Perry et al., 1983) in the pigmented rat The axon diameters range from 0.3 to 3.0 μm (Fukuda et al., 1982) The conduction velocities of the ON axons are classified into three groups, averaging 6.3, 11.4, and 16.8 m/sec (Fukuda, 1977). These groups based on conduction velocity corresponded to the three size groups of RGC somata in the retina, also reported by Fukuda, (1977)

The ON contains non-neuronal cells as well, namely microglia, oligodendrocytes and astrocytes. Microglia are regularly distributed in the ON, similar to the retina The ON axons are arranged in bundles of varying size, surrounded by astrocytic processes (Skoff, 1975). Vascularization intraocularly is supplied by the retinal artery, which travels adjacent to the ON from the optic disc (Ruskell, 1964) Intracranially, the ON receives its blood supply from small arterioles in its meningeal covering (Kiernan, 1985).

At the optic chiasm, 97 to 98% of the RGC axons in the rat cross to the contralateral optic tract (Lund, 1965; Dreher, 1985). The somata of the small percentage of axons that project ipsilaterally are located in the inferotemporal retinal quadrant (Cowey and Franzini, 1979). In addition, there are a small number of axons that project bilaterally (Jeffery et al., 1981).

C. Retinal Ganglion Cell Targets

The RGCs project to six different brain nuclei: 1) the superior colliculus (SC), the main target, receiving 95% of the axons (Linden and Perry, 1983); 2) the dorso-lateral geniculate nucleus (dLGN), which receives the second major

projection, 37% of the RGCs (Martin, 1986). The branching of axons to innervate more than one nuclei explains the overlap of RGC targets (Sefton, 1968); 3) the ventro-lateral geniculate nucleus (Legg and Cowey, 1977); 4) the accessory optic nuclei in the midbrain (Simpson, 1984); 5) the pretectum (Scalia and Arango, 1977); and 6) the hypothalamus (Kita and Oomura, 1982).

V. RESEARCH PROJECT: RATIONALE

The first step in the process of regeneration after neuronal axotomy is the sprouting and regrowth of the severed axon. CNS axons, however, do not respond to axotomy by regeneration but rather, by undergoing retrograde degeneration and often, cell death. The responses to neuronal injury are complex, involving morphological, biochemical and molecular events within the cell in addition to changes in non-neuronal cells near the damaged neuron. An understanding of the events which occur during CNS degeneration and the factors which control this process may enable the prevention or delay of degenerative events and increase the effectiveness of treatments which promote regeneration.

Two of the many variables (reviewed by Lieberman, 1974, section I C) that influence the response of neurons to injury are the location of the injury and the type of injury Several recent reports have examined in detail how these variables influence certain aspects of the neuronal response to injury. Villegas-Pérez (1992) investigated the effect of the soma-injury distance on the survival of RGC's in the adult rat. RGC loss occurred in two phases, a rapid early loss, followed by a later, more protracted phase of cell loss. The patterns of RGC loss were dependent on the location of the lesion, where lesions closer to the eye resulted in greater RGC loss. Lunn et al. (1990), reported that the type of lesion influenced the rate of

Wallerian degeneration in the mouse sciatic nerve, where cut injury resulted in more rapid degeneration than crush injury. These observations of peripheral nerve were made by silver staining and noting the presence or absence of a compound action potential

These studies prompted the questions: does the type of injury, cut versus crush, influence the survival of neurons of the CNS? and if so, are there morphological or molecular events which occur after cut and crush lesions which could contribute to the difference in cell survival? These questions are addressed in this thesis in an attempt to define further the events which compose the neuronal response to injury and to determine how these events are controlled. The adult rat retino-tectal system is ideal for such a study because the RGCs can be accurately identified and counted by applying a retrogradely transported fluorescent tracer to the target tissue, the axons of the RGCs travel in a uniform direction in the ON and both the retina and the ON are easily accessible for morphological and molecular investigation In addition, the effect of soma-injury distance on RGC survival has been previously documented in this laboratory (Villegas-Pérez, 1992), forming a basis for the present study

In the initial experiment, therefore, I determined the time course for RGC survival after ON cut or crush at a fixed distance from the eye. In the second experiment, to determine if there was a correlation between the pattern of cell loss and the extent of retrograde degeneration, I investigated the morphological events which occur in the RGC axons after cut or crush. To determine if the expression of growth factors contributed to the survival of RGCs after cut and crush injury, the final experiment involved the measurement of the level of BDNF expression in the normal retina and ON.

The results reported in this thesis. 1) establish that cut and crush injury result in different neuronal responses (including cell death axonal damage and non-neuronal responses) in the RGCs of the adult rat, 2) describe several of the differences between the events occurring after ON cut and crush which may be determinants of the extent of RGC death, 3) support the suggestion that brain derived neurotrophic factor, the neurotrophic factor proposed for RGCs, mRNA is expressed in the ON and 4) further characterize the rat retino-tectal system as a paradigm for studying neuronal responses to injury in the CNS.

CHAPTER 2: METHODS

In this thesis, I compare the responses of retinal ganglion cells (RGCs) to two types of injury, cut and crush. In this chapter I describe in detail the methods used to investigate responses to RGC injury. The application of retrogradely transported neuronal markers accompanied by fluorescence microscopy enabled the measurement of retinal ganglion cell survival after axon interruption. The morphological changes occurring in the RGC axons after injury were investigated by immunohistochemistry, light and electron microscopy. The expression of brain derived neurotrophic factor, the neurotrophin proposed for RGCs, in the normal optic nerve was determined by Northern blot hybridization.

I. EXPERIMENTAL DESIGN

The experiments described in this thesis are divided into three groups, each addressing a separate aspect of the events which occur after axotomy of CNS neurons, specifically the RGCs of the rat visual system. In the **first experimental group**, I investigated the death of CNS neurons after two different methods of axotomy. The survival of RGCs was quantitated after intracranial ON cut and crush. The RGCs are identified by a retrograde tracer, Fluorogold(R) which is applied to the superior colliculus prior to the ON injury. RGC densities were determined at several times after injury between 1 week and 6 months.

To determine if morphological changes in the axonal portion of axotomized neurons corresponded to the extent of cell death, in the **second experimental group** I investigated the retrograde morphological changes which occur after

intracranial ON cut or crush. The ON stump and the intraretinal axons were examined by immunohistochemistry, light and electron microscopy at several times after injury between 3 days and 3 months.

To determine if the proposed RGC neurotrophic factor, brain derived neurotrophic factor (BDNF) is expressed in the normal ON, in the **third experimental group** I determined the level of BDNF expression in the retina and ON in normal animals. The BDNF mRNA was examined by the Northern blot technique using a rat BDNF DNA probe.

II. ANIMALS: GENERAL PROCEDURES

Female Sprague-Dawley rats, weighing 180-200 grams were used in all experiments. During surgical manipulations, the rats were deeply anesthetized with intraperitoneal 7% chloral hydrate in saline (0.42 mg/g body weight) Following surgery the rats were supplied with analgesia in the form of subcutaneous buprenorphine (0.02 mg/250g body weight). The animals were sacrificed by intraperitoneal injection of a lethal dose of chloral hydrate (0.7 mg/g body weight). Animals were housed in groups of 1-4 per cage on a 12 hour light/dark schedule. Food and water were provided *ad libitum*.

III. Group I: RGC SURVIVAL AFTER INTRACRANIAL ON CUT OR CRUSH

A. Surgical Procedures

1. Retrograde Labelling of RGCs

The fluorescent tracer, Fluorogold(R), was used to retrogradely label RGC somas in the retina, allowing the subsequent quantitation of cell density. The Fluorogold(R) was applied directly to the superior colliculi, target of the majority

of RGCs (Linden and Perry, 1983). The skull was exposed by making a midline incision in the overlying skin. The fascia was scraped away from the skull and a partial craniotomy performed using a Dremel Cordless Moto-Tool, Model 850. The superior colliculi were exposed bilaterally by removal of the overlying cortex. Small perforations were made in the pia mater using fine forceps. The tracer was applied using small pieces of gel foam (approximately 3 mm^3) soaked in a solution of 2% Fluorogold in 10% dimethylsulfoxide (DMSO) and saline. Two applications were applied to each colliculus prior to closure and the final application remained on the superior colliculus. The area was covered with a dry piece of gel foam prior to closure of the wound. The fascia was sutured with 6-0 silk and the skin with 4-0 silk. Secondary surgical procedures were performed at least 7 days after Fluorogold(R) application when all RGCs were labelled (Grant Robinson, personal communication).

2 . Fluorogold(R) as a Retrograde Fluorescent Tracer

Fluorogold(R) was utilized as a retrograde tracer because: 1) it has a rapid labelling time the RGCs are labelled 6 days following surgery, 2) the tracer is persistent, remaining in the RGCs for approximately 6 months after application of the tracer, 3) it withstands immunohistochemical processing of the retinal tissue following sacrifice and 4) is observed as a punctate label in the cytoplasm of the RGCs, allowing visualization of the soma boundaries facilitating easy identification during quantitation (G. Robinson, G.M. Bray and A.J. Aguayo, unpublished observations). Due to these characteristics, Fluorogold(R), as a retrograde tracer in the rodent visual system, is superior to dil, the dye previously used in this laboratory. Dil requires three weeks to complete RGC labelling

(Vidal-Sanz et. al., 1988), it is lost from the RGCs during immunohistochemistry and does not fill the cell soma as completely as Fluorogold(R)

3. Intracranial Interruption of RGC Axons

The left ON was exposed using a dorsal intracranial approach by a partial craniotomy and removal of the overlying cortex. The axons within the nerve were interrupted approximately 9 mm from the back of the eye (1 mm from the optic chiasm) by one of two surgical procedures: 1) the ON was cut with scissors producing a proximal (attached to the eye) and distal (attached to the brain) ON stump, or, 2) the ON was crushed twice for 10 seconds each time at the same position using fine forceps (JFS-3). The area was covered with gel foam, the fascia overlying the skull was sutured with 6-0 silk and the skin was sutured with 4-0 silk. Opthalmic polysporin (Burroughs Wellcome Inc., Kirkland, Quebec) was placed over the eyes during recovery to prevent dehydration.

4. Intracranial Injury Followed by Fluorogold(R) Labelling

To establish that the cut and crush procedures resulted in complete interruption of retinal ganglion cell axons, in 3 animals the ON was cut (n=1) or crushed (n=2) followed immediately by the application of Fluorogold on the superior colliculus. These animals were sacrificed 1 or 4 weeks after the injury

B. Tissue Processing

1. Fixation and Dissection

Animals were sacrificed at several times after ON injury: 7 days, 10 days, 14 days, 1 month, 3 months and 6 months. The number of retinas analyzed at each of these time points is summarized in the following table:

NUMBER OF RETINAS							
Type of Injury	TIME AFTER INTRACRANIAL ON INJURY						Total Number
	7 day	10 days	14 days	1 month	3 months	6 months	
CUT	4	4	5	4	4	1	22
CRUSH	4	4	4	4	4	1	21

At the time of sacrifice, the animals were perfused through the heart with 0.9% NaCl followed by 4% paraformaldehyde in 0.1M phosphate buffer. The ONs were removed and postfixed in 2.5% gluteraldehyde/0.5% paraformaldehyde for 2-24 hours and then rinsed in 0.1M phosphate buffer. The retinas were removed and four radial cuts made to facilitate flat-mounting onto a slide with the vitreal surface up. A piece of filter paper was placed on top of the flattened retinas and they were postfixed in 4% paraformaldehyde for one hour. After fixation, the retinas were removed from the filter paper and rinsed in 0.1M phosphate buffer. These retinas were flatmounted, vitreal side up, for viewing of the retrograde tracer, Fluorogold(R).

2. OX-42 Immunohistochemistry

OX-42 (Serotec, U.K.) is a monoclonal antibody that recognizes epitopes on the surface of microglia (Robinson et. al., 1986). Two retinas, one control and

one 2 weeks after intracranial ON cut, were removed from their slides following Fluorogold (R) counts. They were washed and incubated for one hour at -4°C with the OX-42 antibody, diluted 1/10 in 5% bovine serum albumin (BSA) in saline, followed by incubation with a secondary antibody coupled to rhodamine (goat anti-mouse IgG tetramethyl rhodamine isothiocyanate). These retinas were examined in the Leica Ortholux II fluorescent microscope using the True Blue filter (excitation filter: 355-425 nm, supression filter: Long Pass filter 460 nm) to observe the cells labelled with Fluorogold (R) and the rhodamine filter (excitation filter: 530-560 nm, supression filter: Long Pass Filter 580 nm) to observe microglial cells.

3. Light Microscopy of Optic Nerves

The ONs were osmicated, dehydrated and cut into 1 mm segments. These segments were embedded in epoxy resin (Epon(R)) and 1 µm sections were cut for light microscopy. Sections were examined on the Zeiss light microscope to establish the complete interruption of RGC axons after ON cut or crush.

C. Measurement of RGC Densities

The RGC density for each retina was determined by counting Fluorogold(R) labelled neurons in each quadrant in three areas (0.23 x 0.35 mm each), located 1, 2 and 3 mm from the optic disc. The total number of labelled RGCs in these 12 areas were used to calculate the mean density of RGC's per mm^2 for each retina. This method, which determines the number of labelled RGC's in approximately 1.6% of the retina, was previously described for determining RGC density with dil labelling (Villegas-Pérez et. al., 1992).

Fluorogold(R) labelled cells were counted using a Leica Ortholux II fluorescent microscope using the True Blue filter (excitation band 355-425 nm, suppression filter: Long Pass filter 460 nm). Fluorogold(R) labelled RGCs were recognized by the fine, punctate fluorescence in their cell bodies. After injury, cells with bright fluorescence filling the cell body were also observed. These cells were microglial cells which had phagocytosed labelled RGCs. This phenomenon was previously reported in motoneurons (Riniman et al., 1991) and in RGCs labelled with dil (Villegas-Pérez et al., MSc. Thesis, McGill University, 1991)

D. Statistical Analysis of RGC Densities

The patterns of RGC survival after ON cut and crush were analyzed with the Sigmaplot(R) 4.1 curve fitting module that uses the Marquardt-Levenberg algorithm. Regession lines were calculated using the GB Stat program (IBM). Individual RGC densities, rather than mean densities, were used in the curve fitting program and in the calculation of regression lines. The two-sided Student t-test was used to compare groups of densities

IV. Group II: RETROGRADE AXONAL CHANGES AFTER ON CUT OR CRUSH

A. Surgical Procedures

The left ON of Group II experimental animals was either cut or crushed as described in the Group I methods. Twenty-seven animals received ON cut and 25 animals received ON crush A third method of axon interruption was used on 2 additional animals in Group II. The ON was crushed intracranially and a 6-0 silk suture was tied snugly around the ON at the position of the crush. The suture remained in place until the animals were sacrificed.

B. Tissue Processing

1. Fixation and Dissection

The animals were sacrificed at several times after ON injury: 3 days, 1 week, 2 weeks, 1 month and 3 months. The number of animals analyzed at each time is summarized in the following table:

NUMBER OF ANIMALS						
Type of Injury	TIME AFTER INTRACRANIAL INJURY					Total Number
	3 days	7 days	14 days	1 month	3 months	
CUT	4	4	6	9	4	27
CRUSH	4	5	5	7	4	25

The animals were perfused with fixative solution and the ONs and retinas removed. The ONs were post-fixed as described in the Group I methods. One retinal quadrant from each retina was removed and post-fixed in 2.5% gluteraldehyde/0.5% paraformaldehyde for two hours. The remaining three quadrants were affixed to filter paper and post-fixed in 4% paraformaldehyde as described in the Group I methods. After post-fixation, the retinas were rinsed in 0.1M phosphate buffer to prepare them for immunocytochemistry (3 quadrants) or EM processing (1 quadrant).

2. Light and Electron Microscopy of Optic Nerves

The ONs were osmicated, dehydrated and cut into 1 mm segments. These segments were embedded in Epon(R) and sections were cut for light (1 μm) and electron (100 nm) microscopy. Light microscope sections were stained with

Malloy's azure II-methylene blue and examined on the Zeiss microscope while EM sections were examined on the Philips CM10 electron microscope.

3. Immunohistochemical Visualization of Intraretinal RGC Axons

In three quadrants of each retina RGC axons were visualized with RT97, a monoclonal antibody that recognizes phosphorylated 200kD neurofilaments or a closely related protein (Anderton et al., 1982). The incubation was completed at room temperature with RT97 diluted 1/1000 with 0.01 M phosphate buffered saline (PBS) containing 2% BSA and 1% Triton-X 100. Twelve to 18 hours later, the retinas were rinsed with 0.01M PBS for 1 hour

Two of the quadrants were incubated for 1 hour at room temperature with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) diluted 1/100 with 0.01M PBS containing 3% BSA and 1% Triton-X 100. The third quadrant was incubated for one hour at room temperature with biotinylated horse anti-mouse immunoglobin G (IgG) (Vector Laboratories) diluted 1/200 with 0.01M PBS containing 3% BSA and 1% Triton-X-100 followed by reaction with ABC avidin conjugation system for 1 hour and incubation with diaminobenzidine and hydrogen peroxide for ten minutes

After the immunohistochemistry, the retinas were flatmounted onto slides and examined with the Leica Ortholux II fluorescent microscope using the fluorescein filter (excitation filter: 450-500 nm, suppression filter: Band Pass filter 515-560)

4. Electron Microscopy of Intraretinal RGC Axons

The fourth retinal quadrant was sectioned radially into 3 segments, followed by sectioning each segment transversly, 2 mm from the disk. These retinal pieces

were dehydrated and embedded in Epon and 100 nm sections were cut for electron microscopic viewing of intraretinal RGC fiber bundles in cross-section 2 mm from the disk.

C. Measurement of Intraretinal Axon Bundle Size

Control and experimental retinas incubated with RT97, followed by diaminobenzidine, were examined by light microscopy to determine the distribution of the axon bundle size near the optic disc. A micrometer was used to measure the width of each RGC axon bundle located along a 0.22 mm segment of flatmounted retina, 0.22 mm from the optic disc.

V. Group III: BDNF EXPRESSION IN THE ON AND RETINA

A. RNA Isolation

Total RNA was isolated was using a guanidinium thiocyanate and chloride procedure modified from Chomczynski and Sacchi (1987) The animals, female Sprague-Dawley rats weighing 180-200 g were sacrificed with an overdose of chloral hydrate and perfused briefly with saline to remove blood from the tissues The tissue was removed, weighed and placed on ice in a glass-glass (optic nerves) or glass-teflon (all other tissues) homogenizer containing 1 ml of the denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7, 0.5% sarcosyl and 0.1M 2-mercaptoethanol) per 100 mg of tissue The tissue was then homogenized by hand (glass-glass) or with a Fisher electric homogenizer (glass teflon).

The tissue was transferred to Eppendorf tubes and 0.1 volume sodium acetate, pH4, 1 volume phenol and 0.6 volumes chloroform-isoamylalcohol were

added sequentially The tube was mixed, cooled on ice for 15 minutes and centrifuged at 10 000 X g for 20 minutes at 4°C The upper aqueous layer, containing the RNA, was transferred to a fresh tube One volume of isopropanol was added and the tube was placed at -20°C for 2 - 16 hours to precipitate the RNA

The tube was spun at 10 000Xg for 20 minutes and the pellet air dried. The pellet was resuspended in water and 2 volumes of ethanol were added. The tube was placed at -20°C for 2 - 16 hours to precipitate the RNA The tube was centrifuged as before, the pellet was washed gently with ethanol and resuspended in 20-50 μl of DEPC H_2O

The concentration of RNA was measured by the absorbance ratio, A_{260} : A_{280} and 1-2 μl of each sample were run on a small agarose gel to determine the quality of the RNA and to further ensure equal RNA loading on the Northern blot.

After isolation, the RNA was stored in ethanol or water at -80°C until used.

B. <u>Agarose-Formaldehyde Gel</u>

The total RNA samples diluted in a sample buffer (1.3X MOPS, 8% formaldehyde and 65% deionized formamide) such that to every 5 μg of RNA 6 μl of sample buffer was added The samples were heated to 75°C for 15 minutes and cooled on ice Immediately before loading the samples, 1/10 volume of loading dye and 1/10 volume of ethidium bromide (1mg/ml) were added.

The gel apparatus (BRL Horizon 11.14) was washed for 1 minute with DEPC water and rinsed twice with RNase-free water. The gel was prepared by boiling 1.3 g agarose and 75 ml RNase free H_2O, cooling to 60°C and adding 10 ml 10X MOPS (0.2M MOPS, 50 mM sodium acetate, 1 mM EDTA, pH 8) and 14 ml 37% formaldehyde The gel was poured immediately and left to set for 30

minutes. Ten µg of RNA was loaded per lane. The gel was run at 90 V for 5 minutes followed by 3-4 hours at 80 V using 1X MOPS as a running buffer. The amount of RNA loaded per lane was verified by photographing the gel on a UV light box (Foto/Prepl by Bio/Can Scientific).

C. Transfer to Nylon Membrane

The RNA was transferred to a Nytran(R) filter (Schleicher & Schuell) using the Vacugene(R) XL vacuum blotting system. The Nytran(R) was soaked in 20X SSC for 5-10 minutes prior to transfer. The transfer was mediated by several washes: 5 minutes with water, 5 minutes with 50 mM NaOH, 10 mM NaCl, 5 minutes with 0.1 M Tris-Cl pH 4.0 and 2-3 hours with 20X SSC. The Nytran(R) membrane was blotted and air-dried followed by ultraviolet-crosslinking in the Stratalinker (Stratagene). The ribosomal RNA bands and lanes were marked and the filter was stored in the dark until needed.

D. Hybridization with Probe

The protocol for Northern blots with a DNA probe, as described by Maissonpierre (1990), was followed. The prehybridization buffer consisted of 0.5 M $NaPO_4$, 1% BSA, 7% sodium dodecyl sulphate (SDS), 1 mM disodium ethylenediamine tetraacetate (EDTA) and 100 µg/µl salmon sperm DNA. The filter and prehybridization solution were placed in a sealed bag at 68°C in a shaking water bath for 1-4 hours.

The probe (5 million counts per ml) was added and the bag resealed. The filter was hybridized at 68°C overnight. The filter was then removed from the bag and washed twice at room temperature in 2X SSC, 0.1% SDS and twice at 68°C in

2X SSC, 0.1% SDS for 15 minutes each wash. The blots were rinsed with wash solution and placed on film with 1 or 2 intensifying screens for 3-7 days.

E. BDNF Probe

The rat BDNF probe was obtained in the lab of Dr. P. Richardson using the polymerase chain reaction with upstream and downstream primers corresponding to pig BDNF sequences. The sequence obtained was cut with EcoRI within the primer regions to enable insertion into the pGEM 7Z vector at the Sma site.

To prepare a fragment for labelling of a DNA BDNF probe, the entire BDNF sequence was isolated by cutting with EcoRI and separating the 750 base fragment on a low melting point (LMP) agarose gel. A Pharmacia oligolabelling kit was used to produce a DNA probe. One hundred ng of DNA from the gel slice was heated to 95-100°C for 7 minutes followed by 37°C for 10 minutes. The sample was centrifuged and the following reagents added: 50 μCi ^{32}P-dCTP, 10 μl reagent mix, 9 μl H_2O and 1 μl Klenow fragment. The tube was mixed and placed in a 37°C water bath for 4 hours.

Free nucleotides were removed using a Nuctrap Push Column. The column was prewetted with STE buffer (0.01M EDTA, pH8, 0.02M TrisHCl, pH7.6, 1M NaCl) and the sample, brought up to 70 μl with H_2O, was added. The probe was eluted using 90μl of STE buffer and collected in an Eppendorf tube. One μl was placed in a scintillation tube and counted on the tritium channel. The counts per minute (cpm) obtained multiplied by four yielded the Cherenkov counts, a measure of the ^{32}P cpms. The labelling reaction yielded approximately 120μl of probe at 1×10^6 cpm/μl.

CHAPTER 3: RESULTS

In this chapter, I present the results of the experiments in this thesis, which examine the responses of adult rat retinal ganglion cells to injury. In the first section I report the patterns of cell loss observed after cut or crush of the optic nerve. In the second section I describe the morphological changes occurring in the retinal ganglion cell axons located in the optic nerve and retina after cut or crush injury. Finally, in the third section I describe the expression of brain derived neurotrophic factor in the normal rat optic nerve.

I. RGC SURVIVAL AFTER INTRACRANIAL ON CUT OR CRUSH

A. Fluorogold Labeling

In control retinas, Fluorogold(R) labeled RGC's were recognized by the fine, punctate fluorescence in the perinuclear cytoplasm and some proximal dendrites. After injury, many RGC's appeared to be identical to control RGC's. Other axotomized RGC's retained the punctate labeling but were only faintly fluorescent compared to the bright labeling observed in control RGC's. In addition, cells were also identified which contained very bright, coarse Fluorogold(R) labeling. These cells were often elongated and were located in a deeper plane of focus than the punctate labeled RGC's. This coarse fluorescence was previously described for microglia and macrophages in motoneuron nuclei after injury (Rinaman et al., 1991). To investigate the possible non-neuronal nature of the Fluorogold labeling, several retinas were reacted with OX-42, an antibody which recognizes microglia. Most cells coarsely labeled with Fluorogold(R) were also immunostained with OX-42, suggesting that microglia had phagocytosed the

Fluorogold(R) from degrading neurons, as previously reported for dil labeled retinas (Villegas-Pérez, MSc thesis, McGill University, 1991)

B. RGC Densities in Control Retinas

The mean density of Fluorogold labeled RGCs in control retinas was 2272 $\pm$ 61 cells/mm^2 (Table I, Figure 1a) Control values did not change with the age of the animal Villegas-Pérez et al (1992) reported dil-labeled cell density in control retinas as 2288 $\pm$ 66 cells/mm^2

C. RGC Densities After Intracranial ON Cut or Crush

1 RGC Loss is Delayed

Seven days after ON cut or crush, mean RGC densities were not significantly different from control values ($p > 0.05$; two-sided Student t-test. Mean densities at one week were 2145 $\pm$ 120 and 2218 $\pm$ 92 cells/mm^2 for ON cut and crush respectively representing cell survivals of 94.4 and 97.6% Thus, the cell loss after intracranial ON injury is delayed for at least 7 days, this delay is independent of the type of ON injury

2 Two Weeks to Six Months Different Patterns of RGC Loss After ON Cut and Crush

After the initial one week delay in RGC loss, the patterns of cell loss were different after ON cut and crush Cut injury was characterized by two phases of cell loss, an early, rapid loss followed by a later, more protracted loss RGC loss after crush injury continued at a constant rate from one week to six months (Figure 2) From 10 days to 6 months, mean RGC densities after ON cut were significantly different than after ON crush ($p > 0.05$; two-sided Student t-test).

a. ON Cut: Early Rapid and Later Protracted RGC Loss

There appeared to be an abrupt loss of cells in the second week after cut (cell densities at 10 and 14 days were 1398 $\pm$ 27 and 1364 $\pm$ 80 cells/mm^2 respectively) followed by a more protracted loss which continued until 6 months (Table I, Figure 2). An attempt was made to characterize this loss by making the assumption that the surviving cells were subject to one or more processes of loss, each exerting a uniform effect over time on the survival of different populations of cells. This assumption is described by the equation: $C(t) = C_1e^{-at} + C_2e^{-bt} + C_3e^{-ct} + \ldots$ where C(t) is the number of surviving cells at time t after axotomy and C_1, C_2 etc. are the number of cells subject to cell death with time constants 1/a, 1/b etc.

The data were fit to equations of this nature using the Sigmaplot 4.1 curve fitting program which uses the Marquardt-Levenberg algorithm. Because there was no apparent loss between time zero and 1 week, the time zero points were excluded from this analysis.

The trajectory of RGC survival from 1 week to 6 months after intracranial ON cut was best fit by a curve with two exponentials (Table III) which is plotted in Figure 3. The initial loss of cells is succeeded by a later phase of exponential loss with a calculated half survival time of 2.3 months. 1 502 RGCs/mm^2, or 66% of the original population were subject to this latter phase of loss.

As a check on this calculation, regression lines were separately determined for the cell survival data from 7 days, 10 days, 2 weeks or 1 month to 6 months (Table III). In the latter three cases the regression lines indicated a half survival time of 2.4 or 2.6 months, the R^2 value improving slightly as the earlier data were eliminated, consistent with the inflection at about 10 days on the survival curve calculated from the curve fitting.

b. ON Crush: Single Rate of RGC Loss

Unlike the cut injury, RGC loss after crush injury did not appear to result in a rapid early loss of cells, but rather, the cell loss from 1 week to 6 months after crush injury was slow and protracted. RGC densities at all time points after ON crush were greater than after ON cut and the densities at all time points after 1 week were significantly different for the two types of injury (Table I, Figure 2). The RGC density after ON crush was qualitatively greater than after ON cut from 2 weeks to 6 months after injury (see Figure 1 b and c for pictures of 1 month Fluorogold(R) labelled retinas).

Curve fitting, as described above, was performed on the RGC survival data after ON crush. The trajectory of RGC survival from 1 week to 6 months after intracranial ON crush best fit a curve with one exponential (Table III) which is plotted in Figure 3. The calculated half survival of the single rate of cell loss was 5.3 months. As a check on this calculation, regression lines were determined for the cell survival data from 7 days, 10 days, 2 weeks or 1 month to 6 months (Table III). In the latter three cases, the regression lines indicated a half survival of 5.3 or 5.5 months with R^2 values of 0.85 to 0.88. The rate of cell loss after crush injury (one-half survival time of 5.3 months), therefore, is more than two times slower than the rate of cell loss in the second, protracted phase after ON cut (one-half survival time of 2.3 months).

D. Validation of Axonal Interruption following ON Crush

To validate the completeness of axonal interruption after ON crush, two animals were backlabelled with Fluorogold immediately after ON crush and one animal was labelled after ON cut. These animals, sacrificed at 1 week (1 crush, 1 cut) and 6 weeks (1 crush), did not display Fluorogold(R) labelling in the retina contralateral to the injury while the control retina, ipsilateral to the injury, contained a normal population of Fluorogold(R) labelled cells

TABLE I
FG- LABELLED DENSITIES IN RETINAS AFTER ON CUT OR CRUSH

Cell Density cells/mm^2	Time After Injury	Type of ON Interruption	
		CUT	CRUSH
Mean ± SEM	control	2272 ± 56 (n=21)	
	7 days	2247 2200 2309 1825	2123 2303 2406 2038
Mean ± SEM % control		2145 ± 109 94.4%	2218 ± 84 97.6%
	10 days	1473 1405 1356 1358	1803 1948 2055 1841
Mean ± SEM % control		1398 ± 27 61.5%	1912 ± 57 84.2%
	14 days	1347 1578 1462 1099 1336	1905 1803 1980 2064
Mean ± SEM % control		1364 ± 80 60.0%	1938 ± 56 85.3%
	1 month	897 1033 936 1220	1767 1657 1689
Mean ± SEM % control		1022 ± 72 45.0%	1704 ± 33 75.0%
	3 months	688 592 668 626	1622 1437 1465 1511
Mean ± SEM % control		644 ± 22 28.3%	1509 ± 41 66.4%
	6 months	263	829
% control		11.6%	36.5%

Figure 1

Fluorescence photomicrographs of a portion of one retinal quadrant showing retinal ganglion cells retrogradely labelled with Fluorogold(R). The optic disc is located in the lower right corner. Bar = 250 μm.

a. Control Retina: Retinal ganglion cells densely cover the retina and are recognized by the punctate nature of the fluorescence. Blood vessels are visible as striations in the retina where no RGCs are located.

b. Four Weeks After Intracranial ON Cut: The density of RGCs is markedly reduced. Many of the labelled cells do not display punctate labelling and are not rounded in shape: these are phagocytic cells that have engulfed dying RGCs.

c. Four Weeks After Intracranial Crush: The density of RGC's is reduced compared to control retinas but is greater than after ON cut. Phagocytic cells containing Fluorogold(R) are less frequent than after ON cut.

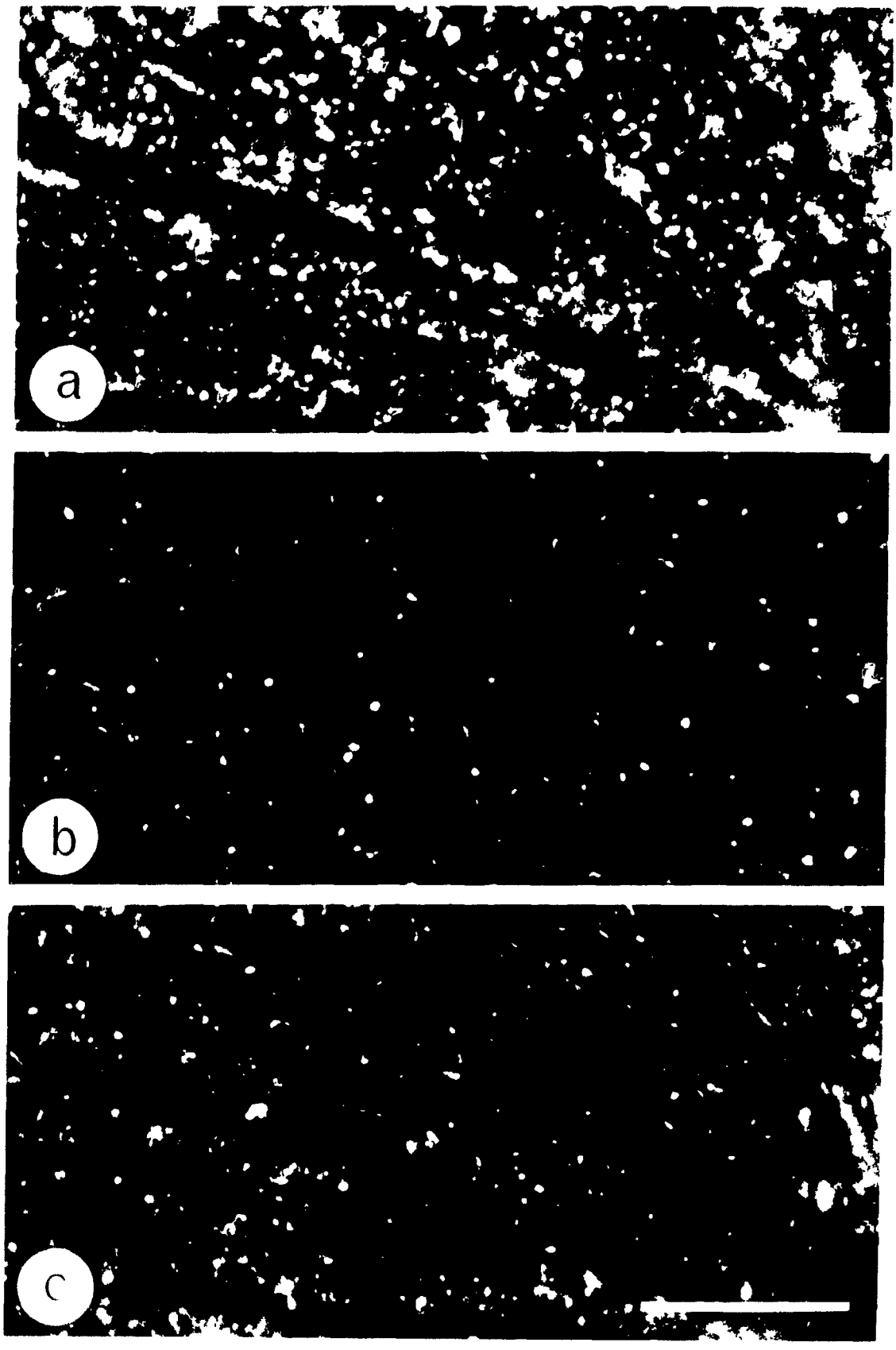
a
b
c

TABLE II
MATHEMATICAL ANALYSIS OF RGC LOSS AFTER CUT OR CRUSH

INTRACRANIAL ON CUT		INTRACRANIAL ON CRUSH	
RGCs SUBJECT TO PROTRACTED LOSS		RGCs SUBJECT TO SINGLE RATE OF LOSS	
Number of cells	1502	Number of cells	2103
1/time constant (months^{-1})	0.30	1/time constant (months^{-1})	0.13
Half survival time (months)	2.3	Half survival time (months)	5.3

TABLE III
LINEAR REGRESSION ANALYSIS OF RGC LOSS AFTER INJURY

Type of Optic Nerve Lesion / Time After Injury(months)	Linear Regression R^2	Slope	One-half Survival Time (months)
CUT			
0.25 - 6	0.86	- 0.33	2.1
0.33 - 6	0.94	- 0.29	2.4
0.5 - 6	0.93	- 0.29	2.4
1 - 6	0.95	- 0.26	2.6
CRUSH			
0.25 - 6	0.86	- 0.14	5.0
0.33 - 6	0.88	- 0.13	5.5
0.5 - 6	0.88	- 0.13	5.3
1 - 6	0.85	- 0.13	5.3

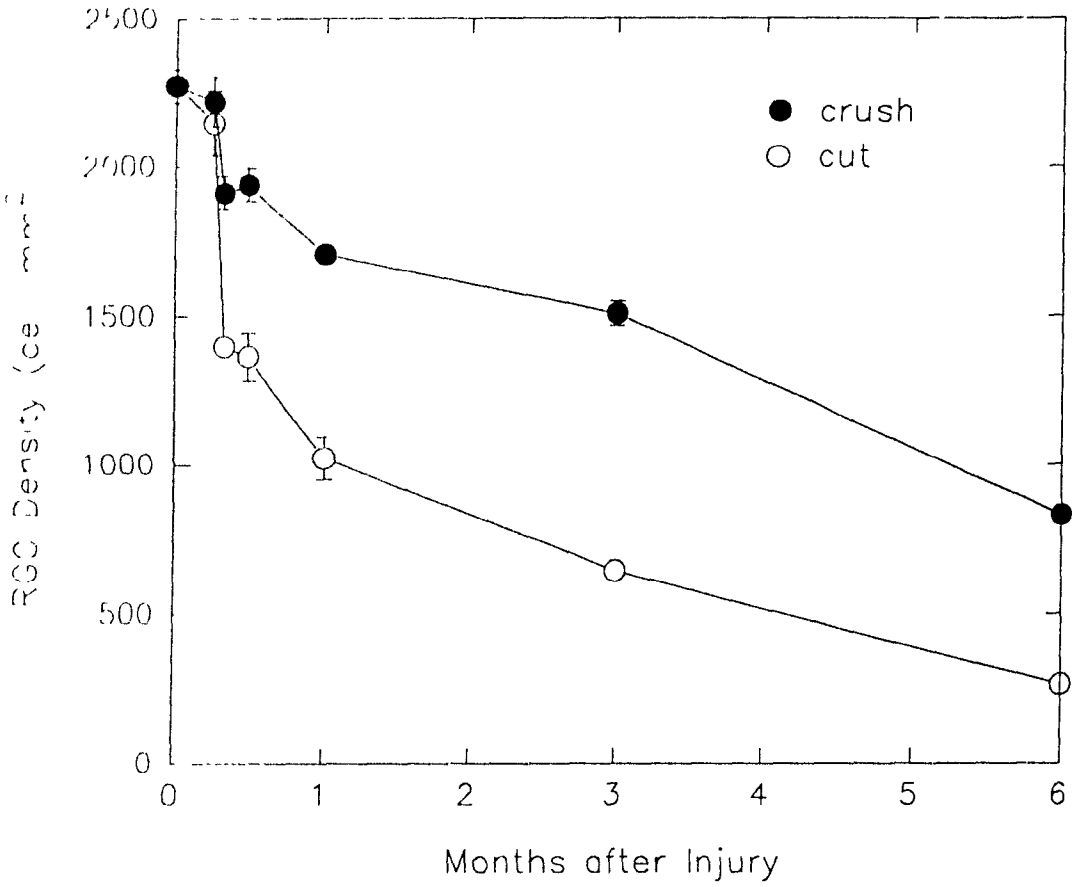

Figure 2

Graph of mean RGC density ± standard error after intracranial ON cut or crush versus time after injury plotted on a linear scale. Mean RGC densities are joined by straight lines. Loss of RGCs is delayed for 1 week after ON cut and crush. Cell loss after cut injury occurred rapidly during the second week followed by a more protracted RGC loss. Cell loss after crush occurred at a slower rate than after ON cut.

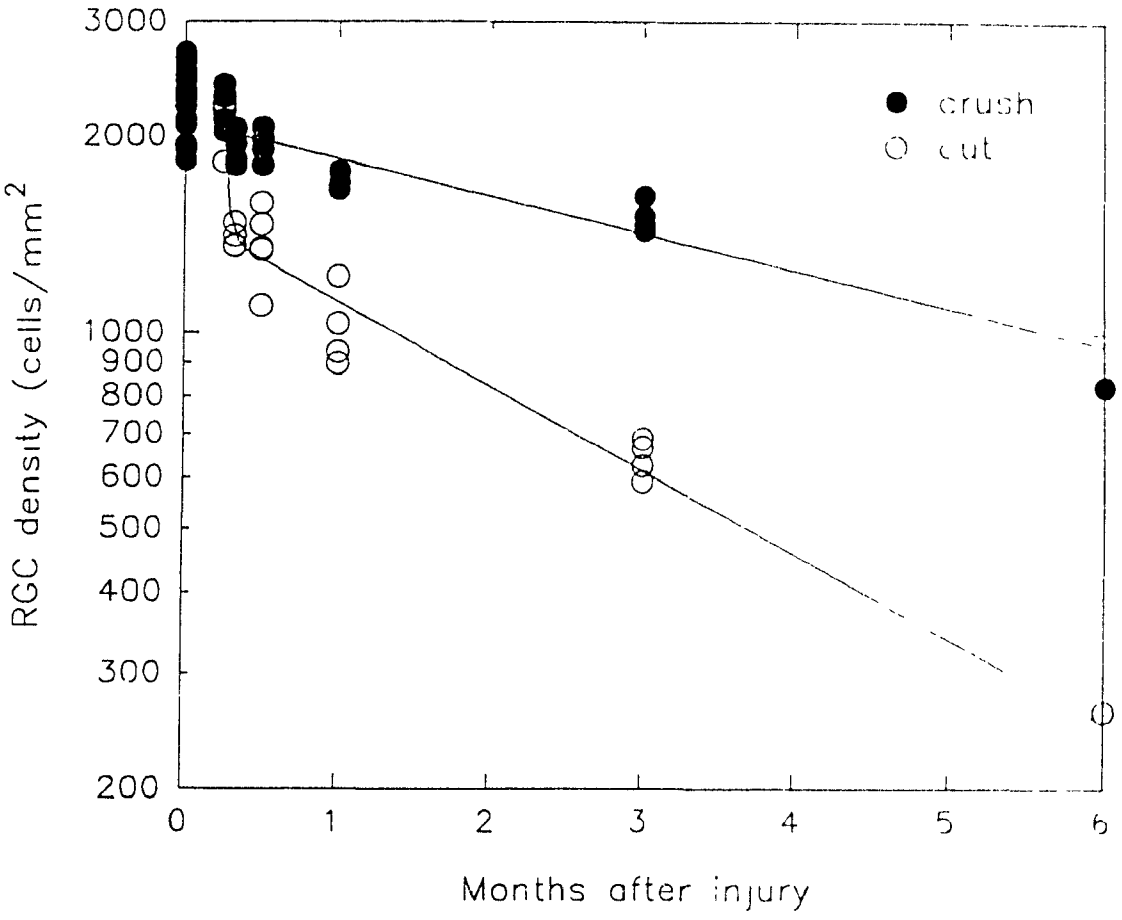

Figure 3

Graph of RGC density after intracranial ON cut or crush versus time after injury plotted on a semilogarithmic scale. The curves plotted were calculated as the best fit exponential decays for the data from 1 week to 6 months. The data at time zero were excluded because the cell loss was delayed until 1 week after injury. After ON cut, RGC loss was best fit by a curve with two exponentials describing an early rapid cell loss followed by a later, more protracted cell loss. After ON crush, RGC loss was best fit by a curve with one exponential describing a single rate of cell loss.

II. GROUP II RETROGRADE AXONAL CHANGES AFTER INTRACRANIAL ON CUT OR CRUSH

A. Retrograde Changes in the Optic Nerve

1 Control ON

Control ONs viewed in cross-section by light and electron microscopy contained axons of various sizes grouped together into fascicles The fascicles varied in size and were delineated by the astrocytic processes which surrounded them Almost all of the axons were myelinated and were irregular in shape (Figure 4a, Figure 5)

2 ON After Cut Injury

After intracranial cut injury, the ON was characterized by a core of extensive myelinated fiber (MF) loss which was observed from the injury site to within 2-3 mm from the eye At **three days**, the cut site contained no intact axons and many axons in the central area of the nerve from the site of injury to within 2-3 mm appeared to be disintegrating The area near the eye contained only normal axons. No macrophages were present. At **one week,** the nerve in cross section 1 mm from the eye contained an occasional disrupted MF (Figure 6a). Four mm from the eye, MF loss was pronounced with a core of macrophages and astrocytic processes The outer rim of the nerve contained an occasional disrupted MF (Figure 6b) Six mm from the eye the central region contained few axons while in the rim of the nerve, more than one half of the axons were lost. Eight mm from the eye (within 1 mm of the cut site) there were numerous macrophages and astrocytic processes but no intact axons (Figure 6c).

Two weeks after cut, the core of disintegrating MFs had not extended closer to the eye but 1 mm from the eye there were more disrupted MFs than

observed at 1 week. The core occupied a larger portion of the nerve in cross-section; at 4 and 6 mm from the eye, the central area contained no axons while the outer edge of the nerve contained a small population of intact axons

Four weeks after cut, at 4 mm (Figure 7b), 6 mm (Figure 4b) and 8 mm (Figure 7c) from the eye there were no intact axons observed in 4 of 6 animals One mm (Figure 7a) and 2 mm from the eye, over half the MFs were disrupted and were replaced by astrocyte processes **Three months** after ON cut, a few intact axons were present at the peripheral area of the ON 1-2 mm from the eye The ON from the cut site to 2 mm from the eye contained no intact fibers and contained many macrophages and astrocytic processes

Cut injury, therefore, caused rapid, extensive retrograde damage extending 6-7 mm toward the eye within 1 week of injury The core area containing many macrophages and few MFs did not extend closer to the eye by four weeks after injury This core occupied a greater portion of the ON in cross section over the time course observed such that at 4 weeks, there were no intact axons from 1 9 mm from the eye Within 3 mm of the eye, the ON progressively lost MFs from 1 week, when only occasional MFs were disrupted, to 4 weeks, when more than half of the MFs were lost No macrophages entered the area within 2 mm of the eye up until 4 weeks after injury, as observed by morphological assessment in LM sections MF loss was accompanied by astrocytic fiber invasion and loss of the fascicular nature of the ON (Figure 9).

3. ON After Crush Injury

Retrograde changes in the ON after crush injury were characterized by mild loss of MFs. The crush site in all animals contained no intact MFs and consisted mainly of astrocytic processes, and in some animals, an occasional macrophage

This complete loss of axons observed at the crush site did not extend more than 1.5 mm from the injury. **Three days** after ON crush, the crush site contained no intact axons and the nerve was filled with astrocytic processes. No axon loss was observed in the remainder of the ON. **One week** after ON crush, severe loss of MFs extended less than 1 mm toward the eye. The remaining ON proximal to the injury appeared normal or contained only an occasional disrupted MF. **Two weeks** after crush, the loss of MFs extended up to 1.5 mm from the injury site but the remaining ON stump showed only occasional disrupted MFs. **Four weeks** after crush, there was a mild loss of MFs 1 mm (Figure 8a), 2 mm, 4 mm (Figure 8b) and 6 mm (Figure 4c) from the eye. The crush site contained no intact axons but, similar to 1 week after injury, contained mainly debris and astrocytic processes (Figure 8c). **Three months** after crush, some axons in the ON were disintegrating, but most appear intact. No core area of increased axon loss was observed after crush injury. As after cut injury, the loss of MFs was accompanied by an increased area occupied by astrocytic processes. Intact MFs appeared to be smaller 4 weeks and 3 months after crush when compared to control nerves.

Crush injury, therefore, causes severe retrograde loss of MFs only 1-2 mm from the site of injury. The remainder of the ON stump undergoes a very slow, progressive loss of axons but by 4 weeks after injury, most of the ON shows only a mild loss of MFs (Figure 9).

Figure 4

Light micrographs of the ON in cross-section, 4 mm from the eye.
a, c, e: Low power micrographs of the whole ON. Bar = 100µm.
b, d, f: Higher power micrographs of a portion of the ON. Bar = 30µm.

a and b: Control Optic Nerve: Most axons are myelinated and vary in size. They are organized into bundles that are separated by astrocytic processes

c and d: Four Weeks After Intracranial Cut: The central portion of the ON is occupied by macrophages and myelin debris. The peripheral area of the ON contains disintegrating axons and some intact axons

e and f: Four Weeks After Intracranial Crush. The ON appears to be similar to the control except for a small population of degenerating axons scattered throughout the cross-section.

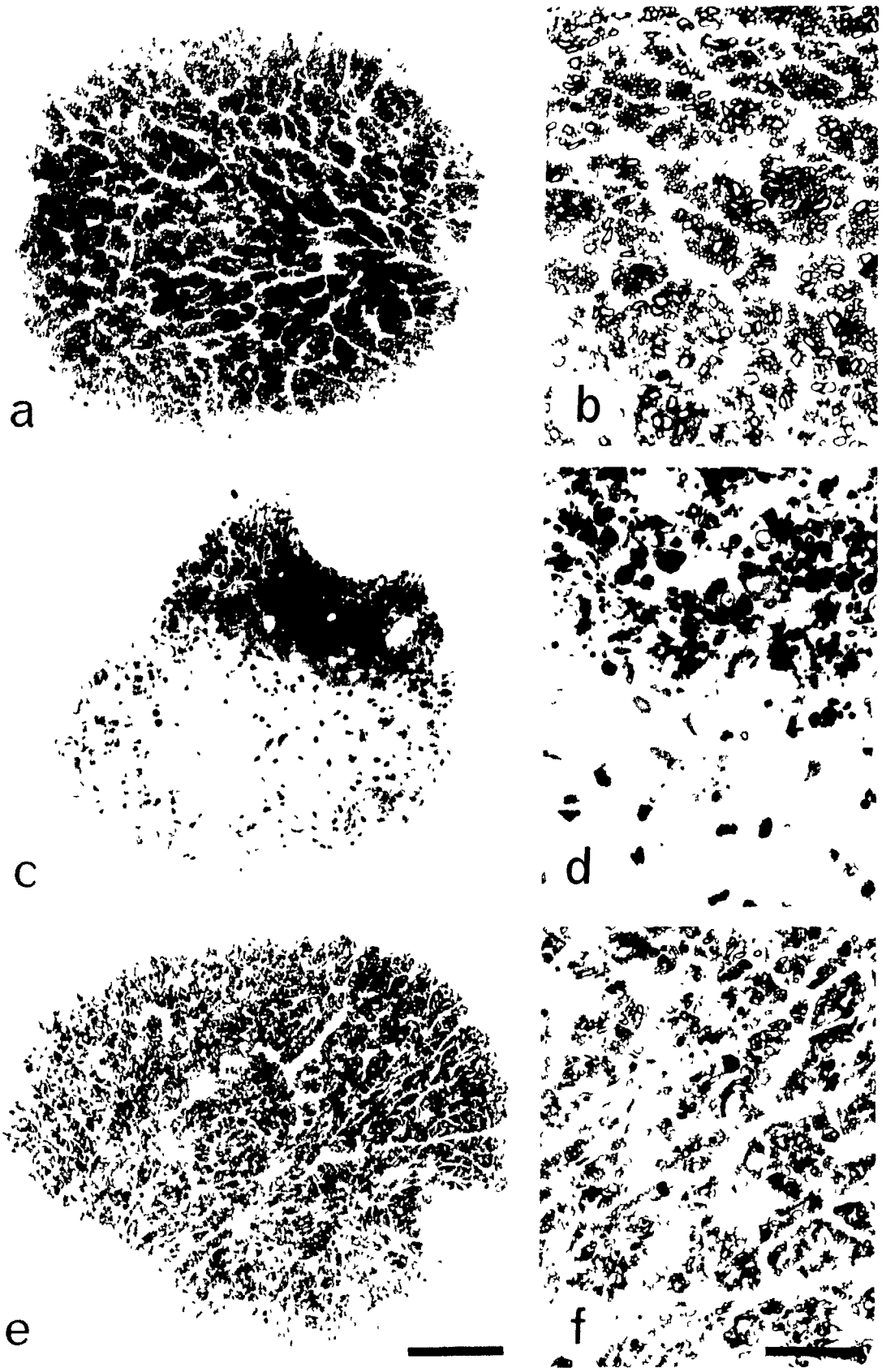
a
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Figure 5

Low power electron micrograph of a control optic nerve 4 mm from the eye. Most axons are myelinated and vary in size. The axons are grouped into bundles that are separated by astrocytic processes. Bar = 10 μm

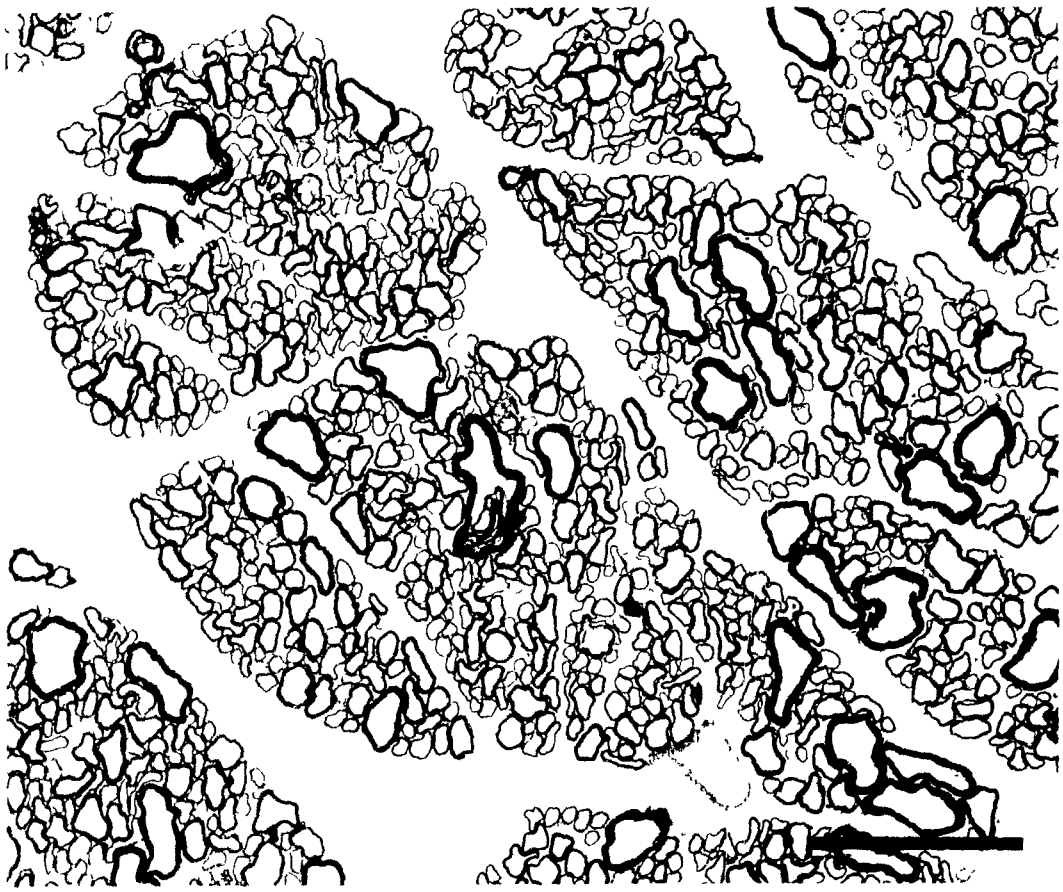

Figure 6

Low power electron micrographs of the ON stump in cross-section at different distances from the eye one week after intracranial ON cut. Bar = 10 μm

a. One mm from the eye: The fibers appear similar to control. An occasional disintegrating fiber is observed.

b. Four mm from the eye: The fibers in the periphery of the ON seen on the right side of the micrograph are mostly intact with a few disintegrating fibers while the central region of the ON, seen on the left side of the micrograph, contains very few intact fibers and is occupied by macrophages and astrocytic processes.

c. Eight mm from the eye: Near the cut site, the entire nerve in cross-section is filled with macrophages and contains no intact fibers. The macrophages contain degrading myelin debris.

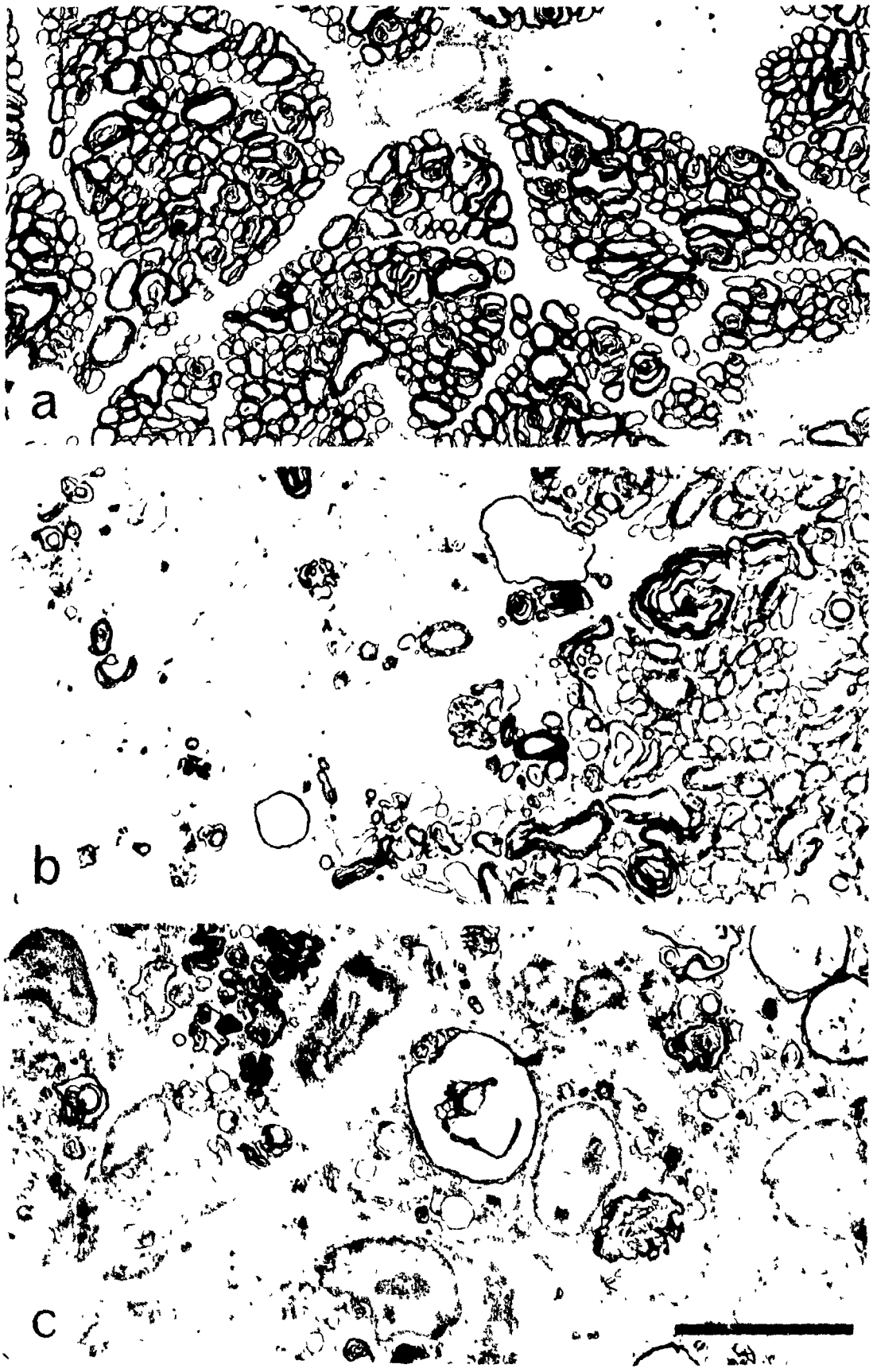
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Figure 7

Low power electron micrographs of the ON stump in cross-section at different distances from the eye four weeks after intracranial ON cut. Bar = 10 μm.

a. One mm from the eye: Most fibers are disintegrating and the bundles of axons are not clearly delineated.

b. Four mm from the eye: No fibers are intact in the central ON. The periphery of the nerve may contain a few intact fibers. The ON contains many astrocytic processes and macrophages engulfing myelin debris.

b. Eight mm from the eye: No fibers are intact. The ON is occupied by astrocytic processes and macrophages engulfing myelin debris.

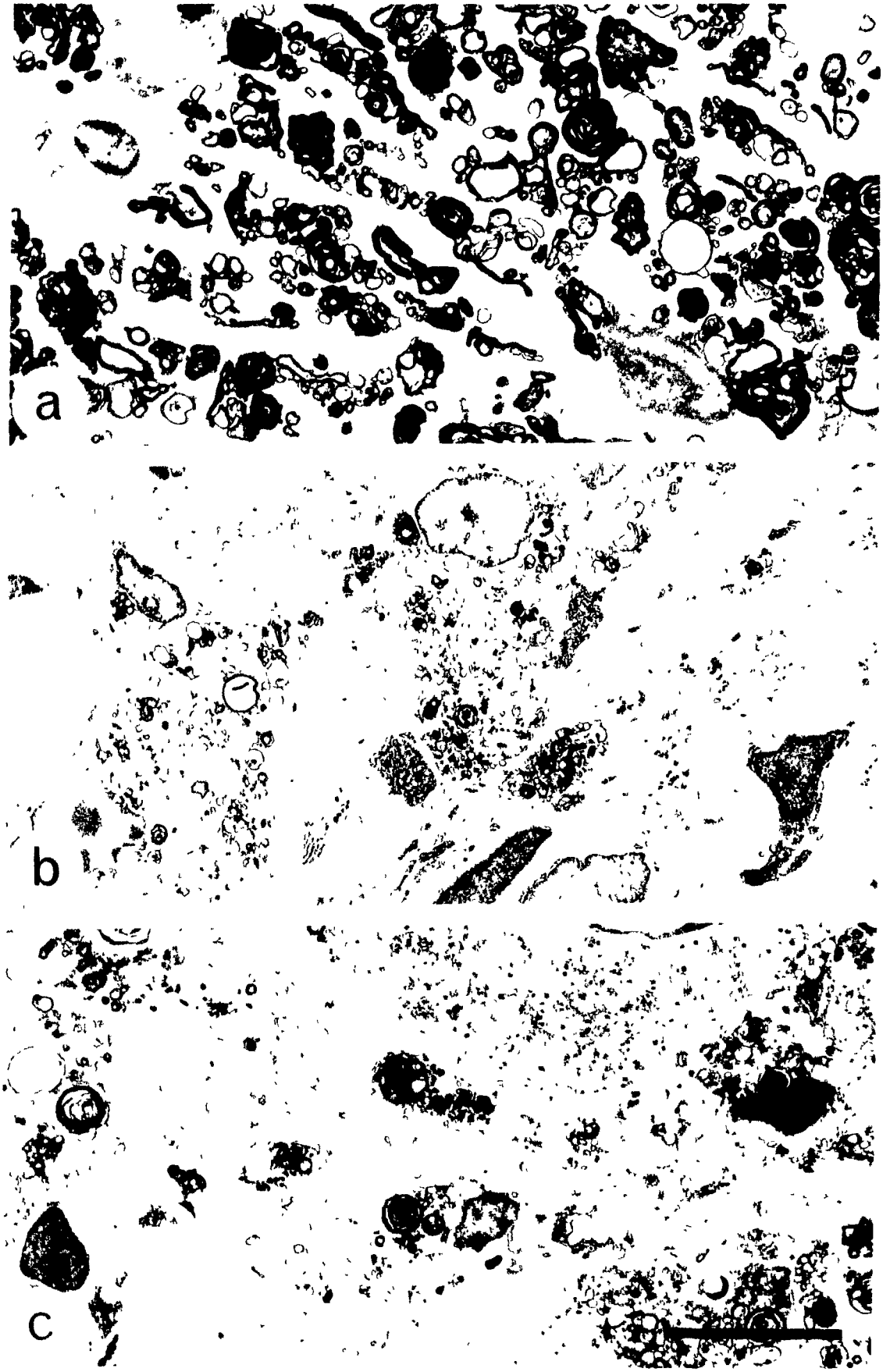
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Figure 8

Low power electron micrographs of the ON stump in cross-section at different distances from the eye four weeks after intracranial ON crush. Bar = 10 μm.

a. One mm from the eye: A few fibers are disintegrating. Axon bundles are clearly delineated.

b. Four mm from the eye: A few fibers are disintegrating. Axon bundles are clearly delineated.

c. Eight mm from the eye, the crush site: No intact axons are observed. Disintegrating fibers and astrocytic processes occupy the nerve.

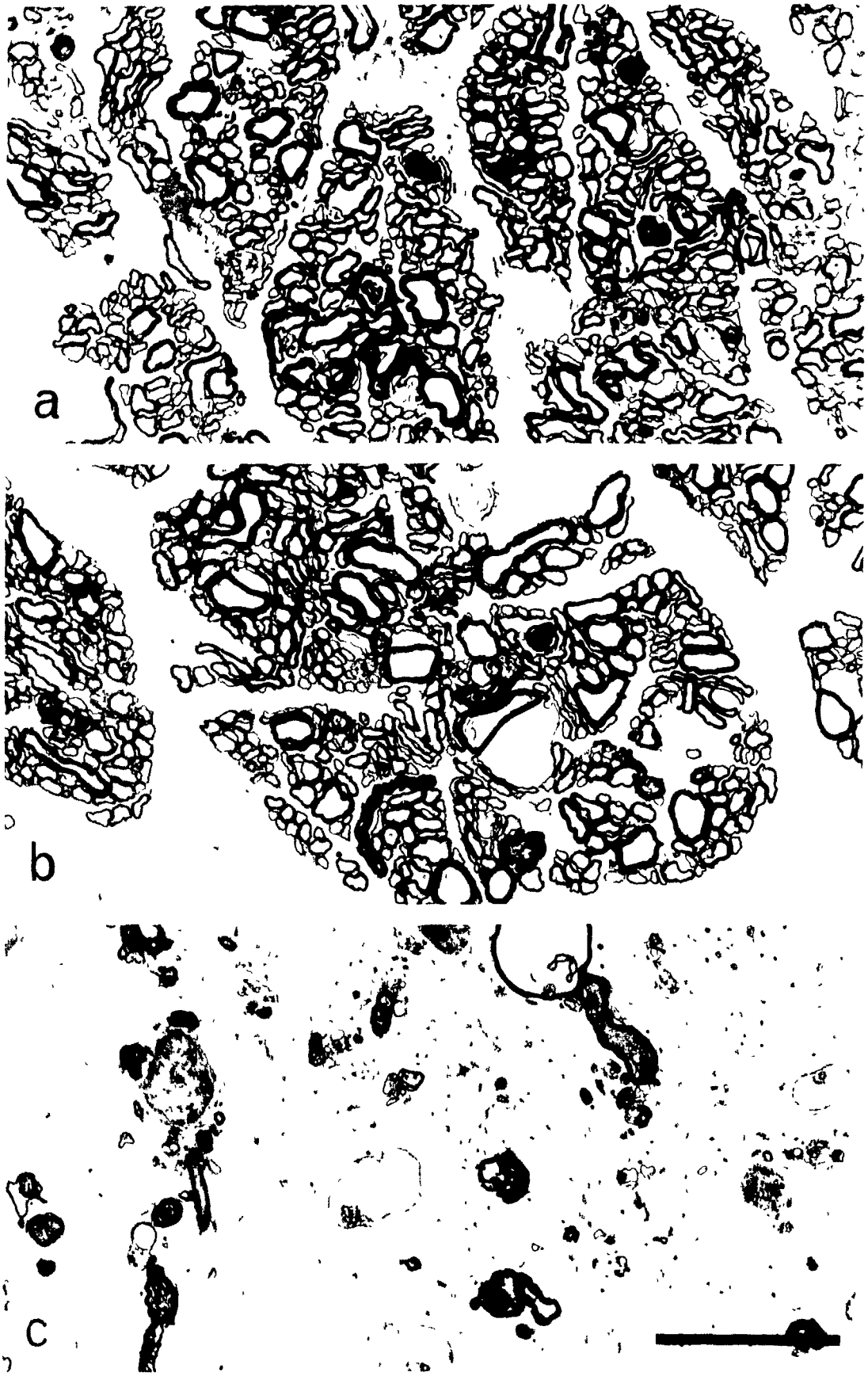
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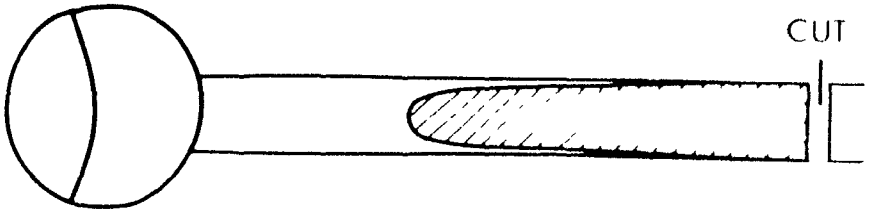
Figure 9

Schematic diagrams of the eye and optic nerve after injury. The area of severe tissue disturbance, axon loss and macrophage invasion is shown by hatched lines.

a. Intracranial Cut: A core of severe axon loss and macrophage invasion was observed from the injury site to within 3 mm of the eye.

b. Intracranial Crush: Severe axon loss was observed at the crush site to approximately 1-1.5 mm from the injury. Phagocytic cells were occasionally observed.

a



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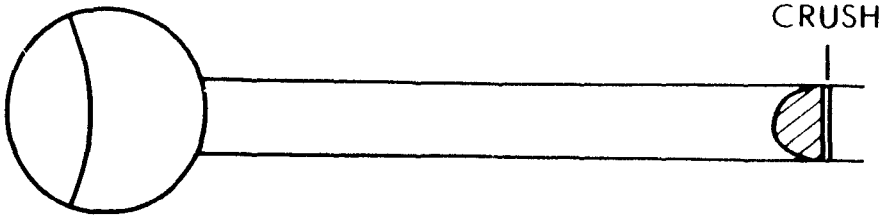


B. Retrograde Changes in the Retina

1 RT97 Immunoreactivity of Intraretinal Fibers

a RT97 Immunoreactivity in the Normal Retina

Retinal ganglion cells send their axons from the cell body to the optic disc in large axon bundles which traverse the fiber layer of the retina. The axons within the retina are not myelinated and can be viewed on flatmounts with immunohistochemical staining with RT97 (provided by Dr. John Wood) which recognizes the 200 kDa intermediate filament subunit (Anderton, 1982). Fluorescent RT97 labeling with FITC on a wholemounted control retina shows the large dense axon bundles converging at the optic disc (Figure 10a).

RT97 immunoreactivity can be observed in the light microscope by using a DAB secondary reaction resulting in a dark brown reaction product. Retinal flatmounts reacted in this way were used to visualize and measure the size distribution of RGC fiber bundles within the retina. In control retinas, the peak of bundle size distribution was in the 5-20μm range, 25% of control bundles were in each of the 5-10, 10-15, and 15-20 μm size classes (Figure 11).

b RT97 Immunoreactivity in the Retina After Cut Injury

After cut injury, the loss of RGC axons within the retina was observed as a decrease in the size and density of the RT97 immunoreactive axon bundles in flatmounted retinas. One week after intracranial ON cut, the axon bundles did not appear qualitatively different from control retinas. However by two weeks after ON cut, the bundles appeared thinner and less dense than control retinas. There was a progressive decrease in axon bundle size and density so that four weeks after cut, the axon bundles appeared to occupy less than half the retinal area occupied in a control retina (Figure 10b).

In addition, some axons or small bundles of axons did not follow the normal straight course within the retina. They occasionally turned and overlapped adjacent axons until they joined another axon bundle and continued their path to the disc (Figure 10b).

The distribution of RGC fiber bundle widths was also determined after cut injury. A shift in bundle diameter distribution to smaller bundle sizes was observed at 4 weeks after ON cut. 45% of axon bundles were in the 0-5 μm size category 4 weeks after cut compared to 4% of control bundles in the same size class (Figure 11). It was noted that the number of bundles measured per unit retinal distance increased after ON cut.

c. RT97 Immunoreactivity in the Retina After ON Crush

The changes in intraretinal axon density, qualitatively determined by RT97 immunoreactivity, were more gradual after ON crush than ON cut. After crush injury, the density and size of intraretinal axon bundles appeared the same as control retinas until four weeks after injury. At four weeks, the axon bundles appeared slightly smaller, but of equal density as control retinas (Figure 10c). The path of the axon bundles, unlike those after cut lesion, did not deviate from normal.

The distribution of intraretinal bundle widths was also measured after crush injury. Again, the decrease in axon bundle width was more gradual after ON crush than ON cut. Four weeks after crush, the peak of bundle size distribution had shifted to smaller bundle sizes; 35% of bundles were in the 5-10μm class and 27% of bundles were in the 0-5μm class (Figure 11). A trend toward smaller axon bundles was observed after both types of axon interruption but the shift to smaller bundles was not as pronounced after ON crush.

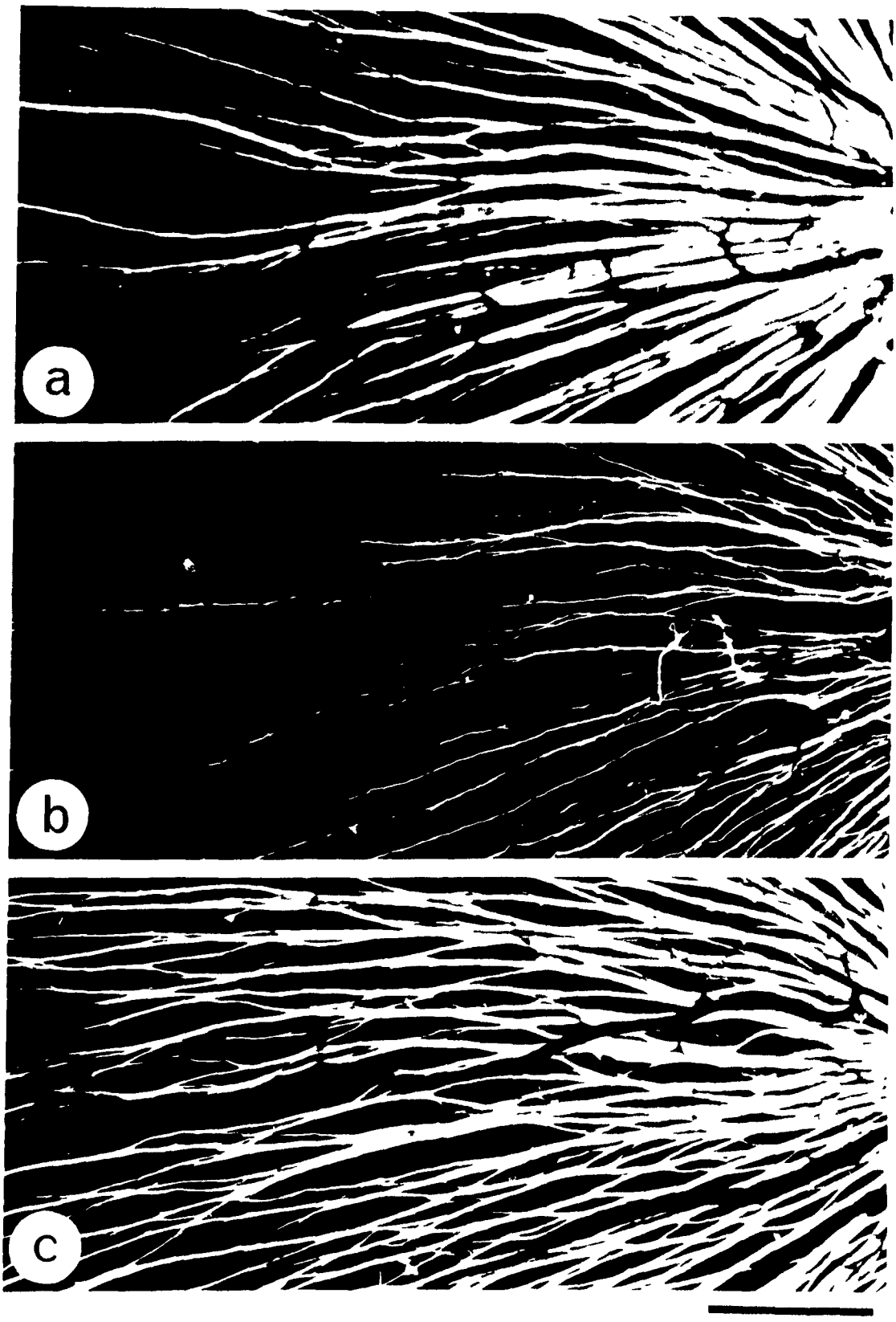
Figure 10

Fluorescence photomicrographs showing RT97 immunoreactivity in a portion of a retinal quadrant. The RGC axons, arranged in bundles, traverse the retina from their cell bodies to the optic disc, located to the right in each micrograph. Bar = 500μm

a. Control Retina: The axon bundles are large in diameter and their projection is straight.

b. Four Weeks After Intracranial ON Cut: The bundles appear reduced in diameter compared to controls and some axons or groups of axons traverse the retina in an aberrant direction.

c. Four Weeks After ON Crush: The axon bundle size is similar to the control retina.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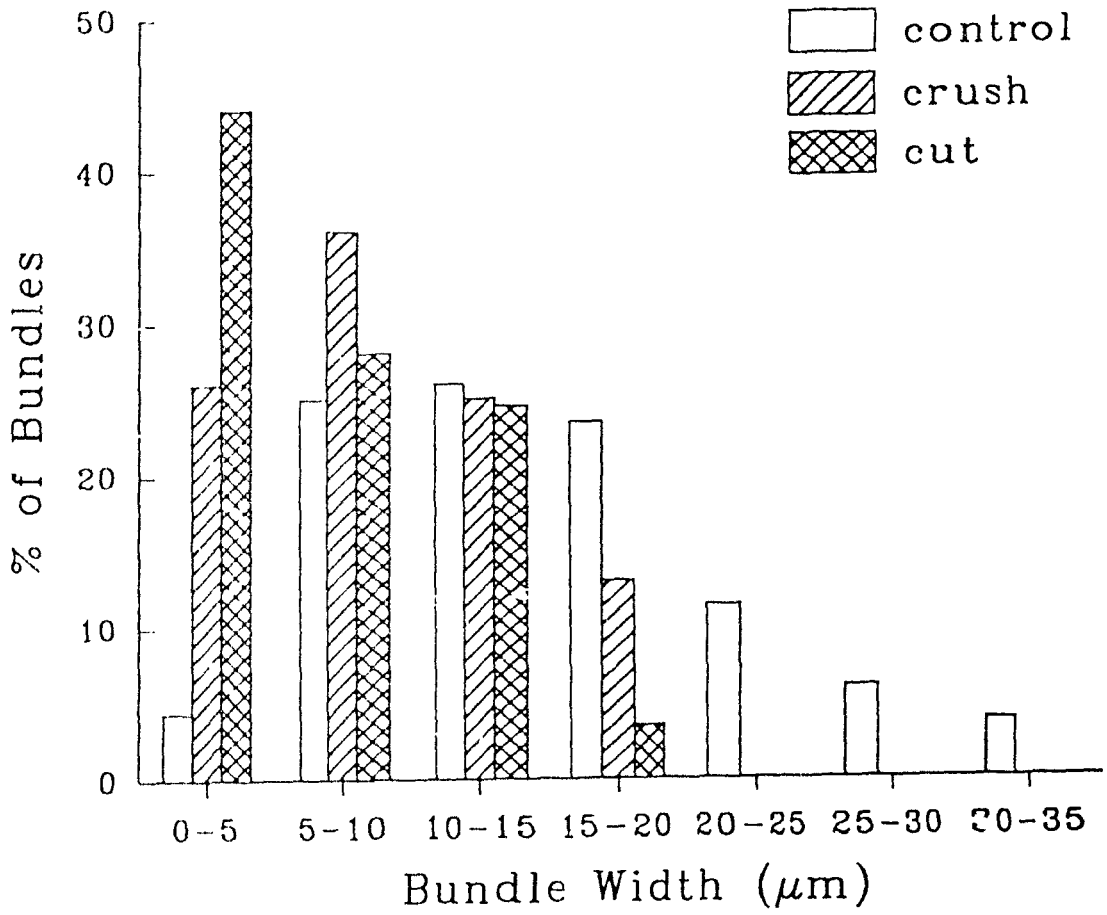


Figure 11

Histogram illustrating the size distribution of intraretinal RGC axon bundles measured 0.22 mm from the disc in control retinas and four weeks after intracranial cut or crush. The bundle size distribution shifts to smaller bundle widths following ON crush (hatched bars) and to even smaller bundle widths after ON cut (cross hatched bars).

2 Electron Microscopy of Intraretinal Axon Bundles

a Control retinas

Control retinas were examined in LM in cross section to view the bundles of axons traversing the retina to the optic disk. Control bundles were round and varied in size, containing approximately 100 to 1000 axons. The axons in a bundle were tightly apposed to each other and the bundles were separated by Muller cell processes and blood vessels, however the boundary of each bundle was not clearly delineated. The identity of the bundle contents as a pure population of axons could not be confirmed. As a result, quantitation of the axon number or cross sectional area was not possible (Figure 12a)

b. Intraretinal Axon Bundles After ON Cut

No changes in the intraretinal axon bundles had occurred at one week after ON cut. However, at two weeks after cut, the axons within each bundle were not as closely associated with each other and the boundaries of each bundle were less apparent. Four weeks after ON cut, fibers were separated further from each other, the bundles appeared to split into smaller patches of axons and the boundaries of each bundle were indiscernible (Figure 12b). These observations of bundle breakdown using electron microscopy correlate well with the observations of RT97 immunoreactivity in Flatmounted retinas. Both methods revealed a qualitative change in the size of axon bundles at 2 weeks after ON cut that became more marked at 4 weeks.

c Intraretinal Axon Bundles After ON Crush

No change in the axon bundles was observed until 4 weeks after ON crush. Four weeks after crush, the axons were not densely packed and the bundle

boundaries were less distinct than in control retinas (Figure 12c) The retinal fiber layer appeared similar to those observed 2 weeks after ON cut These observations using EM also correlate well with the axon loss observed using RT97 immunoreactivity Both methods revealed that qualitative losses in RGC axons were not apparent until 4 weeks after ON crush

C. Crush and Suture

The ON of animals receiving crushes and ligatures were observed by light microscopy 2 weeks after injury The ON degenerative changes were characteristic of a cut lesion, 2 weeks after injury a central area of severe fiber loss and macrophages was observed and the fiber loss was pronounced near the eye

Figure 12

Low power micrographs of the vitreal surface of the retina showing the RGC fiber layer and the inner nuclear layer. Bar = 5 μm.

a. Control Retina: The RGC fibers are arranged in large bundles of approximately 100 to 1000 fibers located near the vitreal surface. One bundle is seen in this micrograph.

b. Four Weeks After Intracranial ON Cut: Large axon bundles are not visible. Small groups of fibers and individual fibers are scattered along the fiber layer.

c. Four Weeks After Intracranial ON Crush: Large axon bundles are observed, similar to control retinas.


a
b
c

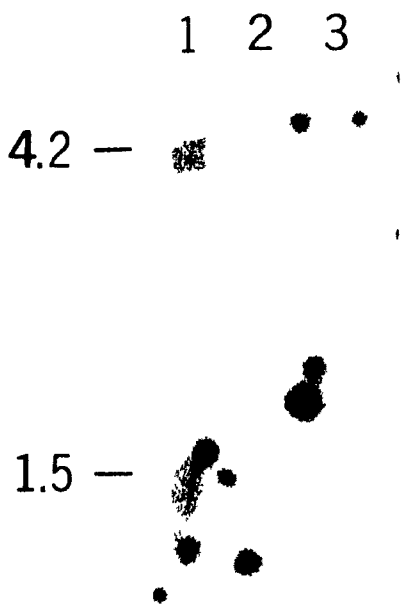
III. EXPRESSION OF BRAIN DERIVED NEUROTROPHIC FACTOR IN THE OPTIC NERVE AND RETINA

Northern blot analysis of total brain RNA with a rat DNA probe recognizing brain derived neurotrophic factor revealed two transcripts, approximately 1.5 and 4.2 Kb in size. No expression was observed in liver. These results were previously reported (Hofer et al., 1990, Marsonpierre et al., 1990) and were repeated to establish that the rat DNA probe recognized these BDNF transcripts. This rat DNA probe was used to probe RNA from normal ON and retina. Two BDNF transcripts (1.5 and 4.2 kb) were clearly visible in brain mRNA preparations (figure 13, lane 1). In retinal (lane 2) and ON (lane 3) mRNA preparations, faint bands were detected in the same regions. The level of expression was higher in the retina than in the ON. Expression of BDNF in the adult retina was previously reported by Marsonpierre et al. (1990b), who found a low level of expression compared to adult brain.

Additional BDNF probes were utilized during the course of the experiment. a mouse BDNF mRNA sequence, obtained from M. Hofer, was used to prepare RNA and DNA probes. Both probes failed to recognize BDNF in rat RNA preparations using various hybridization and washing conditions, despite the identical sequences of rat and mouse BDNF mRNA. The rat BDNF sequence used to make a DNA probe for the experiments reported in this thesis was also used to prepare an RNA probe. Northern blots prepared with this probe showed non specific binding to the ribosomal RNA which interfered with the BDNF bands located directly below the rRNA bands. Thus, the rat BDNF DNA probe was determined to be the most effective probe for Northern hybridization of rat CNS tissue.

Figure 13

Northern blot of brain (lane 1), retina (lane 2) and optic nerve (lane 3), probed with a ^{32}P-labelled rat brain derived neurotrophic factor (BDNF) DNA probe. Two transcripts (1.5 and 4.2 kb) were clearly visible in brain mRNA preparations and faint bands were observed in retinal and ON mRNA preparations.


1 2 3
4.2 —
1.5 —

IV. SUMMARY OF RESULTS

The pattern of RGC loss after axotomy is influenced by the method of ON interruption. There is an initial delay in cell death after both cut and crush injury. RGC densities one week after injury are not significantly different from control values. RGC loss after ON cut occurs in two phases, an early rapid cell loss followed by a later, more protracted cell loss, while RGC loss after crush injury occurs at a single slow rate.

The retrograde morphological changes that occur in the RGC axons after injury are also influenced by the type of ON interruption. After ON cut, the axons within the ON degenerate rapidly and the central region of the ON, extending from the injury to within 2-3 mm of the eye contains mainly debris and invading macrophages. The axons in the retina are also lost rapidly. qualitative changes, as observed by RT97 immunoreactivity and electron microscopy, are apparent 2 weeks after injury and at 4 weeks, the bundle size and density has decreased to less than half of controls.

ON crush is characterized by a less intense, gradual loss of axons in both the ON and the retina. The fiber loss in the ON appears to be uniform along the length of the ON stump and severe MF loss and macrophage invasion is observed only at the crush site. The loss of intraretinal axons is not qualitatively observable using RT97 and electron microscopy until four weeks after injury when slight decreases in bundle size and density are apparent.

Northern blots using a rat BDNF DNA probe suggest that BDNF is expressed in low levels in the normal ON. Two BDNF transcripts, 1.5 and 4.2 kb were observed in the brain, retina and ON, but not in the liver.

CHAPTER 4: DISCUSSION

In this chapter, the possible mechanisms of axotomy-induced retinal ganglion cell death are discussed. In the first section I examine the different patterns of cell death observed after axotomy by cut or crush and suggest mechanisms of cell death that may be responsible for the delayed nature of the cell loss after both types of injury, the rapid and protracted loss after cut and the single, slow rate of cell loss after crush. In the second section I discuss how the axonal changes observed in the ON and retina after cut and crush may contribute to the different patterns of cell loss. In the third section I discuss the possible role of growth factors derived from non-neuronal cells in the maintenance of RGC survival after axotomy.

I. RGC SURVIVAL AFTER INTRACRANIAL ON CUT OR CRUSH

A. RGC Survival After ON Crush or Cut: Comparison with Earlier Studies

Several previous studies have investigated RGC survival after ON injury. Early reports established that neuronal loss occurred after ON interruption (James, 1933; Leinfelder, 1938, Mantz and Klein, 1951, Fayrs, 1952, Polyak 1958, Stone, 1965; Quigley et al., 1977, Radius and Anderson, 1978) and suggested that the location of the injury (Leinfelder, 1938, Mantz and Klein 1951) and the type of intraocular injury, cut versus ligature (Mantz and Klein, 1951) were important determinants of the extent of RGC loss.

Later, quantitative studies of RGC loss in the rodent examined the effects of a single type and location of ON lesion (Grafstein and Ingogha, 1982, Allcutt et al., 1984; Misantone et al., 1984; Barron et al., 1986). These studies relied on

conventional histological stains to identify RGCs. Villegas-Pérez et al (1992), who used a retrograde fluorescent tracer, dil, to distinguish RGCs from displaced amacrine cells (Perry, 1981), examined the effect of the location of the injury on the RGC loss. However, no single study quantitated the effect of the type of ON lesion on the loss of RGCs. In the PNS, Lunn et al (1990) showed that the type of injury influenced the morphology of the ON during Wallerian degeneration: nerve section and prolonged ligature produced more rapid and more pronounced degenerative changes than crush injury or temporary ligature.

The present study documents that the method of axonal interruption, cut or crush influences the pattern of neuronal loss in the CNS, specifically in the RGCs of the adult rat. With either lesion there is an initial delay prior to RGC loss; control densities are maintained for one week after both cut and crush injuries. RGC loss after cut injury occurs in two phases: an early, rapid phase followed by a later, protracted phase, and is more severe than RGC loss after crush injury, which is mild and occurs at a single rate. At all times after injury, RGC survival was greater after ON crush than cut.

The extent of cell loss after these two types of injury can be compared to earlier quantitative reports. Grafstein and Ingoglia (1982) reported that RGC survival after intracranial ON cut in the mouse was 80% at 3 days and 50% at 65 days (present study: 94.4%, 45.0% and 28.3% at 7 days, 1 month and 3 months respectively). Misantone et al (1984), investigated the effect of intracranial ON crush on rat RGC survival. They reported no loss of RGCs up to 3 months after injury and 60% survival at 230 days (present study: 66.4% and 36.5% at 3 months and 6 months respectively). A comparison of these two earlier reports suggests that RGC loss is greater after ON cut. The percentages of surviving RGCs are not the same as those obtained in this report perhaps because the earlier studies

involved different species and used different morphological criteria to identify RGCs. In addition, the lesion type and location may not be identical. However, in spite of these differences, the same trend toward greater cell loss after ON cut can be deduced from the earlier studies.

Villegas Perez et al. (1992) quantitated the loss of RGCs after ON lesions at different distances from the eye. Intracranial ON interruption was performed by multiple crushes in the same site causing the nerve to be severed while intraorbital lesions were transections with scissors. The multiple crush caused an early rapid loss of RGCs as observed after intracranial cut in the present experiment, such that at two weeks the RGC density after multiple intracranial crushes was similar to intracranial cut (57.4%-65.6%, Villegas-Pérez et al., 1992, 60.0%, ON cut present study). However the rate of RGC loss from 2 weeks to 15 months after multiple crush was slower (one-half survival time = 6.1-6.7 months) than ON cut from 2 weeks to 6 months (one-half survival time = 2.4 months) or ON crush from 2 weeks to 6 months (one-half life = 5.3 months). Despite the different pattern of cell loss observed after multiple crush, the RGC density at all times after multiple crush injury was greater than the RGC density after intracranial cut and less than the RGC density after intracranial crush. These different patterns of cell loss suggest that the multiple crush injury technique of Villegas Perez et al. (1992) may represent an injury intermediate between ON cut and the double crush used in the present study.

Thus, a comparison of earlier quantitative studies and the present report of RGC survival after intracranial ON injury supports the idea that the type of injury influences cell survival where ON cut results in greater RGC loss than ON crush. In addition, the type of crush also influences the pattern of RGC loss, where multiple crush results in greater RGC loss than double crush.

The pattern of RGC loss after intracranial cut can also be compared to the patterns of cell loss after intraorbital cut observed by Villegas-Pérez et al (1992). Intraorbital cut at 0.5 mm yielded an RGC loss to 24.7% survival at two weeks and a one half survival time of 0.8 months, calculated from 2 weeks to 3 months. The RGC density at two weeks after intraorbital cut at 3 mm was 38.5 % of control and the one half survival time was 1.2 months, suggesting that lesions close to the eye result in more rapid cell loss (Villegas-Pérez et al., 1992). In the present study, after intracranial cut at approximately 9 mm from the eye, RGC survival was 60.0% at 2 weeks and the one-half survival time was 2.4 months calculated from 2 weeks to 6 months. Thus, the present results support the observation by Villegas-Pérez et al (1992) that increasing the distance from the soma to the injury results in less rapid RGC loss.

The idea that the location of the injury influences the extent of RGC loss is further supported by a comparison of earlier reports of RGC survival after intraorbital ON crush and the present report of survival after intracranial ON crush. Two earlier reports quantitated RGC survival after intraorbital ON crush, in the mouse, Allcutt et al (1984), reported 20-40% survival and 0-20% survival 20 and 80 days while in the rat, Barron et al (1986), showed 64% survival at 7 days and 32% survival 6 months after the same injury. Although these studies used different species and methods of identifying RGCs, the RGC survival after intraorbital crush in these two reports are generally lower than the RGC survivals reported after intracranial ON crush in the present study, suggesting that RGC loss is greater after intraorbital crush than intracranial crush. Thus, for both cut and crush lesions, the loss of RGCs decreases with lesions further from the eye.

B. RGC Loss is Delayed After ON Cut and Crush

The loss of RGCs after both intracranial cut and crush is delayed until approximately one week after ON injury. Villegas-Pérez et al. (1992) did not document a delay prior to cell loss because the retrograde tracer, dil, used in their study, did not allow accurate RGC quantitation prior to 2 weeks after injury. This delay in RGC loss was suggested earlier by McKerracher et al (1991) after they showed that two cytoskeletal proteins, tubulin and neurofilament, continue to be expressed normally for nearly 10 days after intracranial axotomy In addition, these proteins continue to be transported normally for one week after injury, followed by a 10-fold decrease in the rate of slow transport (McKerracher et al , 1990b). Cytoskeletal proteins also continue to be expressed after axotomy of rubrospinal neurons: mRNAs for tubulin and neurofilament are increased during the first week, followed by a decrease in mRNA during the second and third weeks after axotomy, coincident with their atrophy (Tetzlaff et al , 1991)

This delay prior to cell loss suggests that RGCs do not die as a direct result of mechanical damage, which would be expected to occur rapidly Rather, RGCs may die via a mechanism requiring a delay, that possibly permits retrograde transport of a signal, gene expression and protein synthesis Although retrograde axonal transport occurs rapidly, the time required for gene expression, protein synthesis and induction of degenerative changes in the cell body is presently not known. Singer et. al. (1982) reported that a blockade of retrograde axonal transport in motor nerves delayed axotomy-induced chromatolysis Thus, the induction of chromatolysis in these motor neurons may be dependent on retrograde transport of a signal If a retrograde signal is required for the death of RGCs after injury, one would expect that the distance from the lesion to the cell body would influence the length of the delay This possibility is presently being examined (S

Mansour-Robaey, personal communication). Although the morphological changes in the soma of RGCs were not addressed in the present report and the suggestion of an active mechanism of RGC death is purely speculative, several recent studies propose the involvement of gene expression in neuronal death. E. M. Johnson and colleagues, for example, suggest that sympathetic neurons, when deprived of NGF in culture, die by an active suicide-like process that can be prevented by inhibitors of RNA and protein synthesis (Martin et al., 1988) The role of gene expression and protein synthesis in neuronal death is discussed in detail in Section I D

An alternative explanation for the delay in cell death is the transient expression of growth factors in the ON that maintain RGC survival for this period. Lu et al (1991) reported a transient increase in expression of NGF in the distal ON stump after enucleation in the adult rat. An 8-fold increase occurred at day 1, followed by a decrease to normal levels by 1 week. Such a transient increase may explain the delay in RGC death, however, the expression of NGF or other growth factors and their receptors has not been investigated after intracranial injury. The possibility that trophic factors are expressed after injury is examined in Section III.

C. RGC Loss After ON Cut Occurs in Two Main Phases

The RGC loss after ON cut occurs in two phases: an early, rapid cell loss followed by a later, more protracted cell loss. This pattern of cell loss is comparable to the data of Villegas-Pérez et al (1992) They reported RGC loss after intraorbital cut or multiple intracranial crush: there was an early, rapid phase dependent on the soma-injury distance followed by a later, protracted phase of RGC loss.

Different phases of cell loss have also been documented after other types of injury. Ischemic neuronal death occurs in two steps, immediate and delayed

In these two examples, the injury is caused by a single event (hypoxia) or agent (AMPA), yet the death does not occur at a single rate. Each phase of neuronal death may be influenced by different variables. For example, the delayed phase of ischemia induced death, but not the immediate phase, is dependent on protein synthesis (Goto et al., 1990, Shigeno et al., 1990) and can be prevented by NMDA receptor antagonists (reviewed by Siesjo et al. 1989, Choi, 1990, Choi and Rothman, 1990). These examples of a single agent causing more than one pattern of cell death, where each phase can be independently influenced by external factors, suggest that more than one mechanism of cell death can be triggered by one agent or event. The neuronal loss after ON cut, which occurs in two phases, may result from a single factor acting via two or more mechanisms of cell destruction.

D. Early, Rapid RGC loss After ON Cut

The first phase of RGC loss after intracranial ON cut is rapid, such that 34.3% of the cells are lost between 1 and 2 weeks after injury. This rapid phase of cell loss is not observed after the type of ON crush injury used in the present study. In addition, this rapid cell loss begins approximately one week after cut injury and therefore, is not preceded by the retrograde degeneration of the axons from the site of injury to the soma. Rather, the axons 1 mm from the eye appear normal 1 week after ON cut except for a very small population of disintegrating axons. This observation eliminates the possibility that the loss of the soma results from the dying back of the axon.

Villegas-Pérez et al. (1992) also showed an early, rapid loss of RGC's after intraorbital ON cut and intracranial ON interruption by crush. The severity of this early loss was dependent on the location of the injury where lesions close to the

axons. If confirmed by quantitative studies of surviving RGC axons, this observation eliminates the possibility that the loss of the soma results from axons dying back.

Villegas-Pérez et al. (1992) also showed an early, rapid loss of RGCs after intraorbital ON cut and intracranial ON interruption by crush. The severity of this early loss was dependent on the location of the injury where lesions close to the eye resulted in more severe RGC loss in the early phase. In the present study, the early phase of cell loss ended at 10 - 14 days after intracranial ON cut.

The observed delay prior to the rapid cell loss suggests that the events initiating this RGC death may require several days. The continued expression of cytoskeletal component mRNA during this delay in cell death (McKerracher et al, 1991) suggests that mechanisms involving gene expression and protein synthesis could be responsible for this delay. Protein and RNA synthesis have been shown to be essential for cell death in many systems: developmental, target-removal induced, and axotomy-induced cell death in chick motoneurons (Oppenheim, 1990), ischemic brain injury (Goto et al., 1990, Shigeno et al., 1990), NGF deprivation in sympathetic neurons (Martin, 1988) and in sensory and parasympathetic neurons (Scott and Davies, 1990). In addition, genetic control of cell death by specific "killer genes" has been reported in invertebrates such as C. elegans (Driscoll and Chalfie, 1991).

Cell death with the morphological features of apoptosis also requires protein synthesis (Walker et al., 1988, Bursch et al., 1990). Although not examined in the present study, several observations by other investigators support the possibility that RGCs die by apoptosis. Barron et al.,(1986) reported chromatin condensation, one of the early events in the process of apoptosis, in RGC nuclei after ON injury. Apoptosis has also been observed during retinal

developmental cell death in rats (Harvey et al., 1990) and chickens (Ilschner and Waring, 1992) and has been associated with cell death after trophic factor deprivation (Martin et al., 1988) or excitotoxicity (Garthwaite and Garthwaite, 1991). Thus the observation that RGC death after ON cut is delayed followed immediately by rapid cell loss suggests that this rapid cell death may occur by delayed processes, possibly requiring RNA and protein synthesis.

In addition to gene expression, the activity of protein kinases has also been associated with cell survival in culture. Rukenstein et al. (1991) reported that PC12 cells, which die rapidly in a serum free medium unless NGF is present, can be rescued from cell death by adding a variety of agents, including forskolin, permeant cAMP analogs, insulin and insulin-like growth factors I and II to the serum free medium. Each of these agents appeared to mediate cell survival by regulating protein kinase activity, suggesting that protein kinases may play an important role in the prevention of cell death. Apoptotic cell death has also been linked to the activity of endonuclease, an enzyme that cuts the nuclear DNA into fragments of DNA, detected by the production of a DNA 'ladder' when run on an agarose gel. Sympathetic neurons can be rescued very late in the process of NGF-deprivation-induced apoptosis by adding NGF, at a time when protein synthesis inhibitors are ineffective in rescuing these cells (Edwards et al., 1991). These cells can also be rescued by the addition of aurintricarboxylic acid, a suppressor of endonuclease activity (Batistatou and Greene, 1991), suggesting that endonuclease activation is a key event during the later phases of cell death by apoptosis. Thus, although mRNA and protein synthesis are involved in many types of cell death, the regulation of enzyme activities may also be an important determinant of cell death (reviewed by Altman, 1992). In addition to molecular events, the retrograde

transport of a signal prior to the initiation of cell death, as mentioned previously, may contribute to the delay before RGC loss.

E. Later and Protracted RGC Loss After ON Cut

The early phase of rapid cell loss after ON cut was followed by a protracted cell loss that continued until the end of the study. This later phase of RGC loss was also reported by Villegas-Pérez et al. (1992). They showed that the rate of this loss was slow after multiple intracranial crush injury. However, the rate of RGC loss in the later phase of ON cut in the present study was three times greater than the rate obtained by Villegas-Pérez et al. (1992), for intracranial repeated crushes. This difference may be related to: 1) the longer duration of the study by Villegas-Pérez et al. (1992), 2) the possible inaccuracy of the 6 month time point in the present study, where only one animal was available, or 3) the type of injury, cut versus multiple crush. This latter explanation is supported by the observation that the single rate of RGC death after intracranial ON crush in the present study is less than half the rate of cell loss after ON cut. The other possible explanations (1 and 2 above) can be addressed by extending the present study and increasing the number of animals at 6 months.

Lunn et al. (1991) suggested that the different morphological changes observed after PNS cut and crush may be related to the integrity of the nerve sheath since cut injury severs the sheath while crush injury does not interrupt the sheath. The prolonged connection of the two nerve stumps after crush may allow trophic factors from the target to reach the axons, or in contrast, may prevent the axons from exposure to harmful factors in the external environment. Serum, for example, has been shown to be required for excitotoxic cell death in rat cerebrocortical cell cultures (Erdo et al., 1990).

The importance of target-derived factors for RGCs in the adult rodent has been investigated. Removal of the visual cortex and the superior colliculus in the adult rat does not result in RGC loss (Perry and Cowey, 1979), suggesting that, unlike neonates (Perry and Cowey, 1979; Carpenter et al., 1986) RGCs in the adult do not rely strictly on target-derived factors. In contrast, in cats the removal of the dorsal lateral geniculate nucleus by kainic acid lesion causes RGC loss for up to 6 months after injury (Pearson and Stoffler, 1992). Target removal may also result in reformation of synaptic contact with other neurons (Zwimpfer, 1989,1990) or alternatively, RGCs may reacquire dependency on trophic support after axotomy. This latter suggestion is supported by observations that the survival of regenerating RGCs is maintained after peripheral nerve grafts are inserted into the superior colliculus (Vidal-Sanz et al., 1991) while grafts left unconnected showed a decline in number (Kierstead et al., 1985). In addition, 1) trophic factors have been found to be expressed in the superior colliculus, the main RGC target (BDNF, Hofer et al., 1990; bFGF, Ernfors et al., 1990b), 2) BDNF (Thanos et al., 1989) and bFGF (Bahr et al., 1989) have been shown to improve adult RGC survival in culture 3) aFGF and bFGF, placed at the injury site can rescue RGCs after axotomy *in vivo* (Sievers et al., 1987), and 4) NGF, placed in the vitreous, can rescue RGCs after axotomy (Carmignoto et al., 1989). Trophic factor support may also be involved in the enhanced survival of RGCs *in vitro* in the presence of Schwann cells (Baehr and Bunge, 1989) or lesioned peripheral nerve (Thanos et al., 1989) and *in vivo* after Schwann cells are placed in the vitreous (Maffei et al., 1990) or embryonic targets are placed on the ON stump (Sievers et al., 1989).

The importance of target-derived trophic support and the possibility that crush injury maintains this trophic supply has not been defined at present. Another possible source of trophic support for neurons is the non-neuronal cells. For

example, Lu et al. (1990) reported an 8-fold increase in NGF mRNA in the distal ON stump after enucleation in the adult rat. The possible role of non-neuronal production of trophic factors will be discussed in Section III.

F. Single Rate of RGC Loss After ON Crush

RGC loss after ON crush, following an initial delay, occurs at a single rate from one week to six months. It is difficult to postulate if this RGC loss corresponds to one of the two phases of cell loss after ON cut. It is not likely to correspond to the early, rapid cell loss after ON cut since this loss is not prolonged and occurs at a rate approximately 250 times faster than the single rate of cell loss after ON crush However, the initial delay after both cut and crush, suggests that the RGC losses after this delay may involve a mechanism requiring a retrogradely transported signal and/or the synthesis of mRNA and protein. The second phase of cell loss after ON cut is more protracted and prolonged, similar to the cell loss after crush. The rate of cell loss after crush is twice as slow as this prolonged loss after cut. This difference suggests that the rate of prolonged cell loss may be dependent on the type of injury, as discussed in section I E.

The very slow nature of the loss of RGCs after ON crush is reminiscent of the loss of RGC's observed after ON cut in the C57BL/Ola mouse. In this mouse, Wallerian and retrograde degeneration in both the PNS and CNS is slow (Lunn et al., 1989; Perry et al., 1990b, 1991). In the ON, 63 days after ON lesion, the cell density in the ganglion cell layer was 6.1 x $10^3/mm^2$ in the C57BL/Ola and 2.9 x $10^3/mm^2$ in the BALB/C control. The C57BL/Ola mouse was reported to have deficient macrophage recruitment, which appeared to be genetically controlled (Perry et al., 1990a). The slow retrograde degeneration and loss of RGCs associated with the lack of invading macrophages in this mouse raises the

possibility that macrophages influence the rate of degeneration after injury. The recruitment of macrophages after cut and crush injury and their possible role in determining the extent of retrograde degeneration and RGC death is discussed in Section II.

II. RETROGRADE AXONAL CHANGES AFTER INTRACRANIAL ON CUT OR CRUSH

Several previous studies examined retrograde changes in the axons of RGCs after ON injury. Richardson et al. (1982) described an area of central necrosis following intracranial ON cut, accompanied by a rapid loss of axons in the ON stump. Kiernan (1984) qualitatively examined retrograde axon loss in the ON after intraorbital crush, observing decreases in axon number from 2 to 34 weeks. Allcutt et al. (1984b) qualitatively described axonal loss in the retina and proximal ON stump after intraorbital ON crush: the majority of axons in the retina and ON had degenerated by 30 days after injury.

The influence of the type of injury on morphological axon changes has only recently been addressed in the PNS. Lunn et al. (1991) described the effect of cut, crush, ligature and freezing on Wallerian degeneration in the rat sciatic nerve. Cutting, freezing or prolonged ligature resulted in more rapid degeneration than crushing or transient ligature. Four days after injury, the axons, as assessed by silver-staining, were largely intact after crush while after cut they had uniformly degenerated. The loss of a compound action potential was also more rapid after cut injury: at two days the mean size of the compound action potential was 39 ± 6.9% and 11.5 ± 7.8% of control for crush and cut respectively. The degenerative

changes in these peripheral axons began at the distal end of the nerve and progressed toward the injury site (Lunn et al., 1990).

The present study documents that the method of axonal interruption, cut or crush, influences the retrograde morphological changes occurring in CNS axons, specifically in the RGCs in the adult rat. Cut injury results in more rapid fiber loss, characterized by a central core of severe axon breakdown and macrophage invasion in the ON while crush injury results in very mild axon loss in the ON and retina. In addition, in the present study a prolonged intracranial ligation produced a core of severe tissue disruption like a cut injury, similar to Lunn et al. (1991) who observed a rapid, cut like degeneration following prolonged ligature.

In this section I discuss the possible cause of the central core of axon degeneration in the ON after cut injury, and how the difference in the rate of retrograde degeneration after ON cut and crush may contribute to the patterns of RGC loss

A. RGC Axons in the ON: More Rapid Retrograde Degeneration After ON Cut than Crush

1 Possible Causes of Rapid Retrograde Degeneration After ON Cut

The rapid degeneration after ON cut is characterized by a central area of severe axon loss that is observed from the injury to within 2-3 mm of the eye. Richardson et al. (1982) also reported this central core of degeneration and described it as a necrotic area Three days after injury, this core area showed axonal breakdown and at one week, contained only a few normal axons and was filled with macrophages, suggesting that this area of severe tissue breakdown develops in the ON early after injury. Similar "necrotic" changes have been observed in the spinal cord after injury (Barnett et al., 1965). This core may be

caused by **ischemia**, resulting from interruption of the blood supply to the ON. Intracranially, the ON blood supply is in the form of small branches from the sheath of the nerve (Kiernan, 1984). This supply would be interrupted following ON cut but would only be transiently perturbed during ON crush. Zochodne and Ho (1990), who used the technique of hydrogen clearance to measure peripheral nerve blood flow, reported that sciatic nerve crush does not result in ischemia. The possibility of the core being caused by ischemia is supported by the preservation of intraorbital axons near the eye that receive their blood supply from a different source, the retinal artery (Kiernan, 1984).

It is possible that the core of altered tissue observed in the cut ON could influence axonal and RGC survival through **exposure to factors in the external milieu** derived from serum and cells such as macrophages. A. Cohen (personal communication) has shown that the addition of serum to cultures of neonatal or adult RGCs causes the breakdown of neurites. It is possible that certain components of the serum are toxic to the neurons or are necessary triggers for cell death. For example, Erdo et al. (1990) has reported that serum is required for excitotoxic cell death in rat cerebrocortical cell cultures. Cut injury, by interrupting the nerve sheath, allows the axons to be exposed to the external environment longer than ON crush, where the sheath remains intact. The role of factors such as serum components and cellular secretory products in determining neuronal damage *in vivo* is presently being investigated. In addition, a more extensive loss of non-neuronal cells in the cut ON could deplete RGCs of an important source of trophic molecules.

2. Events Leading to Greater RGC Loss After ON Cut: Role of Rapid Retrograde Axonal Degeneration

The different patterns of cell death observed after ON cut and crush may be related to the rapid loss of axons in the central area of the ON after ON cut but not crush. In this section, several possible mechanisms of RGC death, related to the rapid loss of axons after ON cut will be discussed.

a. Ischemia-Induced RGC Death

If the core of severe axon degradation after ON cut is caused by ischemia, RGCs may undergo ischemia-induced cell death or death secondary to ischemia. In other cells, ischemia-induced cell death is associated with glutamate neurotoxicity and calcium influx (reviewed by Choi, 1990). The neuronal death after ischemia is delayed, similar to the death of RGCs after ON cut, but usually occurs within 2-4 days of injury (e.g. hippocampal CA1 neurons, Hoffman et al., 1992). Nakano et al (1990) reported that ischemia-induced death can also occur in a slowly progressive manner over weeks or months, as they observed in the dorsolateral striatum and neocortex after transient middle cerebral artery occlusion. These observations suggest that the mechanisms which are responsible for ischemic death might contribute to the loss of RGCs after ON cut. Thus, the presence of ischemia after ON cut, but not crush may induce certain mechanisms of cell death, ultimately resulting in different patterns of cell loss after these two types of injury.

b. Axon Length as a Determinant of RGC Survival

The core of severe axon loss apparent after ON cut could also influence the pattern of RGC death by altering the length of the axon proximal to the lesion site. Villegas-Pérez et al. (1992) documented that axon interruption near the soma

causes more severe loss of axons than distal injury. Thus, the rapid loss of a large proportion of the proximal axons and their associated non-neuronal cells after intracranial cut injury may lead to a more severe loss of neurons than the intracranial crush, where the axons remain intact for months after injury

Although most axons retrogradely degenerate to within 3 mm of the eye after intracranial ON cut, RGC survival is greater than after an intraorbital cut at 3 mm from the eye (Villegas-Pérez et al., 1992). For example, two weeks and three months after intracranial cut, RGC survival was 60.1% and 28.3% of control respectively while after intraorbital cut, cell survival was 38.5% and 11.8% of control at the same times after injury (Villegas-Pérez et al., 1992) This difference in cell survival may be caused by the preservation of some axons in the periphery of the ON stump after intracranial cut or, alternatively, it may indicate that the length of axon is not the only determinant of RGC survival.

Greater neuronal loss after injuries which destroy large portions of the axon may result from: 1) the increased likelihood that events such as calcium influx and depolarization, which extend retrogradely from the proximal tip (Strautmann, 1990) will affect the cell soma; 2) a decrease in trophic support (e.g. BDNF) from the non-neuronal cells of the ON stump; or 3) the loss of surface membrane, and therefore, receptors (e.g. trkB) for trophic molecules such as BDNF. In this way, the destruction of the axons in the central area of the nerve after ON cut could indirectly result in more severe RGC loss by destroying a longer length of axon

c. Role of Macrophages in Neuronal Regeneration and Regrowth

The core of severe axon loss after ON cut is accompanied by the invasion of large numbers of macrophages. In contrast, ON crush does not result in pronounced macrophage invasion and they are only observed at the crush site.

The extent of macrophage invasion could contribute to the different patterns of cell loss after ON cut and crush. The time course of macrophage invasion, occurring between 4 and 7 days, also supports the suggestion that they are responsible for the early, rapid phase of RGC death after ON cut. Macrophages and microglia are complex cells, having many functions and capable of secreting over 100 different substances (Nathan, 1987; Perry and Gordon, 1988; Streit et al., 1988; Papadimitriou and Ashman, 1989; Thomas, 1992).

In the PNS, macrophages play an important role in both the degeneration and regeneration of neurons after injury. After sciatic nerve injury, Schwann cells upregulate their expression of NGF. This increase occurred in two peaks and the second peak, between the second and seventh day after transection, was induced by IL-1 secretion by macrophages (Heumann et al., 1987b; Lindholm et al., 1987; Heumann et al., 1990). The IL-1 also induces the expression of the low affinity NGF receptor (Heumann et al., 1987b). These increases in NGF and its receptor support the regeneration of the axons. In addition, C57BL/Ola mice, which have reduced macrophage recruitment, do not show increased NGF mRNA after the saphenous nerve is cut. The sensory axons, which require NGF, show impaired regeneration while motor axons are unaffected. The degeneration of the sensory fibers is also slow in this mouse, suggesting a relation between the macrophages and the extent of axon degradation. Thus, in the PNS, macrophages are a critical component in the events that follow injury.

In the CNS, the role of macrophages after injury is not as clearly defined. There is some evidence to support a positive effect via the clearing away of myelin debris that is inhibitory to axonal growth (reviewed by Perry et al., 1987; Perry and Gordon, 1988). In addition, David et al. (1990) suggested that macrophages can cause the ON to become permissive to neurite growth after transection.

In contrast, observations in the C57BL/Ola mouse suggest that macrophages may be involved in the events that lead to neuronal death after CNS injury. These mice, which have impaired macrophage recruitment, also have improved RGC survival after ON injury (Perry et al., 1991). Some of the molecules that macrophages are known to secrete are toxic to cells (e.g. tumor necrosis factor, Beutler and Cerami, 1989). However, many of the factors macrophages secrete have multiple functions (Nathan, 1987). For example, nitric oxide is known to be involved in tumor and bacterial killing and neurotoxic death (Dawson et al., 1991) but has also recently been found to act as a neuronal messenger, similar to a neurotransmitter (Bredt and Snyder, 1992). Thus, the role of macrophages in the CNS is not clearly defined. Macrophages are capable, nonetheless, of mediating cell death and they are a possible candidate for triggering the more severe loss of RGCs after ON cut.

The greater loss of axons in the ON stump after ON cut compared to ON crush may be caused by ischemia or by the exposure of the cut axons to harmful factors in the external milieu. The resulting core of severe axon loss could, in turn, result in a greater loss of RGCs via ischemia-induced cell death, the loss of large portions of the axon or the invasion of macrophages.

B. Intraretinal Axons: More Severe Loss of Axons After ON Cut Than Crush

The time course of intraretinal axon loss correlates with the loss of axons in the ON. After cut injury, axon loss in the retina is qualitatively observable two weeks after injury when axon loss in the ON near the globe is also observable. After crush injury, axon loss in both the retina and the ON is very slow and is not qualitatively detectable until four weeks after injury. The correlation between

axonal loss in the retina and ON suggests that the RGC axons are progressively dying back toward the soma or that the loss of axons at both levels is secondary to the death of the neuron

However, no correlation is observed between the loss of axons after cut injury and the early, rapid loss of RGCs. This rapid RGC death that occurred between 1 and 2 weeks after injury was not accompanied by a period of rapid retrograde degeneration in the axons within the retina and in the ON near the globe. In contrast, a progressive loss of axons was observed. This suggests that the early phase of rapid RGC loss does not result from neuronal die-back, but is more likely caused by an internal mechanism triggered by the injury that directly affects the perikaryon.

The later protracted loss of RGCs after ON cut and the slow rate of RGC death after ON crush may be caused by the retrograde death of the neuron, progressing from the injury site to the soma. The rate of the protracted phase of cell death after ON cut was approximately twice the rate of cell loss after ON crush. The loss of axons in the ON and retina after ON cut was also faster than the loss of axons after ON crush. It is, therefore, possible that the protracted loss of RGCs after ON injury is caused by neuronal die-back

In addition to a decrease in the number of intraretinal axons after ON cut, the axons were observed to deviate from their straight retinal course. These deviations in axonal direction were previously noted by Vidal-Sanz (Ph. D Thesis, 1990) Additional experimentation is required to determine the cause of these irregular axonal paths however, they may result from axonal sprouting or alterations in axon-axon adhesion following cut injury.

In summary, the morphological axonal changes after ON injury are also dependent on the type of injury; these retrograde changes occurring in RGC axons after ON cut and crush may contribute to the difference in cell survival.

III BDNF EXPRESSION IN THE ON

The survival of many neurons during development and after injury is dependent on trophic support. Several aspects of the patterns of RGC loss after ON cut and crush may be related to the supply of trophic factors received by the RGCs: 1) the maintenance of nerve sheath continuity after ON crush may allow trophic support from the target to reach the injured axon, 2) the preservation of axons for months after ON crush, in contrast to ON cut, may result in increased cell survival by allowing these axons to receive trophic support from non-neuronal cells in the ON, and 3) the transient production of growth factors in the ON after injury may mediate the observed delay in RGC death after ON cut and crush

The present study suggests that BDNF is expressed in the normal ON, although as others have reported (Herzog and Barde, 1992), the mRNA is difficult to demonstrate by Northern blot analysis. Since there are no neuronal cell bodies within the ON, this expression appears to be located in the non neuronal cells of the ON. Several earlier reports support the suggestion that BDNF is the neurotrophic factor for RGCs In culture, BDNF supports the survival of E17 RGCs (Johnson et al., 1986) and adult RGCs (Thanos et al , 1989) In addition, the main RGC target, the superior colliculus, expresses BDNF mRNA (Hofer et al., 1990). Finally, *in vivo*, multiple intraocular injections of BDNF enhance RGC survival by 20% 2 weeks after intraorbital transection compared to control injections of saline or PBS/ BSA and by 40% in comparison to the RGC

populations in rats with cut ON that were not injected with BDNF, BSA or PBS (S. Mansour-Robaey, personal communication). Thus, the present observation that BDNF is normally expressed in the ON endorses the possibility that BDNF is an RGC trophic factor. My attempts to determine the level of BDNF in the ON after injury were hindered by the low levels of RNA present in the ON and the accompanying difficulty of measuring the amounts of RNA accurately to create a Northern blot with equal RNA in each lane. However, since I left the laboratory, others have found evidence for increased BDNF mRNA expression after ON injury (A. Jelsma, personal communicat. n). Lu et al. (1991) reported an 8 fold increase in NGF mRNA in the ON after enucleation. NGF injected into the vitreous has been shown to rescue RGCs and their axons after intracranial axotomy. These observations raise the possibility that a sub population of RGCs are responsive to NGF after injury or that NGF is able to mediate RGC survival via the BDNF receptor Presently, the majority of the available data on RGC trophic support suggests that BDNF is a likely candidate for supporting RGCs. Thus, the expression of BDNF in the normal ON endorses this idea and raises the possibility that BDNF may be responsible for the greater neuronal survival after ON crush compared to cut, or may mediate the delay in neuronal death after ON injury.

SUMMARY OF FINDINGS

The experiments in this thesis document the survival of RGCs after two methods of ON interruption, examine the morphological axonal changes to determine if they may contribute to the patterns of RGC death and examine further

the possibility that BDNF is a neurotrophic factor for RGCs by determining if it is expressed in the normal ON. The results support the following conclusions:

1) Although both intracranial cut and crush lesions completely interrupted all RGCs at the same distance from their targets, the pattern of RGC loss varied with the type of lesion and time after injury.

2) The loss of RGCs is delayed for approximately one week after both methods of axon interruption

3) The interruption of the ON by intracranial cut resulted in an early, rapid loss and a later, more protracted loss of RGCs.

4) The interruption of the ON by intracranial crush resulted in a single, slow rate of RGC loss.

5) The morphological changes occurring in the RGC axons within the ON and retina vary with the type of lesion. These different morphological changes may contribute to the different patterns of RGC loss the retrograde degeneration of axons and the loss of RGCs occurs more rapidly after ON cut than crush

6) Non-neuronal cells of the normal ON appear to express BDNF mRNA. This neurotrophic factor may be involved in delaying the loss of RGCs after injury or, alternatively, greater quantities of BDNF may be available to RGC axons after ON crush than cut, resulting in greater cell survival.

CONCLUDING REMARKS

The experiments described in this thesis were aimed at investigating neuronal survival after central nervous system (CNS) injury in adult mammals. The survival of retinal ganglion cells (RGCs) and the accompanying axonal changes were documented after two methods of axon interruption: optic nerve (ON) cut or crush While the severing of axons by cut or crush both interrupt neuronal-target interactions, thus removing nerve cells from their main source of trophic support, my investigations suggest that additional events in the ON play a key role in the timing and severity of the neuronal loss that follows axotomy.

After cutting RGC axons in the ON approximately 9 mm from the retina, I observed an initial abrupt loss of neurons which was followed by a more protracted and gradual reduction in the RGC population, but crushing the ON intracranially gave rise to a slow rate of cell loss. My observations also indicate that the death of approximately 40% of the RGCs in the initial stages following ON cut occurred abruptly between 7 and 10 days after injury and that the loss of these neurons is not preceded by a progressive retrograde degeneration of axons from the optic nerve into the retina. This previously unrecognized delay of approximately one week prior to the onset of RGC loss may result from the induction of a "suicide program" involving gene expression Approximately 6 mm of the ON stump proximal to the site of cut showed a severe tissue disruption characterized by a marked central loss of glial cells and axons and a proliferation of macrophages, such alterations were minimal after crush. Additional observations triggered by these experiments suggest that components of the disrupted tissue, namely serum and macrophages, may be a source of molecules that negatively affect neuronal survival.

In parallel experiments I presented evidence for the expression of two brain derived neurotrophic factor (BDNF) mRNA transcripts (1.5 and 4.2 kilobases) by non-neuronal cells of the uninjured optic nerve. BDNF is a neurotrophin known to support the survival of RGCs. It is possible that the more severe loss of RGC's observed after ON cut results from a difference in the expression of this neurotrophin and/or its receptor following cut and crush injury.

These observations encourage the continued investigation of the involvement of "suicide" gene expression, macrophages, serum components and glial-derived trophic support in the determination of cell survival after neuronal injury in the adult mammalian CNS.

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