PATTERNS OF RETINAL GANGLION CELL LOSS AFTER INTERRUPTION OF THE ADULT RAT OPTIC NERVE AT DIFFERENT DISTANCES FROM THE EYE

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To those who supported my research

ABSTRACT

Using the fluorescent marker dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) as a retrogradely transported persistent label for retinal ganglion cells (RGCs), I have studied RGC survival between 15 days and 12 or 20 months after interruption of the optic nerve (ON) at each of four different distances between the eye and the optic chiasm in adult rats. The patterns of decreases in RGC densities varied with the different levels of axotomy; there was an early rapid loss of cells whose severity increased the closer the lesion was to the eye, and a later more protracted loss of cells that occurred at a similar rate for all distances of ON interruption and continued for the period of the study. The severity of the early rapid loss of cells may have been influenced by factors related to the proximity of the injury to the RGC bodies and/or the length of the ON stump. The later more protracted cell loss may represent a general mechanism by which neurons die after disconnection from their targets.

RESUME

En utilisant le dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) comme marqueur fluorescent persistent transporté de façon rétrograde dans les cellules ganglionnaires de la rétine (GCR) de rats adultes, j'ai étudié la survie des CGR de 15 jours à 20 mois après l'interruption du nerf optique à quatre niveaux différents entre l'oeil et le chiasma optique. Le profil de la diminution de la densité des CGR a varié en fonction du niveau auquel l'axotomie a été efectuée. On observe d'abord une diminution rapide et précoce des densités des CGR dont le degré de sévérité est d'autant plus important que la lésion est proche de l'oeil. On observe ensuite une diminution plus lente des densités des CGR qui est indépendante du niveau auquel on effectue la lésion et se poursuit pour toute la durée de la période étudiée. Le degré de sévérité de la diminution précoce et rapide des densités des CGR semble être sous contrôle de facteurs influencés par la proximité entre le site de la lésion et le corps cellulaire des CGR et/ou la longueur du tronçon proximal du nerf optique. La diminution plus lente et prolonguée des densités des CGR pourrait représenter un mécanisme général de la mort neuronale après l'interruption de la communication nerveuse avec les tissus cibles.

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LIST OF ABREVIATIONS

aFGF	acidic FGF
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA	Analysis of Variance
BDNF	Brain-derived Neurotrophic Factor
bFGF	basic FGF
CGRP	Calcitonin Gene-related Peptide
CNS	Central Nervous System
CNTF	Ciliary Neurotrophic Factor
dil	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
dLGN	dorsolateral geniculate nucleus
DRG	Dorsal Root Ganglia
FGFs	Fibroblast Growth Factors
g	gram
HRP	Horseradish peroxidase
IC	Group of experimental retinas with ON lesion 8 mm from eye
IO	Group of experimental retinas with ON section 3 mm from eye
µm	micrometre
μm^2	Square micrometer
mg	milligram
mm	millimeter
mm^2	Square millimeter
mRNA	messenger ribonucleic acid
NaCl	Sodium Chloride
NGF	Nerve Growth Factor
NMDA	N-methyl-D-aspartate
NT-3	Neurotrophin III, hippocampus-derived trophic factor or NGF-2
NT-4	Neurotrophin IV
OC	Group of experimental retinas with ON lesion 10 mm from eye
OD	Group of experimental retinas with ON section 0.5 mm from eye
ON	Optic nerve
p	probability
PNS	Peripheral nervous system
r	correlation coefficient
RGC	Retinal ganglion cell
RNA	Ribonucleic acid
SC	Superior colliculus
SEM	Standard error of the mean

CLAIMS FOR ORIGINALITY

In Sprague-Dawley rats with four different levels of ON interruption, I have documented that:

1. The pattern of RGC loss differs with distance of ON interruption: There is an early rapid loss of cells whose severity decreases with increasing distances of axotomy from the eye, and a later, protracted loss that proceeds at similar rates for all levels of ON interruption.

2. A small proportion (3 to 4%) of the total RGC population persists in the retina without any appreciable loss from 6 to 20 months after ON section close to the eye.

3. DiI persists within the cytoplasm of axotomized RGCs for at least 12 months after ON interruption; the decreases of dil-labelled cells represent cell death and not leakage of the dye.

In addition, my results suggest that:

1. The severity of the early rapid loss of cells is related to the level of ON interruption.

2. The one-half survival time of RGCs during the later phase of cell death is approximately 6 to 7.5 months.

3. The later phase of RGC loss may represent a common mechanism of neuronal degeneration when the neurons lose contact with their targets.

In normal Sprague-Dawley rats, I have observed that:

1. DiI applied to the main RGC targets -the SC and dLGN-, is retrogradely transported and persists within the cytoplasm of RGCs for periods of up to 21 months.

2. In the conditions reported in this thesis, RGC densities do not decrease in the aged rat.

3. The area of the rat retina increased during the period of study, more noticeably during the first three months (when the rats are between 3 and 6 months of age).

JUSTIFICATION OF APPENDICES

The following paragraph has been reproduced from "Guidelines concerning thesis preparation", McGill University (revised on June, 1988). "Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported".

For this thesis, I have included two of my earlier publications into two appendices (I and II respectively), because I consider them to be of importance for the evaluation of this thesis.

PUBLICATIONS

The work done during the time I have spent in this laboratory eventuated in the following publications. The work presented in this thesis is also presented in publications marked with an asterisk.

ARTICLES

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CHAPTER 1. INTRODUCTION

The experiments to be presented in this thesis analyze quantitatively RGC loss after interruption of their axons at one of four different distances between the eye and the optic chiasm. This introductory chapter presents some aspects related to axotomy and neuronal death. First, I review some of the variables that may affect neuronal loss after axotomy. Secondly, I consider possible mechanisms of neuronal death. Finally, I review the possible role of trophic factors on neuronal survival.

1.1. EFFECTS OF AXOTOMY

The changes that follow the interruption of axons can be classified as orthograde or retrograde. Orthograde effects of axotomy, distal to the injury, consist of the disintegration of axons and their myelin sheaths with subsequent phagocytosis by macrophages (Beuche and Friede, 1984; reviewed by Fawcet and Keynes, 1990). The speed of this process has been reported to be greater after nerve section than after nerve crush in the PNS (Lunn et al., 1990), but is typically slower in the central nervous system (CNS) than in the peripheral nervous system (PNS; Perry et al., 1987; Stoll et al., 1989). Retrograde changes after axotomy involve the proximal axons and parent neurons (see following sections). Finally, there may also be orthograde or retrograde transneuronal changes in neurons proximal or distal to the lesioned neurons (reviewed by: Cowan, 1970).

This thesis analyzes quantitatively RGC loss after ON section at different distances from the eye. Thus, the retrograde changes produced by axotomy and the

factors that influence neuronal death after axotomy are considered in the following sections.

1.1.1. PERIKARYAL RESPONSES TO AXOTOMY

Axonal injury can lead to diverse retrograde changes in the cell bodies of lesioned neurons: i) Morphological changes such as chromatolysis and alterations in neuronal and dendritic shape and size. The reaction known as "chromatolysis" was first described in rabbit motor neurons by Nissl, who observed that after axonal lesions, these neurons showed disintegration of the Nissl bodies, migration of the nucleus to the periphery of the cell, and swelling of the nucleus and cytoplasm (reviewed by: James, 1933; Leinfelder, 1938; Lieberman, 1971, 1974; Torvik, 1976; Barron, 1983a,b, 1989). These changes observed in the perikarya of axotomized neurons presumably reflect molecular changes in neuronal metabolism after the injury; ii) molecular changes such as variations in the production of nucleic acids and proteins and alterations of axonal transport (reviewed by: Lieberman, 1971; Purves, 1976; Barron, 1983a,b, 1989; Grafstein, 1983, 1986); iii) electrophysiologic changes including deafferentation, variations in neuronal membrane potential and neuronal synaptic potentials (reviewed by: Mendell, 1984, Purves, 1976; Titmus and Farber, 1990); and iv) neuron death (reviewed by: Lieberman, 1971, 1974; Barron, 1983a,b, 1989; see below), which is the topic of this thesis.

1.1.2. INFLUENCES ON NEURONAL LOSS AFTER AXOTOMY

The severity of the neuronal cell loss after axotomy is influenced by animal species and age, neuronal group, distance from the lesion to the neuronal cell body, type of lesion and existence of axonal collaterals (for a review see: Lieberman, 1971, 1974; Torvik, 1976; Barron, 1983a,b, 1989).

In relation to the animal species, it is known that axonal lesions (e.g. ON section) cause the death of some parent neurons in mammals (RGCs; Grafstein and Ingoglia, 1982; Allcutt et al., 1984; Misantone et al., 1984; Villegas-Pérez et al., 1988), but that cell death is negligible when the same procedure is carried out in the goldfish (reviewed by: Grafstein, 1986). Concerning age, neuronal death after axotomy in mammals is more extensive during the neonatal period, the "Gudden effect", named for the author, who was one of the first to observe this phenomenon (reviewed by: Lieberman, 1974; Barron, 1983a; for an analysis of this subject in mouse RGCs: Allcutt et al., 1984). However, a few exceptions to this rule have been found. For example, the ventrobasal thalamic neurons have been documented to survive in greater numbers in the cat when hemispherectomy is carried out in neonatal animals (Villablanca et al., 1986; reviewed by: Lieberman, 1974; Barron, 1989).

The amount of nerve cell death after axotomy also varies with the neuronal group studied. For example, in a comparative study in which similar paradigms were used, neurons in the dorsal motor vagal nucleus were more prone to cell death after axotomy than the neurons in the hypoglossal nucleus (Aldskogius et al., 1980).

The type of lesion has also been reported to influence the degree of neuronal loss after axotomy. Crush lesions interrupt the axons in a nerve but continuity of the

nerve sheaths can be preserved; cut lesions interrupt axons and nerve sheaths. After lesions in the PNS, the amount of cell death is less after crush than after cut; transection causes fewer nerve cells to die than a nerve section with capping or ablation of the distal nerve stump (Rich et al., 1989; Murphy et al., 1990; for a review see Lieberman, 1971, 1974; Barron et al., 1983a,b; Aldskogius et al., 1985). Although Himes and Tessler (1989) did not find a decrease in dorsal root ganglion cell numbers 3 months after resection-ligation of the sciatic nerve, most evidence suggests that the long-term survival of peripheral neurons after different types of lesion depends on the regrowth of their axons in the distal peripheral nerve segment (Purves, 1975, 1976; Rich et al., 1989; Murphy et al., 1990; reviewed by: Lieberman, 1974; Barron, 1983a; see below). The demonstration that peripheral nerve lesions are followed by increased expression of Nerve Growth factor (NGF) and Nerve Growth Factor receptor in the peripheral stump (Heumann et al., 1987a,b; see below, section 1.3.) indicates a possible mechanism for this effect, at least for NGF-responsive neurons (see below, section 1.3.).

Other than in the ON, the effects of cut and crush lesions are difficult to assess in the CNS. Mantz and Klein (1951) reported that adult rat RGCs are more sensitive to ON section than to ON ligature. In such responses, mammalian RGCs may differ from lower vertebrates, such as Rana pipiens, in which similar proportions of cell death have been found after crushing or after cutting-resecting the ON (Humphrey, 1987). Ligature may also have additional effects such as the prevention of axonal regeneration; in the frog Hyla moorei, prevention of regeneration in the ON by ligation

or capping delayed the onset of RGC death, but produced greater RGC death over the long-term (Humphrey et al., 1989).

As will be documented further in this thesis, the distance from the site of axotomy to the neuronal somata also influences the amount of cell death. There seems to be agreement among authors that lesions closer to the neuronal cell body cause greater amounts of cell death that more distal lesions, a fact that has been demonstrated in many neuronal populations of the central and peripheral nervous system (Mantz and Klein, 1951; Liu, 1955; Glover, 1967; Egan et al., 1977a,b; Loewy and Schader, 1977; Sofroniew and Isacson, 1988; Ygge, 1989; Murphy et al., 1990). However, few studies have tested the influence of the proximity of the lesion to the cell body on the temporal course of neuronal death. The protective effect of non-lesioned axonal collaterals was suggested by Fry and Cowan (1972) to explain their findings after axonal injury in the lateral mammillary nucleus of the cat, where lesions of the axonal trunk produced a much greater amount of cell death than the sum of cell loss produced by lesions of each axonal collateral. This finding was interpreted by some authors (reviewed by: Lieberman, 1974) as an indication that neuronal death is the consequence of the neuronal disconnection from trophic influences in their targets (see section 1.3.).

1.1.3. DEATH OF RETINAL GANGLION CELLS AFTER AXOTOMY

In 1900, Birch-Hirschfeld studied the morphologic changes that occur in RGCs after ON lesions and showed that the morphological changes in the perikarya described by Nissl for other types of axotomized neurons were also present in these

neurons (reviewed by: James, 1933). The retrograde loss of proximal axons after ON section in the rabbit was studied by Tello (1907) and Ramón y Cajal (1907). Later work in the visual system assessed whether or not neuronal cell death occurred after ON section in mammals (James, 1933; Leinfelder, 1938; Mantz and Klein, 1951; Eayrs, 1952; Polyak, 1958; Quigley et al., 1977). These authors demonstrated retrograde loss of proximal axons and RGCs after lesions of the ON (although some surviving neurons remained in the retina over long periods of time) and that, at least qualitatively, RGC death was more severe when the axotomy was near the eye. However, Radius and Anderson (1978) examined RGC densities in different retinal areas after retinal photocoagulation in monkeys and could not demonstrate a relation between the distance of the lesion to the RGC bodies and the proportions of cell death.

Quantitative studies of the mammalian RGC response to injury were undertaken by Grafstein and Ingoglia (1982), Misantone et al. (1984), Allcutt et al. (1984), and Barron et al. (1985, 1986). Grafstein and Ingoglia studied the number and size of adult mouse RGCs after transecting the ON intracranially and found that RGC densities were decreased to 80% of normal in the first 3 days and to 50% by 65 days after the lesion; average RGC size diminished by 25% of normal in the first 3 days after ON section, but recovered to normal by 90 days after the lesion. Allcutt et al. (1984), who studied RGC response to axotomy after intraorbital ON crush in neonatal and adult mice, found that both RGC atrophy and death were more severe in the neonate, and that in the adult, RGC densities were reduced to 50-80% by 30 days after the lesion, and remained constant thereafter. Misantone et al. (1984) studied

adult rat RGC survival after intracranial ON crush and found that RGC densities were normal during the first three months after axotomy, while RGC size diminished to 50% of control in the first month after the lesion. Barron et al. also studied RGC degeneration in the adult rat after intracranial (Barron et al., 1985) or intraorbital (Barron et al., 1986) ON crush. In the latter study, Barron et al. (1986) report a decrease in RGC size for the period from 28 to 90 days after axotomy, and a decrease in RGC density that started 7 days after ON lesion, when RGC densities were decreased to 65%, and continued throughout the period of the study so that by 180 days after ON crush, RGC densities were reduced to 32% (Barron et al., 1986).

Considered together, these studies suggest that RGC atrophy and death are more severe with ON lesions closer to the eye. However, it is difficult to compare the results more precisely because of the different methods used to produce the injuries and to count RGCs. Moreover, these studies lacked a definitive means of distinguishing RGCs, which project their axons in the ON, from the many non-projecting ("displaced") amacrine cells that coexist with RGCs in the RGC layer of the rat retina (Cowey and Perry, 1979; Perry, 1981). Although morphologic criteria, including size, were used to distinguish these two types of neurons in the RGC layer (Allcutt et al., 1984; Misantone et al., 1984; Barron et al., 1985, 1986), such approaches can be confounded by the atrophy of axotomized RGCs.

The retrograde degeneration of axons in the adult rat ON was investigated by Richardson et al. (1982) who found that after intracranial ON section, at approximately 8 mm from the eye, 90% of the axons had dissappeared from the segment of the nerve 1 mm from the optic disc by 1 month and that 99% of these axons were lost by

8 months. Thus, in adult rats, RGC axons undergo rapid retrograde degeneration after axotomy. Richardson et al. (1982) also reported that the loss of axons in the ON was not affected by grafting a peripheral nerve segment at the intracranial site of axotomy, and that the RGC axons did not grow into peripheral nerve grafts at this location.

Recent investigations indicate that RGC survival after axotomy is influenced by gene expression in neuronal and non-neuronal cells. For instance, it has been reported that RGC survival after intracranial ON section is greater in a strain of mice, the C57BL/Ola, than in two other mouse strains, the C57BL/6J/Ola and the BALB/c/Ola (Perry et al., 1991). The C57BL/Ola strain has a very slow rate of wallerian degeneration in the PNS that is controlled by a single autosomal dominant gene (Perry et al., 1990) and is associated with retarded down-regulation of the Po gene after peripheral nerve section (Thomson et al., 1991). Wallerian degeneration in these animals is characterized by lower macrophage recruitment (Lunn et al., 1989) and, as possible consequence, lower levels of NGF mRNA (Brown et al., 1991). Whether the morphological and molecular characteristics of the anterograde and retrograde changes caused by ON section in this strain of mice differ also from other strains of mice have not been studied.

1.2.- MECHANISMS OF NEURONAL DEATH

In the previous section, I reviewed the retrograde effects of axotomy on neurons, focusing on cell death. In this section, I present a review of the mechanisms that have been shown to play a role in neuronal death during development and after

injury. Although neuronal death is also observed during aging and in neurodegenerative diseases, the mechanisms by which neurons die in these situations are generally not well understood. Finally, I mention a proposed classification of cell death. It is anticipated that a better understanding of the molecular mechanisms involved in determining cell survival and death will shed light on the determinants of neuronal responses to axotomy.

1.2.1. NEURONAL DEATH DURING DEVELOPMENT

A great deal of the current understanding of mechanisms of neuronal death has resulted from developmental studies. During the development of the nervous system, neuronal death has been documented in many neuronal groups. This naturally ocurring neuronal death may depend on factors present in the neuronal targets, afferent inputs, hormones and neuronal interaction with non-neuronal cells and molecules of the extracellular matrix (reviewed by: Cowan et al., 1984; Clarke, 1985; Oppenheim, 1991).

In invertebrates, developmental neuronal death has been shown to be the result of genetically-programmed mechanisms. For example, in the nematode Caenorhabditis elegans, cell lineages are rigidly established, and cell death during development follows a fixed program in which specific cells are always eliminated (Horvitz et al., 1982; Ellis and Horvitz, 1986). Mutations of genes that cause abnormalities in the pattern of cell death (Sternberg and Horvitz, 1984; Ellis and Horvitz, 1986) and even mutations of genes that cause neuronal degeneration (Driscoll and Chalfie, 1991) have been described in these nematodes. However, it is

unlikely that naturally occurring neuronal death in vertebrates is so rigidly programmed because, as Oppenheim (1991) indicates, the proportions of dying neurons can be altered significantly by experimental manipulations that affect cell to cell interactions. For example, the application of curare to chick embryos during the period of naturally occurring neuronal death inhibits the loss of spinal motoneurons (reviewed by Oppenheim, 1991).

In contrast to C. elegans and other invertebrates, developmental neuronal death in vertebrates appears to be more dependent on extrinsic conditions. Such developmental neuronal death usually coincides with the arrival of the axons at their projection territories. Furthermore, experimental increases in the size of the target substantially reduce the amount of neuronal death of certain neuronal populations (reviewed by Oppenheim, 1991). These observations have been interpreted as evidence that naturally occurring neuronal death is the result of neuronal competition for a factor or factors (e.g. trophic factors, see section 1.3.) present in limited quantities in the neuronal targets (reviewed by: Cowan et al., 1984; Barde, 1989; Oppenheim, 1991). However, other variables have been shown to influence the extent of neuronal death during development (reviewed by: Oppenheim, 1991): experimental modifications of afferent input to neuronal groups during the period of naturally occurring cell death, and mainly the reduction of inputs influences the incidence of neuronal death (reviewed by: Oppenheim, 1991). Finally, hormones also regulate neuronal survival during development (e.g. thyroxine influences granule cell survival in the rat cerebellum, Lewis et al., 1976, and possibly the sex-related

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related differences in neuronal numbers in certain dimorphic groups of neurons (reviewed by Oppenheim, 1991).

To understand the molecular mechanisms that govern neuronal death during development, some authors have studied whether neuronal death during development is dependent on RNA and protein synthesis. Oppenheim et al. (1990) have documented that *in ovo* treatment of chick embryos during the period of naturally occurring motoneuron death with inhibitors of RNA and protein synthesis significantly reduces the amount of death of these neurons. These drugs also reduce the amount of neuronal death in chick embryos in two other experimental conditions: i) early in development after experimental ablation of their targets, and ii) later in development (at the end of the period of neuronal death) after axotomy (Oppenheim et al., 1990). Thus, Oppenheim et al. (1990) suggest that these three situations of motoneuron death during development: naturally occurring, after removal of the target, and after axotomy may share a common mechanism that requires RNA and protein synthesis.

Another approach to the study of neuronal death during development was used by Martin et al (1988) and Scott and Davies (1990). These authors cultured either embryonic sympathetic (Martin et al., 1988), or trigeminal mesencephalic, trigeminal ganglion or ciliary ganglion neurons (Scott and Davies, 1990), neurons that require the presence in the culture medium of specific trophic factors for their survival, and tested whether neuronal death after trophic factor deprivation was dependent on RNA and protein synthesis. The addition to the culture of drugs that blocked either RNA and protein synthesis reduced the amount of neuronal death after trophic factor deprivation, suggesting that this type of cell death is the result of an active cellular

program (Martin et al., 1988; Scott and Davies, 1990). However, other studies in cultured PC12 cells have documented that the blockade of RNA and protein synthesis does not affect the survival effects that various trophic factors exert on these cells (Ruckenstein et al., 1991; see below, section 1.3.).

Finally, it has been shown that during developmental cell death, certain cell populations express or synthesize different molecules. Two examples will be cited. The fibers of the intersegmental muscles (ISMs) of the moth Manduca sexta atrophy and die during development because of the decline in the production of a steroid hormone (Schwartz and Truman, 1983). This type of cell death has been documented to depend on gene expression (Schwartz et al., 1990a), and one of the genes that is selectively expressed during this type of cell death is that of polyubiquitin (Schwartz et al., 1990b). In the cat, the neurons of the subplate that die during development are selectively labelled by an antibody that recognizes a polypeptide whose identity is yet unknown (Naegele et al., 1991).

1.2.2. NEURONAL DEATH AFTER INJURY

Although the precise mechanisms by which neurons die after lesions, including those that may be situated far away from their cell body, are not known, recent studies have shown that excitatory amino acids and calcium are involved in this type of neuronal death.

After hypoxia or ischemia, neuronal damage occurs in two phases. Immediately after the injury, there is neuronal swelling that has been shown to be dependent on extracellular sodium and potassium (reviewed by: Choi, 1988a; Choi and Rothman,

1990). After a delay of 48-72 hours the cells start to disintegrate, a process that requires protein synthesis (Goto et al., 1990; Shigeno et al., 1990), and depends on the presence of extracellular calcium (Choi, 1988a). This ion, as well as excitotoxic aminoacids, have been documented to play a role in the death of adult brain neurons after certain injuries such as hypoxia, hypoglycemia and mechanical trauma (reviewed by: Choi, 1988a,b, 1990; Siesjö et al., 1989; Pauwels et al., 1991). Elevated intracellular calcium levels are believed to be responsible, at least in part, for the neuronal degenerative events that occur after the injury (reviewed by: Choi, 1990; Pauwels et al., 1991); the noxious effects of excitotoxins appear to be mediated by calcium entry through NMDA-receptors (MacDermott et al., 1986; Siesjö et al., 1989; Tecoma et al., 1989; Choi, 1990; Michaels and Rothman, 1990; Pauwels et al., 1991). The presence of calcium-binding proteins, such as calbindin and parvalbumin, have been reported to attenuate the toxicity of excitatory aminoacids (Weiss et al., 1990; Mattson et al., 1991). In addition, it has been shown that in hippocampal cultures, calbindin-containing neurons can lower intracellular concentrations of calcium more effectively than other types of neurons (Mattson et al., 1991). However, a recent study has documented that in cultured hippocampal neurons, large elevations of cytoplasmic calcium produced by agents other than excitotoxic aminoacids (e.g. sodium cyanide) do not always determine cell death (Dubinsky and Rothman, 1991). Thus, elevated concentrations of free intracellular calcium may not be the only factor determining cell death after excitotoxic injury to neurons.

1.2.3. MORPHOLOGICAL CORRELATES OF CELL DEATH

Although cell death occurs in diverse circumstances, the ultimate molecular mechanism causing cells to die might not be as diverse. A classification of cell death suggests that there are essentially two morphological types of cell death that may reflect two basic mechanisms by which cells die. These two types of cell death have been called necrosis and apoptosis (reviewed by: Kerr et al., 1972; Wyllie et al., 1980; Searle et al., 1982; Walker et al., 1988). Necrosis is caused by injuries to the cell membrane or metabolism while apoptosis is a self-destructive event that involves gene expression (reviewed by Kerr et al., 1987). Many exceptions to this classification have also been described (see below).

Apoptosis is characterized by the contraction of the cellular volume, condensation of the nuclear chromatin with preservation of the cellular organelles and the plasma membrane (reviewed by: Wyllie, 1981; Kerr et al., 1987; Bursch et al., 1990), and has been considered to be the main type of cell death observed during development (reviewed by Kerr et al., 1987), and in proliferating cells during adulthood (reviewed by Kerr et al., 1987). In adult animals, cell death caused by hormonal or growth factor withdrawal resembles apoptosis (e.g. T-lymphocyte cell death in culture after removal of interleukin-2, reviewed by Kerr et al., 1987).

In contrast to apoptosis, the cellular events that characterize necrosis are cellular edema, mitochondrial swelling, rupture of plasma and nuclear membranes and leakage of the cell contents to the extracellular space (reviewed by: Trump et al., 1981; Wyllie, 1981). Experimental studies have documented that the agents that cause this type of cell death interfere with the mitochondrial oxidative phosphorylation

or damage the cell plasma membrane and the transmembrane transport system (reviewed by: Trump et al., 1981; Dean, 1987). Elevated concentrations of intracellular calcium are believed to play a pivotal role in the pathogenesis of this type of cell death (Kirillina et al., 1979; Snowdone et al., 1985; reviewed by: Trump et al., 1981; Walker et al., 1988).

Finally, it has been reported that the same causative agent can cause different morphological types of cell death. While mild ischaemia and hyperthermia or low doses of radiation may produce apoptosis, more severe applications of the same agents have been reported to produce necrosis in the same tissues (reviewed by: Kerr, 1971; Kerr et al., 1987). Furthermore, "killer" lymphocytes and tumor necrosis factor may cause cell death by both necrosis and apoptosis (Laster et al., 1988; Zychlinsky et al., 1991).

Although the above classification of cell death defined only two types of cell death, Clarke (1990) has reviewed the morphology of cell death during development and proposed that there are at least four different morphological types of cell death. He suggests that neuronal death during development occurs with 3 different morphologies: two of which may correspond to the two types postulated in the above classification and a last type that has been named autophagic degeneration (reviewed by: Schweichel and Merker, 1973; Clarke, 1990). In this form of death, the membranous organelles dilate, sequester and digest parts of the cell cytoplasm (Schweichel and Merker, 1973; Hornung et al., 1989; reviewed by Clarke, 1990). In addition, Pilar and Landmesser (1976) and Clarke and his collaborators (Clarke and Egloff, 1988; Hornung et al., 1989; reviewed by Clarke, 1990) have documented that

the morphology of neuronal death during development is susceptible of change by experimental manipulations such as removal of the target, blockade of axonal transport or elimination of afferents.

Furthermore, a type of cell death different from the types described above has been reported for embryonic rat sympathetic neurons in tissue culture. These neurons, that require NGF in the culture medium for their survival (Chun and Patterson, 1977; Greene, 1977), can be prevented from dying after trophic factor deprivation by drugs that inhibit RNA and protein synthesis (Martin et al., 1988). When these cells are deprived of NGF, they show two types of cell death: one resembles apoptosis, while the other represents a distinct type of cell death characterized by the accumulation of lipid droplets in the cytoplasm (Martin et al., 1988).

Although the classification of cell death on the basis of morphology may imperfectly reflect the underlying mechanisms, the concept of apoptosis, as distinct from necrosis, has fostered an appreciation of the need to consider the molecular basis of cell death.

1.3. TROPHIC FACTORS AND NEURONAL SURVIVAL

The previous sections have reviewed the effects of axotomy and the possible causes and mechanisms of cell death. Although the mechanisms of neuronal death after axotomy are unknown, it has been postulated that axotomy-induced neuronal death might be due to the loss of trophic support from their targets (reviewed by Oppenheim, 1991). The survival of neurons has been shown to be dependent, in certain situations, on neurotrophic factors. This section reviews studies that provide

evidence for the dependency of embryonic, neonatal and adult neurons on neurotrophic factors for their survival.

1.3.1. NEURONAL DEVELOPMENT

Naturally occurring neuronal death during development usually coincides with the arrival of the axons to their fields of innervation. This fact has been considered as an indication that neuronal death is due to neuronal competition for a factor that is present in their targets (reviewed by: Cowan et al., 1984; Barde, 1989; Oppenheim, 1991). The discovery of the neurotrophic factors and in particular the results of experiments with NGF, have prompted some authors to postulate that the cause of the naturally occurring neuronal death during development may be the limited availability of trophic factors in the neuronal targets (reviewed by: Cowan et al., 1984; Barde, 1989; Oppenheim, 1991). Evidence for this theory comes from experiments using NGF or antibodies against this factor. For example, in rat embryos, the administration of NGF reduces the amount of cell death in sympathetic ganglia (Levi-Montalcini, 1966; Hendry, 1977), while the administration of antibodies against NGF leads to the almost complete degeneration of these ganglia (Cohen, 1960).

Three other factors with similar primary structures to NGF have been described, and two of these have been shown to promote the survival of neurons: Brain-derived neurotrophic factor (BDNF, Leibrock et al., 1989; Hofer et al., 1990) supports the survival *in vitro* and *in vivo* of different types of embryonic primary sensory neurons (reviewed by: Barde, 1989; Walicke, 1989; Thoenen, 1991), and also the survival *in vitro* of other types of neurons, such as embryonic rat retinal ganglion cells (Johnson

et al., 1986), basal forebrain cholinergic neurons (Alderson et al., 1990) and mesencephalic dopaminergic neurons (Hyman et al., 1991). Neurotrophin 3 (NT-3, Hohn et al., 1990; Maisonpierre et al., 1990a; Rosenthal et al., 1990, also named hippocampus-derived growth factor, Enfors et al., 1990a; reviewed by Thoenen, 1991) increases the *in vitro* survival of various types of primary sensory neurons (Hohn et al., 1990; Rosenthal et al., 1990). It remains to be documented whether the third factor related to NGF, neurotrophin 4 (NT-4; Halböök et al., 1991) has survival promoting activity, but it has been reported to promote neurite outgrowth from embryonic chick nodose and dorsal root ganglia (Hallböök et al., 1991). Fibroblast growth factors (FGFs, two factors that share 53% homologies and show trophic influences also in some non-neuronal tissues (Baird et al., 1985a; Gosporadovich et al., 1986) can promote the survival *in vitro* of various types of embryonic central and peripheral neurons (reviewed by: Barde, 1989; Walicke, 1989).

During the neonatal period, axotomy causes the loss of most of the injured nerve cells (see section 1.1.). This cell death is assumed to be caused by the neuronal loss of target-derived trophic support, on which neonatal neurons appear to be particularly dependent. The main evidence for the neonatal dependence of neurons on trophic factor support from their targets comes from experiments in which it was demonstrated that the blockade of axonal transport (Hendry et al., 1974) or mechanical and chemical forms of axotomy (Angeletti and Levi-Montalcini, 1970; Hendry and Campbell, 1976), caused an exaggeration of the naturally occurring neuronal death in rat sympathetic ganglia.

The results of recent experiments that investigated neuronal death in developing motor neurons have suggested that the cause of the naturally occurring neuronal death in this system may not be the limited quantities of neurotrophic factor present in the target organs during development, but a restricted number of synaptic sites in the muscle (reviewed by: Oppenheim, 1989, 1991). This evidence does not invalidate the neurotrophic theory because the ability of a neuron to synapse with its target might mean that these neurons receive their trophic support via their axonal arborizations or synapses (Oppenheim, 1989; 1991). Developing motoneurons in the chick have been shown to be dependent on at least one neurotrophic factor: ciliary neurotrophic factor (CNTF; Adler et al., 1979; Manthorpe et al., 1986), a factor recently characterized and cloned (Lin et al., 1989; Stockli et al., 1989). Treatment of chick embryos during the period of naturally occurring motoneuron death with human recombinant CNTF rescues a proportion of lumbar spinal motoneurons from death (Oppenheim et al., 1991). Furthermore, the application of purified rat CNTF to the proximal stump of transected facial nerves in newborn rats resulted in the survival of most facial motoneurons by one week after axotomy (Sendtner et al., 1990). The receptor for CNTF has been cloned (Davis et al., 1991), and is present only in skeletal muscle and the CNS (Squinto et al., 1990). However, CNTF is not considered to be a target-derived neurotrophic factor for developing motoneurons (reviewed by Thoenen, 1991).

1.3.2. ADULT NEURONS

In adult mammals, axotomy (see section 1.1.) and sometimes the removal of neuronal targets cause degeneration of the affected neurons (reviewed by: Oppenheim, 1991). Thus, it has been postulated that adult neurons are still dependent on neurotrophic support from their targets (reviewed by: Purves, 1976; Oppenheim, 1991). Evidence for the neurotrophic dependency of adult neurons comes from studies in which NGF has been shown to rescue a proportion of axotomized nerve cells and treatment with antibodies against NGF causes death of sympathetic neurons (see below). For example, basal forebrain cholinergic neurons, as well as dorsal root ganglion (DRG) neurons, survived in greater numbers if NGF was administered after their axons were lesioned (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Otto et al., 1987; Rich et al., 1987; Koliatsos et al., 1990; Tuszynski et al., 1990). Furthermore, the administration of antibodies against NGF to adult mice for one month caused the death of 25% of the sympathetic neurons in the superior cervical ganglia (Ruit et al., 1990).

There are also situations in which NGF-responsive adult neurons have been demonstrated not to depend so strictly on their targets or on NGF for their survival. For example, Sofroniew et al. (1990) have documented that in the rat, basal forebrain neurons can survive for up to 4 months after injections of excitotoxins that destroy their target neurons in the hippocampus. Embryonic rat DRG neurons, whose survival *in vitro* is dependent on NGF, do not show such a dependency on NGF for survival in adulthood (Lindsay, 1988). In addition, treatment of adult rats and guinea pigs with antibodies against NGF did not produce a decrease in numbers or size of the neurons

in the DRG of these animals (Schwartz et al., 1982). However, these neurons have been shown to require NGF administration for the expression of certain phenotypic functions: *in vitro*, the addition of NGF was needed for these neurons to express the genes encoding two of their neuropeptides (substance P and calcitonin gene-related peptide, Lindsay and Harmar, 1989). Furthermore, the presence of BDNF in the culture medium did not influence the survival of adult DRG neurons but estimulated the regeneration of axons from these neurons (Lindsay, 1988).

Finally, in adult animals, basic FGF (bFGF) can also reduce the amount of photorreceptor death in the retina of mice suffering from a inherited retinal degenerative disease (Faktorovich et al., 1990) and reduce the amount of striatal dopamine neuronal death in mice treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Otto and Unsicker, 1990).

1.3.3. POSSIBLE MODES OF ACTION

Whether the observed effects on survival in all these situations is specific and receptor mediated is not known, and the modes of action of these trophic factors remains undetermined. NGF, and presumably other factors related to NGF (BDNF, NT-3 and NT-4), exert their specific biological effects upon receptive target cells by binding to high-affinity cell-surface receptors. The high affinity receptor for NGF is a 158K complex that is formed by two different proteins that in isolation can both bind NGF with low affinity (Kaplan et al., 1991). The first protein is the 140K proto-oncogene tyrosine kinase trk product (p140*prototrk*), while the second is the 75K low affinity NGF receptor ($p75^{NGFR}$; Hempstead et al., 1991). While it is known that the low

affinity NGF receptor $p75^{NGFR}$ can bind other trophic factors with similar primary structures such as BDNF in adittion to NGF (Rodríguez-Tébar et al., 1990), NT-3 (Enrfors et al., 1990b) and NT-4 (Hallböök et al., 1991), the p140*prototrk* does not bind BDNF, thus discriminating NGF from other family related known neurotrophins (Martín-Zanca, et al., 1990). Presumably, the activation of *trk* by high affinity NGF binding activate tyrosine phosphorylation that, in turn, would also activate intermediate kinases. Another *trk* related tyrosin kinase, trkB, has been reported to be involved in the formation of the high affinity binding sites for BDNF and NT-3 (Squinto et al., 1991; Soppet et al 1991).

The cellular mechanisms by which trophic factors promote neuronal survival are not known. Although various trophic factors induce the expression of intermediate early genes (Squinto et al., 1990; reviewed by: Walicke, 1989; Sheng and Greenberg, 1990), the actions of these genes in promoting neuronal survival are not well understood (reviewed by: Walicke, 1989; Sheng and Greenberg, 1990). Because inhibitors of RNA and protein synthesis have been reported to rescue cultured embryonic sympathetic or sensory neurons from cell death after NGF deprivation (Martin et al., 1988; Scott and Davies, 1990) and supress the naturally ocurring death of embryonic chick motoneurons (Oppenheim et al., 1990), it has been postulated that one of the mechanisms by which trophic factors promote neuronal survival might be the supression of a death program that requires RNA translation (Martin et al., 1988). However, the death of cultured PC12 cells, cells whose survival is dependent on the presence of serum (Greene and Tischler, 1976) or of other various trophic factors (Greene, 1978; Rydel and Greene, 1987; Ruckenstein et al., 1991) in the culture

medium, after serum deprivation cannot be prevented by inhibitors of macromolecular synthesis (Ruckenstein et al., 1991). Furthermore, the blockade of RNA and protein synthesis does not affect the survival effect of various trophic factors (NGF and insulin-like growth factors) in cultured PC12 cells (Ruckenstein et al., 1991). The experiments carried out using these cells have indicated that death caused by trophic factor deprivation may not always require RNA and protein synthesis and conversely, the mechanisms by which trophic factors increase neuronal survival might not require macromolecular synthesis (Ruckenstein et al., 1991).

1.4. RESEARCH PROJECT

Earlier studies documented the capacity of RGCs to regrow their axons along peripheral nerve grafts when their axons were lesioned near the cell bodies (So and Aguayo, 1985; Vidal-Sanz et al., 1987). Such studies showed that up to 10% of the total RGC population could regenerate their axons for distances of 3-4 cm when the optic nerve is cut at its exit from the eye and substituted by a peripheral nerve graft (Vidal-Sanz et al., 1987). However, such regrowth did not occur when the optic nerve was cut 8 mm from the eye (Richardson et al., 1982). This interesting paradox as well as the lack of quantitative studies on the effect of the distance of axotomy on RGC survival prompted some of the studies adressed in this thesis.

1.4.1. PREVIOUS STUDIES OF RGC SURVIVAL

In this section, I summarize the results of experiments that I carried out prior of those to be reported in this thesis and that have been included as appendices.

Following the demonstrating of the regrowth and connectivity of axotomized RGCs (Vidal-Sanz, Villegas-Pérez et al., 1985; Vidal-Sanz et al., 1987), I investigated if peripheral nerve grafts could increase the survival of RGCs after axotomy. For this study, adult rat RGC survival was analyzed after ON section at its exit from the eye with or without grafting a segment of peripheral nerve to the proximal ON stump (Appendix I). The conclusions of this study were that: i) ON section at its exit from the eye caused the death of more than 90% of the RGCs by one month after axotomy; ii) the peripheral nerve graft promoted axonal regeneration of approximately 20% of the surviving RGC population (3-4% of the total RGC population) during the

first three months after axotomy; iii) peripheral nerve grafts also increased RGC survival two to four-fold during the first three months after axotomy; such increased survival could not be explained only on the basis of the number of RGCs that have regenerated their axons.

Several techniques were applied or developed for the above mentioned studies. i) The retinal areas assesed in each animal were standardized. For this purpose we designated twelve areas in each retina, three in each quadrant at one, two and three mm from the optic disc. Each of these areas is within the central region of the rat retina and have similar densities of RGCs (Perry, 1981). ii) To identify retinal ganglion cells, two different techniques were used. The first was to stain the RGC layer with methylene blue (Stone, 1965) and then designate RGCs using the criterion of cell size (Perry, 1981). The second technique involved retrogradely labelling RGCs with the fluorescent tracer dil (1,1'-dioctadecyl, 3,3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, Junction City, OR; Honig and Hume, 1986) applied at the site of ON section. In addition, the RGCs that regenerated their axons along the peripheral nerve grafts were doubly labelled with Fast Blue applied to the end of the graft.

In this previous study, however, dil application to the site of ON section only permitted the labelling of one-third of the total RGC population, probably because of the crystaline nature and insolubility of this dye. Thus, all calculations of RGC survival using this technique had to be corrected for this factor. To label all RGCs, another technique was subsequently developed in which dil was dissolved with 2% Triton X-100 in saline and applied to the main RGC targets: the superior colliculus (SC) and

dorso-lateral geniculate nucleus (dLGN). This technique allows the retrograde labeling of the entire RGC population (Vidal-Sanz, Villegas-Pérez et al., 1988; Appendix II), and permits the identification of RGCs long periods of time after axotomy. The persistence of the dye in the cytoplasm of the RGCs for long periods of time, without apparent leakage or degradation, even 3 months after ON section, was also documented (Vidal-Sanz, Villegas-Pérez et al., 1988).

1.4.2. RESEARCH PROPOSAL

The objective of the experiments to be reported in this thesis was to explore further the responses of RGCs to ON interruption at different distances from the eye and the identification of possible influences that may affect these responses. Thus, the main questions were: Is the temporal pattern of RGC degeneration similar or different for all experimental groups? If so, what are the experimental variables determining the timing of RGC degeneration? Can influences be identified that might be affecting this response and that could be exploited in the future for the rescue of the RGC population after ON section or to enhance RGC regeneration?

For these experiments, the whole RGC population was retrogradely labelled with dil applied to the SC and the dLGN. In four experimental groups of animals, the ON was lesioned at one of four different distances between the eye and the optic chiasm. RGC survival was analyzed from 15 days to 20 months after lesioning the ON. In addition, we have also studied whether changes in retinal area or ageing or leakage of the dye may influence our results.

1.5. THE VISUAL SYSTEM OF THE ADULT RAT

This section describes several aspects of the visual system of the adult rat that are relevant for the methodology used in the experiments reported in this thesis.

The retina is the photoreceptor organ of the visual system that carries out the initial processing of the visual stimuli (Dowling and Werblin, 1971; Levick and Dvorak, 1986).

1.5.1. STRUCTURE OF THE RETINA

As a part of the central nervous system, the retina is composed of neurons and glial cells. There are five classes of neurons in the retina: photorreceptors (rods and cones), horizontal cells, bipolar cells, amacrine cells and retinal ganglion cells. In addition, the retina contains various types of glial cells: Müller cells, microglia, astrocytes, and oligodendrocytes.

The retina is organized in layers that contain the neuronal cell bodies and their processes. From the outer or scleral surface to the inner or vitreal aspect of the retina, nine layers can be distinguished: i) the photorreceptor layer, which contains the outer segments of these neurons that are in direct contact with the retinal pigmented epithelium; ii) the outer limiting membrane; iii) the outer nuclear layer, which contains the photoreptor nuclei; iv) the outer plexiform layer, which is composed of the processes of photorreceptors, horizontal and bipolar neurons; v) the inner nuclear layer that contains the cell bodies of bipolar, horizontal, amacrine and Muller cells; vi) the inner plexiform layer, which contains the processes of bipolar, amacrine and

ganglion cells; vii) the ganglion cell layer; viii) the nerve fiber layer containing the axons of the RGCs, and; ix) the inner limiting membrane.

Ramón y Cajal (1893) suggested that the transmission of the visual input is from photoreceptors to bipolars and from these to ganglion cells, whose axons exit from the retina and make synaptic contacts in different brain regions. This hypothesis was confirmed with the development of the electron microscope which allowed the study of the synapses between retinal neurons (reviewed by Boycott, 1974). It was thought that the neurons that form the visual pathway are photoreceptors, bipolar and ganglion cells, the other retinal neurons acting only as modulators of the transmission between these cells (reviewed by: Boycott, 1974; Kaneko, 1979). However, recent experiments have documented that rod bipolar cells excite both on-centre ganglion cells and amacrine cells that in turn inhibit off-centre ganglion cells (reviewed by Daw et al., 1990).

1.5.2. DISPLACED AMACRINE AND GANGLION CELLS

The main layers of the retina tend to be conserved among species, although there are small differences in the contents of individual layers, even among mammals. In this section, I will review aberrant locations of amacrine and ganglion cells within the retina.

In the last century, Dogiel identified some retinal ganglion cells in the inner nuclear layer and called them spongioblasts (Dogiel, cited by Ramón y Cajal, 1893). Such displaced RGCs were studied in the rat retina by Linden (1987), who has calculated that they represent one percent of the total RGC population.

As described by Ramón y Cajal (1893), there are also displaced amacrine cells in the RGC layer. Studied in rats (Perry 1979, 1981; Perry and Walker, 1980) these displaced amacrine cells represent approximately 50% of the neurons in the ganglion cell layer (Perry and Cowey, 1979; Perry, 1981).

1.5.3. RETINAL GANGLION CELL DENSITIES

Unlike other mammalian species (human, cat, etc), the rat retina does not have a fovea centralis but presents a region of maximal RGC density, called the area centralis (Sefton and Dreher, 1985), in the superotemporal quadrant 1-2 mms from the optic disc. The total number of RGCs in the rat retina was calculated to be 110,000 (Schober and Gruschka, 1977) or 115,000 (Fukuda, 1977); these values are similar to the number of axons in the ON, estimated to be 117,000 (Forrester and Peters, 1977) or 120,000 (Hughes, 1977).

Depending on the region of the retina examined, different authors have calculated that RGC densities in the rat retina vary between 1000 and 6000 cells/mm^2. Fukuda (1977) and Schober and Gruschka (1977), using classic stains for Nissl substance and Dreher et al. (1985), using horseradish peroxidase (HRP) applied to the RGC targets as a means of RGC identification, calculated mean numbers between 1000 and 2000 RGCs/mm^2 for the periphery of the retina and 5000 to 6000 RGCs/mm^2 for the area centralis, the region of maximal density. Perry (1981), who also used HRP to retrogradely label the RGCs from their targets, calculated a mean of 2500 RGCs/mm^2 for the retinal area situated within 3 mms around the optic disc, area that he called central region, and 1600 cells/mm^2 in the more peripheral regions.

1.5.4. OTHER CELLS IN THE RETINAL GANGLION CELL LAYER

Cells other than RGCs and displaced amacrine cells are also found in the retinal ganglion cell layer of mammals. Astrocytes (Büssow, 1980), microglia (Muchnick-Miller and Oberdorfer, 1981; Barron et al., 1986; Robinson et al., 1986), and small numbers of oligodendrocytes (Villegas, 1964; Barron et al., 1986) can also be found in this layer (reviewed by Stone, 1981).

1.5.5. METHODS FOR RETINAL GANGLION CELL IDENTIFICATION

RGC identification after ON section has relayed on three methods that have been used together or separately: i) Morphology. With this method, RGCs are distinguished on the basis of their pale nucleus with dispersed chromatin and their variable amounts of cytoplasm with Nissl substance (Stone, 1981); ii) Cell size. In normal retinas, amacrine cells are smaller than most RGCs (Perry, 1981). Thus, most cells in the RGC layer that are larger than 10 μm in diameter (Perry and Cowey, 1979; Perry, 1981) can be considered to be RGCs; and iii) Retrograde labelling from the RGC targets (Bunt et al., 1974; Perry and Cowey, 1979; Perry, 1981; Dreher et al., 1984, 1985). The two first methods may not be reliable for RGC identification after axotomy, because the retrograde degeneration of neurons is accompanied by changes in cell size and morphology (see sections 1.3.3 and 1.3.6). Retrograde labelling of RGCs may thus be a better method for RGC identification after axotomy, if the tracer persists in the RGC bodies.

1.5.6. RETINAL GANGLION CELL TARGETS

The RGC axons of the rat converge at the optic disc and leave the eye through the ON. From 97 to 98% of the RGC axons decussate at the optic chiasm in pigmented rats and even greater proportions do so in the albino rat (Lund, 1965; Dreher et al., 1985). Thus, less than 3% of the axons in the optic tract come from the ipsilateral retina. The RGCs that project ipsilaterally are situated in the inferotemporal quadrant of the rodent retina (Cowey and Franzini, 1979). A small number of RGCs branch to project bilaterally (Cunningham and Freeman, 1977; Jeffery et al., 1981).

Once in the optic tract, the RGC axons are directed to six nuclei in the brain were synapses are formed: the hypothalamus (Rita and Oomura, 1982); nuclei of the optic accessory system in the midbrain (Hayhow et al., 1960; Kostovic, 1971; reviewed by Simpson, 1984); the pretectum (Scalia and Arango, 1977); the SC (Linden and Perry, 1983); and both the ventro-lateral and dorso-lateral geniculate nuclei (Lund et al., 1974; Rodieck, 1979; Martin, 1986). In the rat, most RGC axons are directed mainly to two nuclei in the brain: i) the SC, where more that 95% of the axons from the contralateral axons converge (Linden and Perry, 1983); and ii) the dLGN, that receives 37% of the axons from the contralateral retina (Martin, 1986). There is a discordance in these numbers because many axons branch to innervate both structures (Sefton, 1968). These nuclei are situated at a distance of 21.5 (SC) and 18 mms (dLGN) from the adult rat retina (Sefton, 1968).

CHAPTER 2. GENERAL METHODS

2.1. ANIMALS

Female Sprague-Dawley rats weighing 200-220 grams were used for this study. For all surgical procedures, the rats were deeply anesthesized using intraperitoneal injections of 7% chloral hydrate in saline (0.42 mg/g body weight as the initial dose for surgery). A lethal dose of the same anesthetic was given intraperitoneally before sacrifice. Before and after surgery, the animals were placed in groups of two to three per cage, in one windowless room with a 12 hour light/dark cycle. Water and food were provided ad libitum. Light intensity, measured inside the animal cages ranged from 2.4 to 3.1 foot candles (ftcd) at the front of the cages and less than one to 1.2 ftcd in the back of the cages. This luminance, which is lower than the 3 to 3.7 ftcd recommended for the front of the cages by the NIH Committee on Care and Use of Laboratory Animals (1985), was chosen to avoid retinal phototoxicity, that has been reported not to occur in specific pathogen free rats when exposed to light intensities of less than 3 ftcd (Weisse et al., 1974).

2.1.1. EXPERIMENTAL GROUPS AND SURGICAL MANIPULATIONS

2.1.1.1. Retrograde labelling of RGCs

Retinal ganglion cells were retrogradely labelled with the fluorescent tracer dil (Sims et al., 1974; Honig and Hume, 1986). The SC and dLGN, which are the targets of most of these neurons (Linden and Perry, 1983; Martin, 1986), were bilaterally

exposed in anesthetized animals. After gently removing the pia mater over these structures, a piece of Gelfoam (Upjohn, Kalamazoo, Mi.) containing 30% dil, and 2% Triton X-100 (New England Nuclear Corp.) in 0.9% NaCl was placed over the SC and dLGN. This procedure has been shown to produce persistent labelling of most RGCs (Vidal-Sanz, Villegas-Pérez et al., 1988; Appendix II).

2.1.1.2. Experimental retinas

Twenty days after dil application, when most RGCs are labelled (Vidal-Sanz, Villegas-Pérez et al., 1988, Appendix II) the experimental animals were divided into four groups and were subjected to a second operation in which the left optic nerve (ON) was interrupted at one of four sites between the eye and optic chiasm (Figure 1): i) at its exit from the eye; ii) intraorbitally as far as possible from the eye; iii) intracranially at half the distance between the optic foramen and optic chiasm; and iv) intracranially rostral to the chiasm. These four lesion sites are referred to as: optic disc (OD), intraorbital (IO), intracranial (IC) and optic chiasm (OC), respectively.

The technique used to transect the optic nerve varied depending on whether the site of axotomy was intraorbital or intracranial. In the two firsts groups (OD & IO) the optic nerve was sectioned intraorbitally by opening its dural sheath and cutting the nerve with scissors. In the other two groups (IC & OC) which received an intracranial lesion of the optic nerve, the nerve and its meninges where repeatedly crushed with jeweler's forceps until a discontinuity between the two stumps of the nerve was observed. This later procedure of transection thus respected the continuity of the dural sheath of the optic nerve, and it was chosen in the two later groups to avoid

damage to other vascular or neural structures present in neighbouring regions of the intracranial course of the nerve.

2.1.1.3. Control retinas

Three groups of control retinas were used:

i) To examine dil-labelling of RGCs twenty days after dye application, I used the retinas from two animals of a previous study (Vidal-Sanz, Villegas-Pérez et al., 1988; Appendix II).

ii) For long-term controls, I used both retinas from two animals in which dil was applied to the RGC targets for 21 months.

iii) The right (non-axotomized) retinas of the experimental animals were examined qualitatively to assess the pattern of dil-labelling. Some of these retinas from the optic disc experimental group were also used to determine the areas of control retinas.

2.2. TISSUE PROCESSING

2.2.1. FIXATION AND DISSECTION

All animals were perfused transcardially with 0.9% NaCl, followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer. After fixation, the length of the interrupted ONs from the eye was measured in at least four animals of every experimental group at one month after ON interruption and determined to be approximately: 0.5 mm for the optic disc group, 3 mm for the intraorbital group, 8 mm for the intracranial group and 10 mm for the chiasm group.

The retinas from the experimental animals were processed at different times between fifteen days and twenty months after ON section. The number of experimental retinas analyzed at each time period is summarized in the following table:

NUMBER OF EXPERIMENTAL RETINAS											
GROUPS	TIME AFTER OPTIC NERVE INTERRUPTION (months)										TOTAL NUMBER
	0.5	1	2	3	6	9	12	15	17	20	
OD	4	8	5	4	4		5		1	2	33
IO	4	6	3	6	6	2	5				32
IC	4	6	4	6	4	4	4	2			34
OC	4	6	4	3	4		4				26

2.2.2. WHOLE MOUNTS OF THE RETINAS

The retinas were dissected and prepared as whole-mounts by making four radial cuts; the superior pole of the eye was indicated by the deepest cut. After dissection, the retinas were placed with their inner side up on a slide and a piece of filter paper was placed in top of them. This procedure flattens the retina and makes it adhere to the filter paper that is then immersed (with the retina attached) in the

fixative solution for one hour. After fixation, the retinas were freed of their attachment to the filter paper, washed in 0.1 M phosphate buffer, mounted on coated slides vitreal side up, and examined within 24 hours by fluorescence microscopy. The mounting medium was composed of a 3/1 solution of glycerol and sodium carbonate buffer (pH 9.0) containing p-phenylendiamine 4 mg/ml (Dodd et al., 1984).

2.2.3. DiI FLUORESCENCE

All control and experimental retinas were examined for dil-labelling in the fluorescence microscope (Leitz Ortholux II model), using the Ploemoback filter set. DiI dissolved in water has a maximal absorption at 540 nm and maximal emission at 556 nm (Sims et al., 1974); its emission has an orange-red appearance with rhodamine filters, and orange-yellow with fluorescein filters. For this study, we used a rhodamine filter that had an excitation band of 530 to 560 nm and a barrier filter of 580 nm.

2.2.4. OTHER TISSUE PROCESSING

2.2.4.1. OX-42 Immunocytochemistry

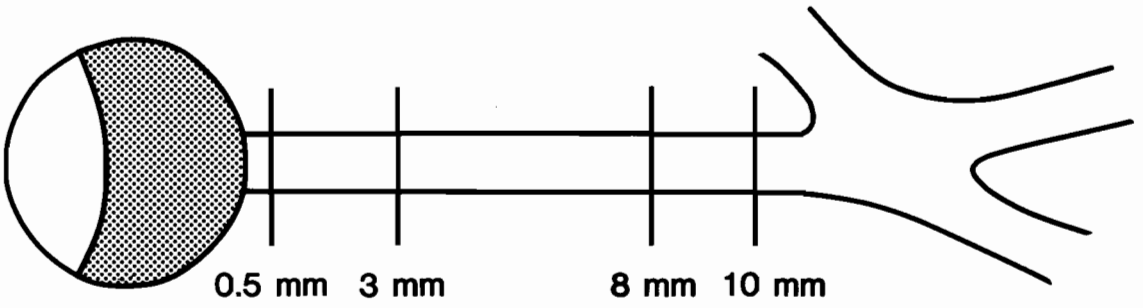
OX-42 (Serotec, U.K.) is a monoclonal antibody (mouse IgG) that recognizes epitopes on the surface of microglia (Robinson et al., 1986). Two retinas from the optic disc group processed 15 days after ON section were removed from the slides, washed and incubated for one hour at -4ºC with this antibody, diluted 1/10 in 5% bovine serum albumin in saline and then with a secondary antibody (Goat anti-mouse IgG, Calbiochem, San Diego, Ca.) coupled to fluorescein (diluted 1/50). These retinas

were again examined by fluorescence microscopy using the fluorescein filter (excitation 450-490 nm, supression band 525/20) of the Ploemoback filter set. To discern whether some dil fluorescence was contained within microglia, we observed the same regions of these retinas alternating the rhodamine and fluorescein filters.

2.2.4.2. Methylene blue staining

To compare the densities of dil-labelled cells with total cell densities in the RGC layer of the experimental retinas, two retinas from the experiments where the ON was interrupted at the optic chiasm at each time period studied (15 days, 1, 2, 3, 6, and 12 months; total n=12) were removed from the slides after examination by fluorescence microscopy, washed, and stained with 0.02% aqueous methylene blue for 10 minutes. This stain was fixed by the treatment of the retinas with 5% ammonium molybdate in water for 5 minutes (Stone, 1965; Villegas-Pérez et al., 1988; Appendix I). After the staining procedure, the retinas were dehydrated for 1 minute in each of the following concentrations of ethanol in distilled water: 50, 75, 90, 95 and 99%, cleared in Hemo-De (Fisher Scientific Co.) and mounted with DPX (BDH Inc., Toronto). Only three quandrants of these experimental retinas (superotemporal, inferotemporal, and superonasal) were stained with methylene blue; the fourth (inferonasal) quadrant was used for the immunohistochemical demonstration of calbindin (data not shown).

Figure 1. *Diagram, with the eye on the left and the optic chiasm on the right, that illustrates the four levels of interruption of the optic nerve in the experimental animals.*


0.5 mm
3 mm
8 mm
10 mm

2.3. MEASUREMENT OF RGC DENSITIES

Because of the central to peripheral gradient of RGC densities in normal retinas (Fukuda, 1977; Schöber and Gruschka, 1977; Dreher et al., 1984), I determined the densities of dil-labelled neurons in 12 standard areas located within the central region of each retina (Villegas-Pérez et al., 1988; Appendix I). For each retinal quadrant (Superotemporal, inferotemporal, superonasal and inferonasal), the numbers of dil-labelled neurons were counted in three areas, which measured 0.23 x 0.33 mm each, and whose center was located at 1, 2 and 3 mm from the optic disc. The number of dil-labelled RGCs in these 12 regions were used to calculate the mean density of labelled cells/mm^2 for each retina.

2.3.1. DiI-LABELLED CELL DENSITIES

DiI-labelled RGCs in each of the twelve areas were counted directly by fluorescence microscopy, focusing at different planes. RGCs were also counted from photographs taken from these areas, using an Orthomat-W camera adapted to the Leitz fluorescence microscope, and printed at a total magnification of x700.

These two methods of counting were used in control and experimental retinas. Photographs were used when the high densities of dil-labelled RGCs that were present in the retinas made it difficult to count the number of cells accurately by eye. Thus, this method was used for control retinas and also during the first six months of the study for the experimental retinas with ON lesion 8 or 10 mm from the eye. Direct counting was used for the rest of the experimental retinas, when dil-labelled cells were

less easily recognized because: i) there were small numbers of dil-labelled cells in the retinas; ii) the cells were only faintly labelled; or iii) cells other than RGCs had accumulated dil within their cytoplasm and were obscuring the dil-labelled RGCs. Usually all of these conditions coincided in the retinas examined by this method, although the third condition was more prevalent with 0.5 or 3 mm ON sections during the first month after axotomy. Thus, the direct counting method was used when it was assumed that accurate cell counting in photographs was not possible.

The counts in each of the twelve retinal areas were converted into dil-labelled cell densities per mm^2, and grouped to obtain a mean cell density for each retina.

2.3.1.1.- Cell densities at different distances from the optic disc

To assess whether RGC losses within the retina varied with distance of axotomy, I studied whether the densities of dil-labelled cells in the experimental and control animals were different in the areas examined at 1 and 3 mm from the optic disc. For this purpose, cell densities obtained in the four standard areas examined at each one of these two distances from the optic disc were grouped to obtain for every retina a mean density of dil-labelled cells at one and three mm from the optic disc. Finally, for each retina, a ratio between these mean cell densities was calculated by dividing the mean cell density at 1 mm from the optic disc by the mean cell density at 3 mm from the optic disc.

2.3.2. CELL DENSITIES IN METHYLENE BLUE STAINED RETINAS

Nine standard retinal areas (three in each quadrant at 1, 2, and 3 mm from the optic disc) were examined and photographed in the three quadrants of the experimental retinas that were used for methylene blue staining (see above, section 2.2.4.2.). Because methylene blue stains all cells in the ganglion cell layer, all cells, except those clearly non-neuronal, such as endothelial cells, were counted in each photograph. These counts were converted to cell densities per mm^2, and a mean density of cells calculated for each retina.

2.4. ANALYSIS OF RETINAL AREAS

The outline of the whole mounted control and experimental retinas was drawn from the slide using a microscope slide viewer (Carl Zeiss Jena, FDR) at a magnification of x35. The areas of these retinas were determined from the camera lucida drawings using an IBAS-1 image analyzer. The areas of twenty five experimental retinas could not be calculated because peripheral areas of the retina were missing.

2.5. ANALYSIS OF THE DATA

Graphs were obtained using Sigmaplot 4.0 (Jandel Corp., Corte Madera, Ca.). Regression lines were calculated from individual RGC densities per retina, rather than from mean densities per time studied.

For statistical analysis of the data, we used: i) the two-sided Student's t test (unless otherwise specified) to compare groups of densities using the software of the

IBAS-1 image analyzer; ii) analysis of variance to analyze regression lines (Sokal and Rohlf, 1981); and iii) the General Linear Model of the SAS program (SAS Institute, Cary, North Carolina) to compare slopes. Differences were considered significant if $p < 0.05$.

CHAPTER 3. RESULTS

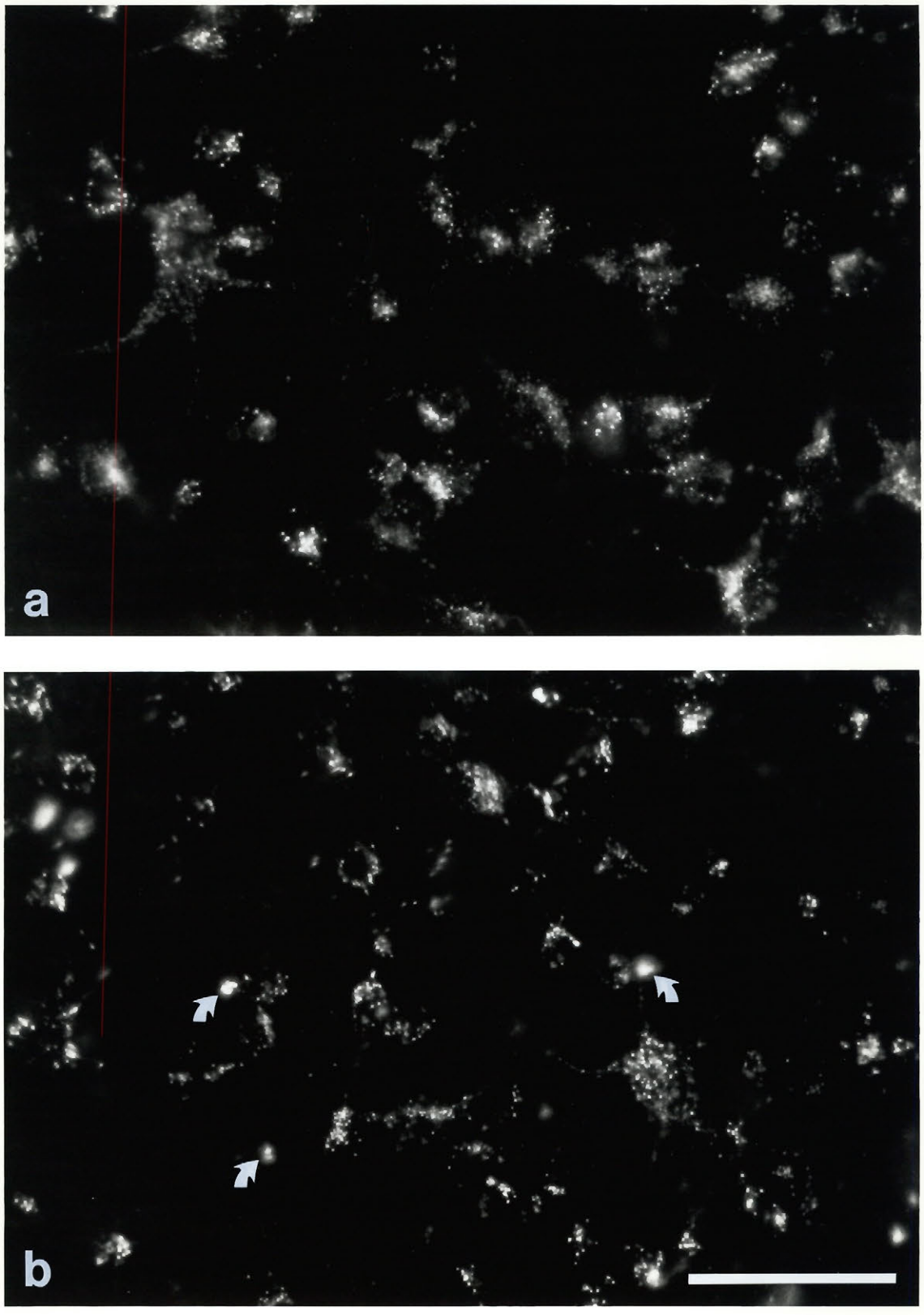
3.1. DII-LABELLING

Two patterns of dil-labelling were identified: i) DiI-labelled RGCs were recognized in the experimental and control retinas by their fine, punctate fluorescence (Figure 2a,b) that was most intense in the perikarya and occasionally delineated dendrites; and ii) After ON lesions, the retinas of the experimental animals also showed a coarse dil fluorescence (Figure 2b) that was more extensive in the animals with the greatest RGC loss during the first month of the study. To investigate the possible non-neuronal nature of the pattern of dil-labelling, retinas from two animals with ON transections at the optic disc fifteen days before processing were examined by fluorescence immunocytochemistry using OX-42, an antibody that has been reported to recognize epitopes on the surfaces of microglia (Robinson et al., 1986; Perry and Lund, 1989). Most of the profiles of coarse dil labelling, and none of the finely-labelled profiles, were also immuno-stained with the OX-42 antibody, suggesting that microglia had phagocytosed the dil from disintegrating neurons, as indicated in a recent report (Thanos, 1991).

3.2. RGC DENSITIES IN CONTROL RETINAS

In a previous study (Vidal-Sanz, Villegas-Pérez et al., 1988; Appendix II), it was determined that the mean density of dil-labelled cells in control retinas 20 days after dil application to their targets was 2288 ± 66 cells/mm^2 (Mean ± SEM; n=4; Range:

Figure 2. *dil-labelled neurons in the central regions of the superotemporal quadrant of flat-mounted retinas from a control (a) and from an experimental (b) rat one month after optic nerve section 10 mm from the eye. The typical punctate dil fluorescence that delineates RGC bodies and sometimes proximal dendrites can be observed in both control and experimental retinas (a, b). The coarse, non-neuronal dil fluorescence can be observed only in the experimental retina (b). Some examples of non-neuronal fluorescence are indicated by arrows. Fluorescence photomicrographs, bar = 50* μ*m.*


a
b

2158-2466; Table I). For the present study, I have not repeated these experiments, because the technique that we use for dil-labelling is essentially the same as that used in the 1988 report. However, because the survival period in the present experiments extended up to 20 months after optic nerve section, we also determined whether RGC densities in control animals diminish with age, as has been suggested by some authors (Cano et al., 1986; Katz and Robinson, 1986). In the four retinas of the animals in which dil was applied to the RGC targets 21 months before processing, we found a mean density ± SEM of 2213 ± 39 dil-labelled cells/mm^2 (Range: 2112-2274; Table I, Figure 3). Although these densities are not statistically different from the densities of dil-labelled cells determined 20 days after dil application (Vidal-Sanz, Villegas-Pérez et al., 1988; Table I; Appendix II), they are significantly smaller ($p < 0.01$) than the maximum dil-labelled cell densities determined one month after dil application (Mean ± SEM: 2436 ± 44; n=8; Range: 2262-2616; Vidal-Sanz, Villegas-Pérez et al., 1988; Appendix II). Thus, between 1 and 21 months after dil-application, there is a decrease in dil-labelled cell densities in control retinas of approximately 9% of the maximum density as determined by this method.

3.3. RETINAL AREA

The areas of the retinas in which dil was applied twenty days before processing in a previous study (Vidal-Sanz, Villegas-Pérez et al., 1988; Appendix II) were used as control areas for the time zero in this study (Table IV). Both the control and experimental retinas increased in size throughout the period of study, although this increase was most marked during the first three months (Table IV, Figure 4).

Figure 3. *Low power view of the central region of a control retina processed 21 months after dil application. The optic disc can be seen in the upper left corner. The numbers of dil labelled cells and the intensity of the labelling were similar to those of retinas processed at earlier times. Bar = 300 μm.*

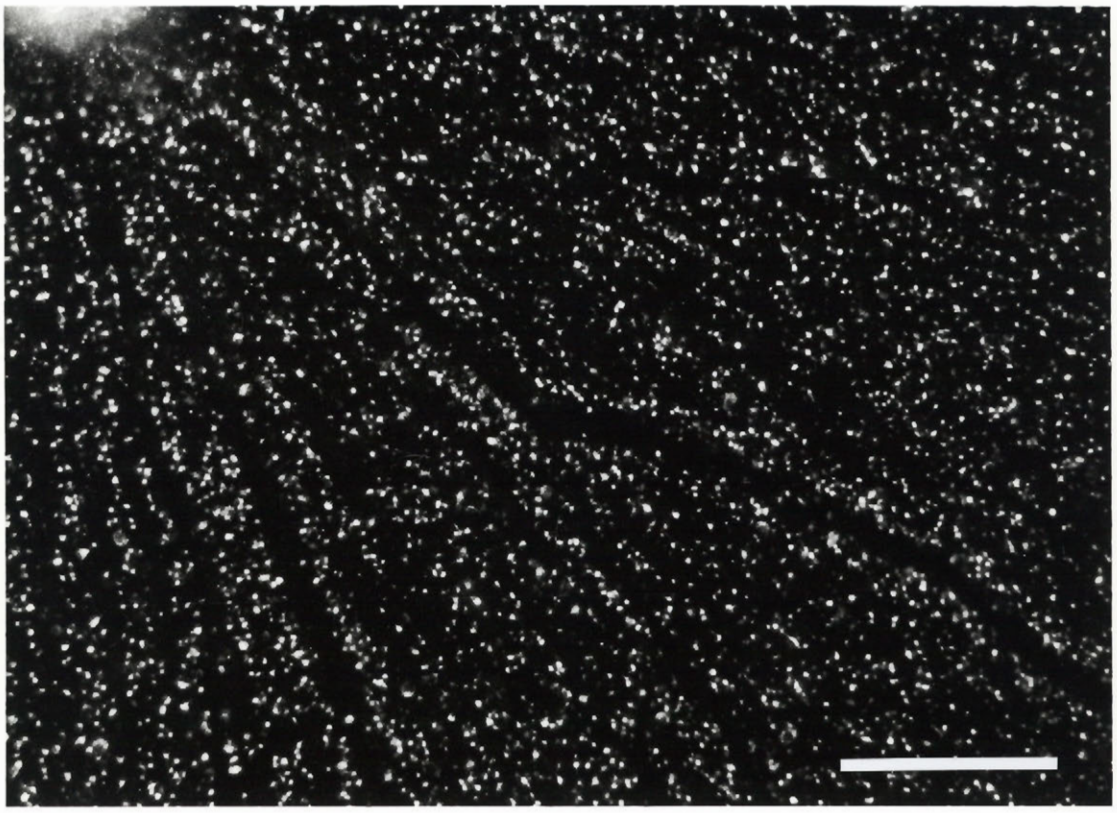


TABLE I
DiI-LABELLED CELL DENSITIES IN RETINAS AFTER AXOTOMY

DENSITY cells/mm²	EXPERIMENTAL RETINAS					CONTROL RETINAS	
	Time After Optic Nerve Interruption	Level of Optic Nerve Interruption				Time After dil Application	
		0.5 mm	3 mm	8 mm	10 mm		
	15 days	596 595 553 521	763 928 911 917	1334 923 1605 1392	1260 1330 1641 1769	20 days*	2158 2466 2232 2298
Mean ± SEM % control*		566 ± 18 24.7%	880 ± 39 38.5%	1313 ± 143 57.4%	1500 ± 122 65.6%		2288 ± 66
	1 month	399 349 509 480 322 452 403 422	732 585 944 1012 450 546	1167 1204 954 1047 1573 1551	1038 1738 1621 1677 1756 1989		
Mean ± SEM % control*		417 ± 22 18.2%	711 ± 92 31.0%	1249 ± 105 54.5%	1636 ± 130 71.4%		
	2 months	158 258 177 240 301	531 454 431	1140 782 985 1510	1419 1090 852 937		
Mean ± SEM % control*		227 ± 26 10.0%	472 ± 30 20.6%	1104 ± 154 48.3%	1074 ± 125 46.9%		
	3 months	109 131 87 130	234 196 257 196 366 376	1303 948 1058 915 1155 1270	1292 1184 1236 1250		
Mean ± SEM % control*		114 ± 10 5.0%	271 ± 33 11.8%	1108 ± 66 48.4%	1240 ± 22 54.2%		
	6 months	131 135 69 101	185 183 161 167 196 157	404 980 619 588	906 987 885 848		
Mean ± SEM % control*		109 ± 15 4.8%	175 ± 6 7.6%	648 ± 120 28.3%	906 ± 29 39.6%		
	9 months		144 110	232 179 226 290			
Mean ± SEM % control*			127 ± 17 5.5%	232 ± 23 10.1%			
	12 months	70 65 64 71 93	141 183 221 55 57	423 349 531 564	436 375 505 514		
Mean ± SEM % control*		73 ± 5 3.2%	131 ± 33 5.7%	467 ± 49 20.4%	457 ± 33 20.0%		
	15 months			359 329			
Mean ± SEM % control*				344 ± 15 15.0%			
	20 months	93 85				21 months	2193 2112 2274 2273
Mean ± SEM % control*		89 ± 4 3.9%					2213 ± 39

* From (Vidal-Sanz et al., 1988).

Between time zero (20 days after dil application) and 21 months after dil application, retinal areas increased 14% in control retinas (Table IV, Figure 4). The experimental (axotomized) retinas also increased in size by approximately 14% from 15 days to twelve months after ON section (Table IV, Figure 4). Thus, the interruption of the ON did not affect the increases in retinal area.

3.4. DII-LABELLED CELL DENSITIES AFTER OPTIC NERVE INTERRUPTION

3.4.1. CELL LOSS IN THE FIRST FIFTEEN DAYS AFTER ON INTERRUPTION

Fifteen days after interruption of the ON, dil-labelled cell densities were significantly lower than control values in all groups. Mean densities at this time period were 566, 880, 1313, and 1500 cells/mm^2, after ON interruption at 0.5, 3, 8, and 10 mm from the eye, respectively (Table I). Thus, the initial RGC loss was related to the proximity of the axotomy to the RGC somas in the retina, being greater with more proximal lesions.

3.4.2. CELL LOSS UP TO FIFTEEN MONTHS AFTER ON INTERRUPTION

Cell loss in the experimental retinas examined for 12 or 15 months after ON interruption followed different patterns depending on the distance of axotomy from the eye. However, in all retinas, there was an early rapid decrease in cell densities followed by a more protracted decrease that continued for the entire period studied (Figures 5,6).

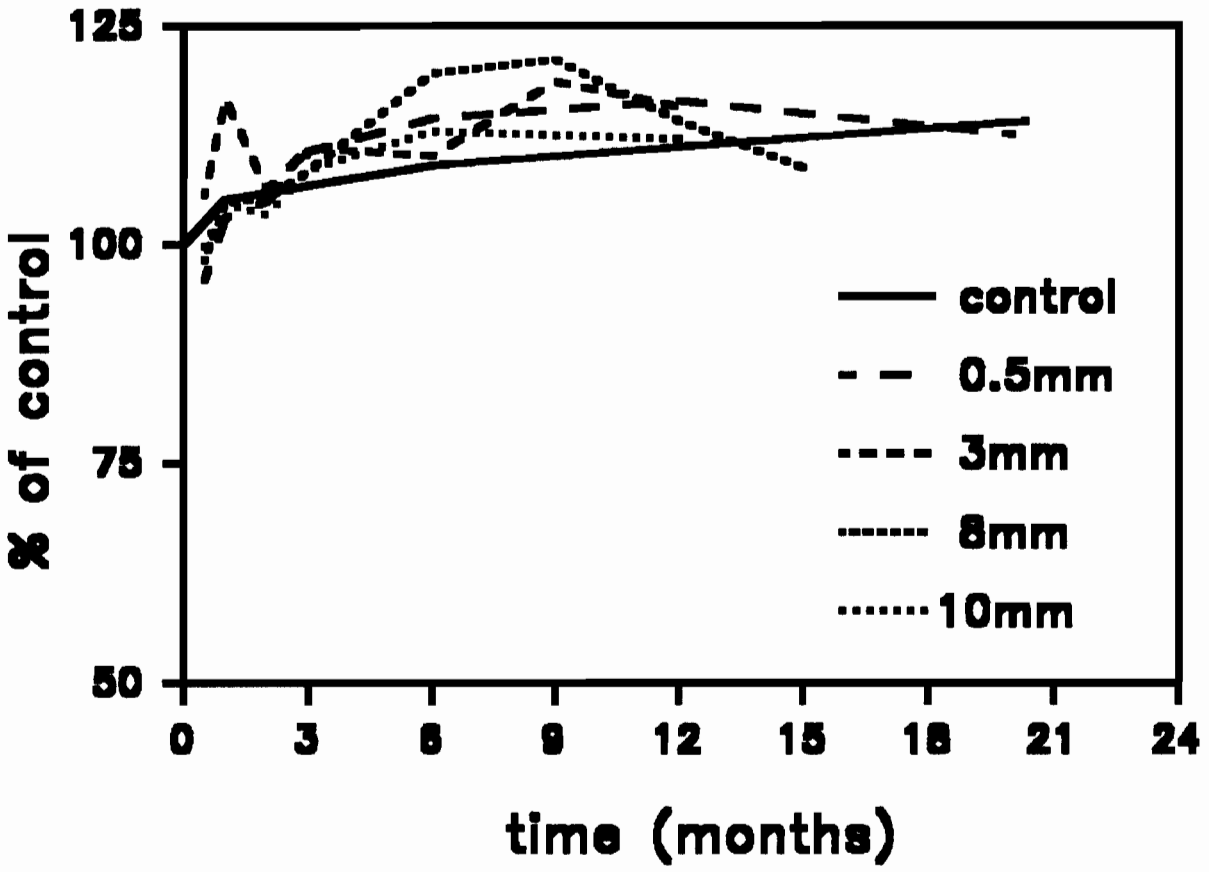


Figure 4. *Proportional increases in area of control and experimental retinas during the period of study. The mean areas at each time analyzed for each of the four experimental and control groups have been joined.*

Early rapid decrease of RGC densities. There was an early rapid decrease in RGC densities in all groups that was more severe the closer the ON lesion was to the eye (Figure 6). With lesions at 0.5 or 3 mm, this rapid decrease of RGC loss could be observed up to 3 months while, with more distal (8 or 10 mm) lesions, RGC losses after 15 days were less marked and appeared to proceed at the slower rate seen for all groups 3 months or more after axotomy (Figure 6).

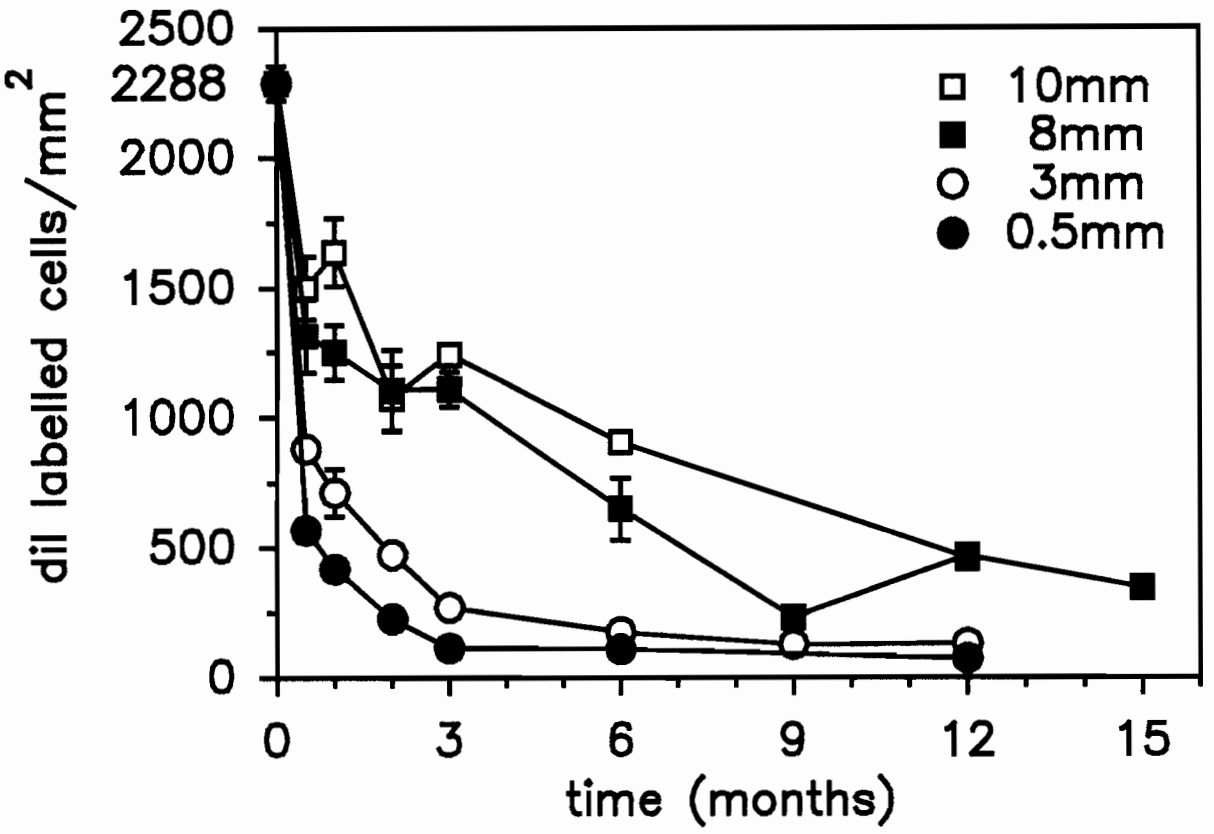


Figure 5. *Densities of dil-labelled cells in the retinas of adult rats examined at different intervals after interruption of the optic nerve. The data points represent the mean number of cells per mm² for groups of 3 to 8 retinas. The bars indicating the standard error of the mean are only shown if greater than the data points. The mean density (2288 ± 66 cells/mm²) of labelled cells in control retinas 20 days after dil application is depicted at time zero. Mean densities in the experimental retinas are illustrated from 15 days to 15 months after the lesion of the optic nerve.*

Later protracted decrease of RGC densities. After the early rapid decrease in RGC densities, there was a more gradual decrease in the densities of dil-labelled cells that continued throughout the period of study in all four groups of retinas (Figure 6).

Although the data did not permit the application of a mathematical model for the analysis of RGC loss in experimental retinas throughout the entire period of study, RGCs densities for the period 3 to 12 or 15 months after axotomy were plotted

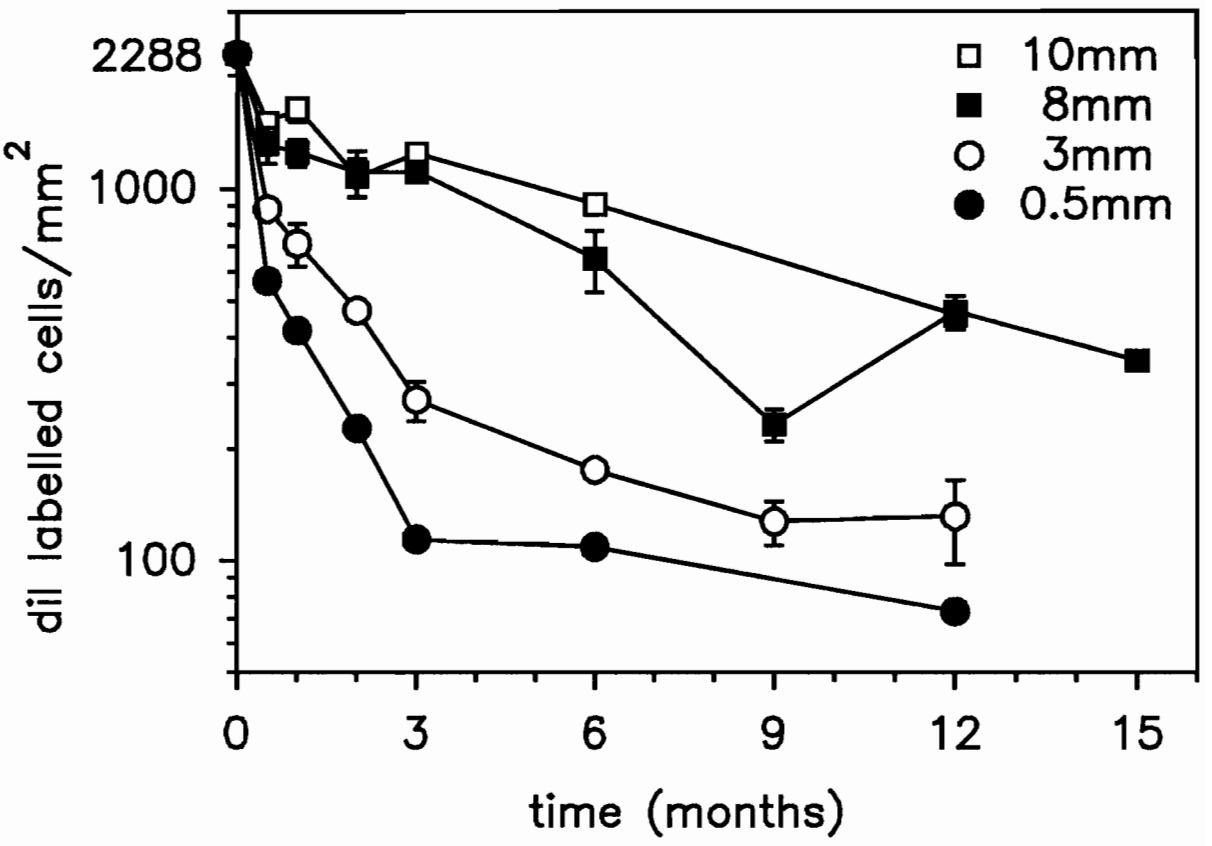


Figure 6. *Mean densities of dil-labelled cells per mm² ± SEM represented on a semilogarithmic scale.*

against time on a semilogarithmic scale (Figure 6) and the rates of cell loss estimated using regression lines (Figures 6, 7).

Regression lines relating RGC density to time between 3 and 12 or 15 months after axotomy (Figures 6,7, Table II) were statistically significant (analysis of variance, $p < 0.01$) for the groups of animals with ON lesions at 0.5, 3, or 10 mm; for the group of animals with axotomy 8 mm from the eye, a significant regression with time could also be demonstrated if the data for 9 months were excluded. For the animals with the most distal axotomies (3, 8, and 10 mm), the calculated one-half survival times for this late period ranged from 6.2 - 7.6 months; for the animals with ON section at 0.5 mm,

the calculated one-half survival time was 13.2 months (Table II). However, the slopes of the regression lines for these four groups (Table II; Figure 7) were not statistically different when compared using the general linear model of the SAS Program ($p > 0.1$), suggesting similar rates of cell loss beyond 3 months for all groups.

TABLE II
LINEAR REGRESSION ANALYSIS OF diI-LABELLED CELL DENSITIES

Level of Optic Nerve Interruption / Time after Axotomy (months)	Linear Regression Parameters			
	r	Significance ANOVA p <	Slope	One-half Survival Time (months)
0.5 mm				
0.5 - 3	0.96	0.0005	- 0.28	1.1
3 - 12	0.71	0.01	- 0.02	13.2
3 - 20	0.54	0.05	- 0.01	28.2
3 mm				
0.5 - 3	0.90	0.0005	- 0.21	1.5
3 - 12	0.69	0.0025	- 0.04	7.4
8 mm				
0.5 - 3	0.32	NS	-	-
0.5 - 15	0.82	0.0005**	- 0.05	6.1
0.5 - 15*	0.89	0.0005	- 0.04	7.5
3 - 15	0.71	0.0001**	- 0.05	6.6
3 - 15*	0.68	0.0005	- 0.04	7.6
10 mm				
0.5 - 3	0.49	0.05	- 0.05	5.6
0.5 - 15	0.91	0.0005	- 0.05	6.7
3 - 12	0.98	0.0005	- 0.05	6.2

* regression line calculated without the data for the nine month period.
** significant deviations from the regression line.

Because the data for the groups of animals with ON lesion at 8 mm from the eye showed significant deviations from the regression line (Table II; Sokal and Rolf, 1981), we also compared the slopes of the four regression lines when the data for 9 months or 15 months was excluded from the 8 mm group, and found that the slopes were still not significantly different. However, it is possible that subtle differences would have become apparent if the number of observations in each group had been larger.

TABLE III
RATIOS OF DiI-LABELLED CELL DENSITIES AT DIFFERENT DISTANCES FROM THE DISC

RATIO A/B	EXPERIMENTAL RETINAS					CONTROL RETINAS	
	Time After Optic Nerve Interruption	Level of Optic Nerve Interruption				Time After dil Application	
		0.5 mm	3 mm	8 mm	10 mm		
	15 days	1.50	1.23	1.59	1.38	20 days[a]	1.35
		1.98	1.46	1.57	1.51		1.48
		2.17	1.26	1.46	1.25		1.26
		1.86	1.22	1.66	1.48		1.49
Mean ± SEM		1.88 ± 0.14	1.29 ± 0.06	1.57 ± 0.04	1.40 ± 0.06		1.40 ± 0.05
	1 month	1.87	1.30	1.39	1.28		
		1.36	1.70	1.39	1.26		
		1.39	1.42	1.25	1.22		
		1.44	1.29	1.43	1.31		
		1.56	1.25	1.44	1.30		
		1.71	0.97	1.19	1.25		
		1.33					
		1.24					
Mean ± SEM		1.49 ± 0.07	1.32 ± 0.10	1.35 ± 0.04	1.27 ± 0.01		
	2 months	1.55	1.15	1.36	1.36		
		1.30	1.21	1.23	1.32		
		0.77	1.29	0.94	1.15		
		1.55		1.10	1.23		
		1.25					
Mean ± SEM		1.28 ± 0.14	1.22 ± 0.04	1.16 ± 0.09	1.26 ± 0.05		
	3 months	0.51	0.84	1.17	0.98		
		1.07	0.71	1.12	1.10		
		0.65	0.78	1.14	1.23		
		0.81	1.18	1.36	1.09		
			0.97	1.32			
			1.25	1.33			
Mean ± SEM		0.76 ± 0.12*	0.95 ± 0.09*	1.24 ± 0.04	1.10 ± 0.05*		
	6 months	0.80	0.65	1.04	1.11		
		1.07	0.63	1.19	1.04		
		1.18	1.10	1.24	1.50		
		0.82	0.56	1.11	1.15		
			0.73				
			0.94				
Mean ± SEM		0.97 ± 0.09*	0.77 ± 0.09*	1.14 ± 0.04	1.20 ± 0.10		
	12 months	0.96	0.81	1.08	0.85		
		0.77	1.12	0.80	0.93		
		0.36	1.39	0.98	0.94		
		1.51	0.17	1.03	1.01		
		0.54	0.74				
Mean ± SEM		0.83 ± 0.20*	0.85 ± 0.21	0.97 ± 0.06*	0.93 ± 0.03*		
	15 months			1.19		21 months	1.45
				0.96			1.35
							1.84
							1.38
Mean ± SEM				1.07 ± 0.11			1.50 ± 0.11

[a] From Vidal-Sanz et al., 1988
A cells/mm² at 1mm from the optic disc
B cells/mm² at 3mm from the optic disc
* significantly less than control, $p < 0.05$

TABLE IV
AREAS OF EXPERIMENTAL AND CONTROL RETINAS

AREA mm^2	EXPERIMENTAL RETINAS[a]				CONTROL RETINAS	
	Time After Optic Nerve Interruption	Level of Optic Nerve Interruption			Time After dil Application	
		0.5 mm	3 mm	8 mm	10 mm	
	15 days	54.8	--	57.3	57.8	20 days[b]	57.4
		56.7	61.3	--	57.3		56.7
		--	61.2	55.4	57.2		58.0
		54.3	60.1	57.1	--		57.0
							58.7
Mean ± SEM		55.3 ± 0.7	60.9 ± 0.4	56.6 ± 0.6	57.4 ± 0.2		57.6 ± 0.3
% control[b]		96%	105.7%	98.3%	99.8%		100%
	1 month	59.3	--	--	59.8	1 month[c]	61.1
		--	65.6	56.9	58.6		67.9
		60.9	--	62.2	61.7		57.8
		--	--	63.9	61.1		49.4
		58.4	67.3	--	--		65.7
		58.4	68.7	58.6			61.3
		--					
		59.9					
Mean ± SEM		59.4 ± 0.5	67.2 ± 0.9	60.4 ± 1.6	60.3 ± 0.7		60.5 ± 2.6
% control[b]		103.1%	116.7%	104.8%	104.7%		105.2%
	2 months	57.4	59.8	59.6	55.2		
		59.4	--	61.2	57.8		
		--	62.4	60.0	60.5		
		62.7		61.7	61.9		
		62.4					
Mean ± SEM		60.4 ± 1.3	61.1 ± 1.3	60.6 ± 0.5	59.5 ± 1.1		
% control[b]		105%	106.2%	105.3%	103.4%		
	3 months	--	61.1	--	--		
		66.2	61.3	--	62.7		
		61.9	66.0	59.2	--		
		63.0	66.5	60.8	62.4		
			64.5	65.4			
			64.2	64.5			
Mean ± SEM		63.7 ± 1.3	64.0 ± 0.9	62.5 ± 1.5	62.5 ± 0.1		
% control[b]		110.7%	111.1%	108.5%	108.6%		
	6 months	68.6	--	65.2	62.7	6 months[c]	61.7
		66.3	63.9	66.7	64.3		63.8
		63.9	63.4	71.1	65.6		63.0
		64.7	--	72.4	67.6		
			63.0				
			63.5				
Mean ± SEM		65.9 ± 1	63.5 ± 0.2	68.8 ± 1.7	65 ± 1.2		62.8 ± 0.6
% control[b]		114.5%	110.2%	119.6%	113%		109.1%
	9 months		68.5	70.2			
			68.2	69.4			
				--			
				69.7			
Mean ± SEM			68.3 ± 0.1	69.8 ± 0.2			
% control[b]			118.7%	121.2%			
	12 months	--	63.7	64.9	67.3		
		65.9	69.5	63.8	66.6		
		62.4	63.7	66.9	61.0		
		70.9	70.2	67.3	63.3		
		69.2	66.3				
Mean ± SEM		67.1 ± 1.9	66.7 ± 1.4	65.7 ± 0.8	64.5 ± 1.5		
% control[b]		116.5%	115.8%	114.2%	112.1%		
	15 months			62.7		21 months[a]	65.9
				62.6			66.1
							66.7
							63.9
Mean ± SEM				62.6 ± 0.2			65.7 ± 0.6
% control[b]				108.8%			114%

[a] Areas of the retinas listed in Table I.
[b] From Vidal-Sanz et al., 1988; Addendum II.
[c] Contralateral to axotomized retinas of the experimental group.
-- Retinas incomplete.

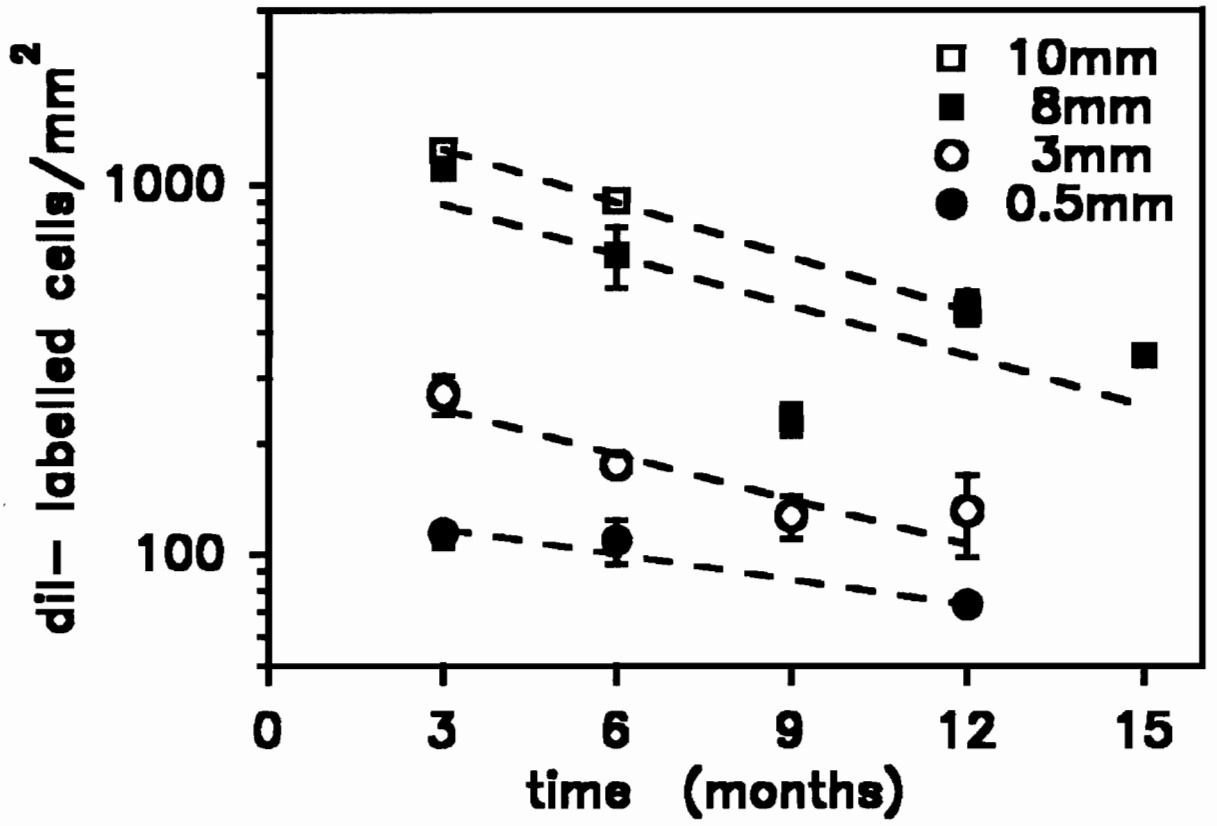


Figure 7. *Regression lines relating log of dil-labelled cell densities to time in the four experimental groups with ON interruption at different distances from the eye. The regression lines, which were calculated using densities for individual experimental retinals processed from 3 to 15 months after axotomy, represent the rates of dil labelled cell loss for during this period of time. The calculated half-survival time for lesions at 10, 8, 3 or 0.5 mm from the eye was 6.3, 6.1, 7.4 and 13.2 months, respectively. Means ± SEM for groups of retinas at each period are also plotted.*

3.4.3. THE LATE PATTERN OF CELL LOSS IN THE OD GROUP

The slopes of the regression lines that relate dil-labelled cell densities to time for the four experimental groups from 3 to 12 or 15 months were not significantly different statistically (see above). However, the one-half survival time for this later more protracted decrease of cell densities in the four groups varied from 6.2 to 13.2 months

(Table II). The one-half survival time for the group with ON section at 0.5 mm was 13.2 months, almost twice the one-half survival time observed for the other groups (6.2 to 7.6; Table II). For the group with ON section at 0.5 mm, we extended the period of study up to 20 months. The densities of dil-labelled cells in three retinas of this group that were analyzed 17 and 20 months after ON section were 71 cells/mm2 17 months after ON section and 93 and 85 cells/mm2 20 months after ON section (Table I). The regression line calculated for this experimental group including all the cell densities obtained from 3 to 20 months after ON section was significant, and indicated a one-half survival time of 28.2 months for the RGCs remaining in these retinas (Table II). However if the regression line was calculated with the densities obtained between 6 and 20 months after axotomy, the slope was not significantly different from zero (data not shown), suggesting that RGC numbers do not decrease during this time period. These observations indicate that there might be a small proportion (3-4%) of RGCs in the rat retina that remains without suffering further losses from 6 to 20 months after ON section near the eye. Alternatively, it could be that the numbers of animals analyzed six months or more after ON section at 0.5 mm are too small and have large variances that do not permit the documentation of small RGC losses.

3.4.4. RGC DENSITIES AT DIFFERENT DISTANCES FROM THE OPTIC DISC

To determine if RGC loss is more marked near the optic disc than in the more distal retinal regions, as a secondary effect of the different distances of axotomy to the RGC somas in the retinas, we calculated the ratio of the mean RGC densities at 1

and 3 mm from the optic disc of each experimental and control retina (Table III). For control retinas, this ratio was 1.39 ± 0.55 twenty days after dil-application and 1.50 ± 0.11 twenty-one months after dil application, a difference that was not statistically significant (two sided Student's t test). In experimental retinas, the ratios diminished with time after ON lesion. Fifteen days after axotomy, the ratios of the experimental retinas were already smaller than the ratios obtained in control retinas 20 days after dil application, but this difference was statistically significant only for the 0.5 and 8 mm groups. Furthermore, the ratios of the retinas examined 12 months after ON lesion for each of the four experimental groups were significantly smaller than the ratios obtained for the same groups 15 days after axotomy, except for the 3 mm group, where the difference was significant only when a one-sided t-test was applied. The decrease in the ratios of the experimental animals with time after ON interruption indicates greater RGC losses in the region close to the optic disc (Table III).

3.5. INTERPRETATION OF RESULTS

3.5.1. VALIDATION OF COUNTING METHOD

The use of retrogradely-transported dil to quantitate neuron survival assumes that the loss of the fluorescent marker is due to cell death. To validate the counting method used for these experiments, RGC densities, determined by dil labelling, can be compared with other methods of estimating RGC populations.

3.5.1.1. DiI-labelling versus Fast blue labelling

A previous study (Vidal-Sanz, Villegas-Pérez et al., 1988; Appendix II) investigated dil persistence within the RGC cytoplasm by comparing the numbers of neurons in the retina that were labelled with dil applied to the SC and dLGN for different periods of time to the numbers of neurons that were also doublely labelled with Fast Blue applied intraorbitally to the ON two days before processing. The proportion of cells labelled with dil that were also labelled with Fast Blue (applied to the optic nerve for 2 days) was between 93 and 96% for control animals for periods up to 9 months, and between 78 and 92% for animals with intracranial ON lesions for up to 3 months (Vidal-Sanz, Villegas-Pérez et al., 1988; Appendix II).

3.5.1.2. Methylene blue staining

Because in the present study dil-labelling was used to quantitate RGC survival for up to 20 months after ON interruption, we have relied on dil persistence within the RGC cytoplasm for longer periods of time after ON lesion than in the previous study. To validate this assumption, RGC densities determined by dil labelling were compared with the densities of methylene blue-stained neurons in the ganglion cell layer of the same retinas from 15 days to 12 months after axotomy. There was close correlation between the densities of dil-labelled cells and methylene blue stained cells in these retinas, the numbers of methylene blue labelled cells falling in linear relationship to the loss of dil cells (Table V, Figure 8). The correlation between these two methods of labelling was statistically significant ($p < 0.001$, $r = 0.96$; Table V; Figure 8).

TABLE V
COMPARISON OF DiI AND METHYLENE BLUE LABELLING

Time After Optic Nerve Section	cells/mm²	
	DiI	Methylene Blue
15 days	1641 1769	5035 5283
Mean % of Control*	1705 75%	5159 92%
1 month	1738 1038	4738 4121
Mean % of Control*	1388 61%	4429 79%
2 months	852 937	3874 3751
Mean % of Control*	894 39%	3812 68%
3 months	1250 1236	4337 4342
Mean % of Control*	1243 54%	4339 77%
6 months	848 885	3460 3995
Mean % of Control*	866 38%	3707 66%
12 months	436 375	3405 3079
Mean % of Control*	405 18%	3242 58%

* Control values (Mean ± SEM):
Methylene blue: 5601 ± 365 (Villegas-Pérez et al., 1988; Addendum I)
DiI : 2288 ± 66 (Vidal-Sanz et al., 1988; Addendum II)

The mean decreases in cell densities with these two methods of labelling were different, however. From 15 days to 12 months after interruption of the ON, mean methylene blue-labelled cell densities decreased in approximately 1900 cells/mm² (from 5159 to 3242, Table V), the mean dil-labelled cell densities decreasing only 1300 cells/mm² (from 1705 to 405, Table V). This difference in cell loss between the two methods can be explained in two ways: i) cell densities are higher in methylene

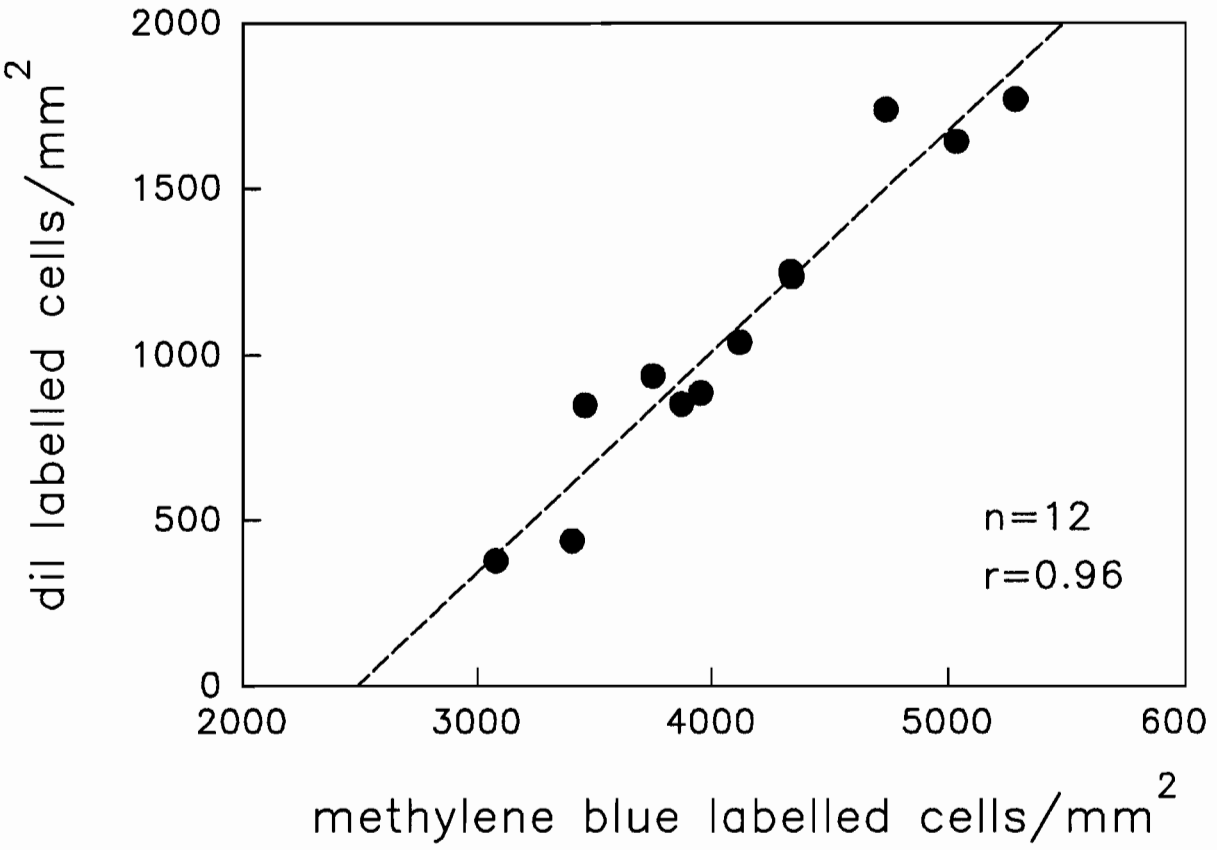


Figure 8. *Correlation between RGC densities determined by retrograde labelling with dil and by methylene blue staining. Densities were compated in two retinas each at 0.5, 1, 2, 3, 6 and 12 months after the ON was injured, approximately 10 mm from the eye. the correlation coefficient for the counts in these 12 retinas is 0.96, significant at p< 0.001. The slope of the regression line is 0.66.*

blue stained retinas, because these retinas were subjected to dehydration; and ii) cells other than dil-labelled RGCs in the RGC layer were also decreasing in numbers during the period of study: these cells could be RGCs that were not dil-labelled or microglia, whose numbers in the mammalian retina have been reported to increase shortly after ON section (Sievers et al., 1987; Schnitzer and Scherer, 1990), and to decrease thereafter (Schnitzer and Scherer, 1990).

3.5.2. RELATIONSHIP BETWEEN CELL DENSITIES AND RETINAL AREAS IN EXPERIMENTAL AND CONTROL RETINAS

The retinal areas of control and experimental retinas were measured to determine if the decreases in cell densities could be due to an increase in retinal area with age.

In control retinas, there was an increase of approximately 9% of the total retinal area between 1 and 21 months after dil-application. This increase in area could account for the approximately 9% decrease in dil-labelled cell densities observed between 1 and 21 months after dil application (see above, section 3.2. in this chapter).

In experimental retinas, there was also an increase in retinal areas with time. This increase accounted between 8.5 and 11% of the area during the first three months after ON interruption, and between 3.5 and 6% during the 3 to 12 months period of the study (Table IV). These increases in area are too small to explain the decreases in dil-labelled cell densities observed in experimental retinas, and could only be in part responsible for the small decreases in densities observed in the period from 3 to 12 months for the optic disc experimental group (Table I).

3.6. SUMMARY OF RESULTS

We describe that the pattern of RGC loss after four different levels of ON interruption between the eye and the chiasm differs with the distance of axotomy from the eye. There is an early rapid decrease in cell densities whose severity depends on the distance of axotomy to the eye and a later, more protracted decrease of cell

densities that proceeds at a similar rate for all distances of ON interruption. Increases in retinal areas in experimental retinas and decreases in RGC densities with the age of the animal have been studied and cannot explain the rates of cell death. A small percentage (3-4%) of the total RGC population remains in the retina from 6 to 20 months after ON section close to the eye. A significant correlation between the numbers of dil-labelled and methylene blue stained cells in a group of experimental retinas documents that dil can be reliably used in cell death studies.

CHAPTER 4. DISCUSSION

The study of neuronal survival after axotomy requires the use of reliable methods for the identification and quantification of the axotomized neurons. Because axotomy can lead to changes in cell morphology and size (reviewed by: Lieberman, 1971, 1974; Barron, 1983a,b, 1989) and neurotransmitter phenotype (Lams et al., 1988; Henken et al., 1990; Dumoulin et al., 1991), methods that rely on morphology or cell size or transmitter immunocytochemistry for purposes of neuronal identification may not accurately estimate the amount of cell death. In the present studies, RGCs were retrogradely labelled with dil from their targets prior to ON interruption. Such labelling has permitted a quantitative study of the effects of distance of axotomy on RGC survival. The results of the experiments presented in this thesis document different patterns of RGC loss after ON interruption. Early after the lesion there is a rapid decline in RGC densities whose severity is related to the distance of axotomy. Later, a more protracted decline in cell densities, whose rate is similar for all levels of ON interruption, is observed for the rest of the period of study (up to 12 or 15 months). In addition, there may be a small population of RGCs in the retina that do not degenerate after ON interruption close to the eye that accounts for 3 to 4% of the total RGC population.

4.1. RGC LOSS AFTER ON INTERRUPTION

Earlier studies had documented RGC loss after different types of ON lesions in several mammalian species (James, 1938; Quigley, 1977; Muchnick-Miller and Oberdorfer, 1981; Grafstein and Ingoglia, 1982; Alcutt et al., 1984; Misantone et al., 1984; Barron et al., 1985, 1986). Other studies had also shown that the distance of axotomy to the RGC soma influenced the amount of RGC death (Leinfelder, 1938; Mantz and Klein, 1951; Radius and Anderson, 1978). The present study documents that the axotomy level of ON interruption not only influences the amount of cell loss but also the pattern of the decreases in RGC densities. Thus, after ON interruption at four different levels between the eye and the optic chiasm, there is an early rapid loss of RGCs and a later more protracted loss that continues for up to 12 or 15 months. The severity of the early loss was greater in the animals with ON interruption closest to the eye (intraorbital), while the rates of the later, more protracted loss were similar for all levels of ON lesion.

Although the pattern of cell loss is different in the various experimental groups, the action(s) of a single agent might be causing these different pattern of cell loss. For example, neuronal death after ischemic injury to the brain *in vivo* takes place in two steps: immediate and delayed (reviewed by: Choi, 1988a, 1990; Choi and Rothman, 1990), and *in vitro* the toxicity of a glutamate receptor antagonist, AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) causes three forms of neuronal death: early and rapid, delayed protracted and late slow (Garthwaite and Garthwaite, 1991a). Only the delayed form of neuronal death observed after ischemic injury to the brain is dependent on protein synthesis (Goto et al., 1990; Shigeno et al., 1990) and

can be prevented by administration of NMDA receptor antagonists (reviewed by: Siesjö et al., 1989; Choi, 1990; Choi and Rothman, 1990). The three forms of cell death described after AMPA toxicity have been shown to depend on the type of neuron and the length of the exposure to AMPA (Garthwaite and Garthwaite, 1991a). Different mechanisms may be involved in each of these three forms (Garthwaite and Garthwaite, 1991b). Since the same agents or molecules may cause different patterns of neuronal death acting through different mechanisms, it is thus possible that the different patterns of RGC loss observed in this study may be caused by a same agent acting with different intensities or through different mechanisms.

4.1.1. EARLY RAPID RGC LOSSES

The amount of cell death during the early rapid decrease of RGC densities is related to the distance of axotomy from the eye. Lesions close to the eye cause greater RGC losses than more distal lesions in same time period, as judged by comparing cell densities in axotomized retinas 15 days after ON interruption (Table I). In the present experiments the intraorbital lesions (0.5 and 3 mm from the eye) were produced by ON transection with scissors while, for technical reasons, the intracranial lesions (8 and 10 mm) were produced by repeated crushes with fine forceps. Thus, because preliminary studies suggest that the extent of the retrograde axonal degeneration is greater after intracranial ON cut than crush lesions (Berkelaar et al., 1991), it is possible that the type of lesion, as well as the distance of axotomy from the cell body, may have influenced the rate of early RGC loss. However, 15 days after ON section at 0.5 or 3 mm from the eye, RGC densities were significantly

greater in the 3 mm group (Table I). Thus, although the type of lesion may have influenced RGC survival, same type of ON lesions also cause different RGC losses when applied at different distances from the eye.

In addition, this early phase is also longer-lasting after more proximal lesions (Figure 6). Results similar to the obtained here were reported by Liu (1955) in the cat Clarke's nucleus after two different levels of spinocerebellar tract section. Liu (1955) documented that the lesions close to the neuronal cell body were followed by a sharp decline in neuronal density in this nucleus that lasted up to 70 days, while after more distal lesions neuronal cell densities decreased less markedly and only during the first month. Liu (1955) suggested that the differences in cell loss observed in his experiments could be due to the preservation of axonal collaterals by more distal lesions. Because in the experiments reported here the axons are lesioned before giving off collaterals, the severity of the early RGC loss cannot be dependent on such a mechanism.

The differences in the severity of the early decreases in RGC densities cannot be explained solely on the basis of deprivation of trophic factors from their targets (Rich et al., 1987; reviewed by Oppenheim, 1991), since all types of ON lesions eliminate RGC contact with their targets. Because the amount of early cell loss is related to the distance of axotomy from the eye, it is possible that it may be influenced by the characteristics of the traumatic injury to the axon. Thus, ON interruptions close to the neuronal somata may cause more damage to these cells by depriving them of larger proportions of axonal membrane and cytoplasm. The removal of larger axonal segments might mean that events such as calcium influx and depolarization

(Strautman et al., 1990), which occur in a graded way from the proximal tip of the axon after axotomy, are more likely to affect the cell somata when the lesion is more proximal. Alternatively, it could be that the differences observed in the duration and severity of RGC losses with different distances of ON interruption may be related to the length of the optic nerve stump, whose non-neuronal population could be protecting the RGCs from retrograde degeneration by means of, for example, the release of neurotrophic substances. In fact, after an injury to the brain, it has been documented that NGF (Ishikawa et al., 1991), acidic FGF (aFGF; Ishikawa et al., 1991), and other trophic substances (Nieto-Sampedro et al., 1983; Gage et al., 1984) are released in the wound cavity. Macrophages migrate into the wound, and they have been shown to secrete a molecule similar to EGF (Hygashiyama et al., 1991), bFGF (Baird et al., 1985b) and Interleukin-1. After peripheral nerve section, Interleukin-1 secreted by macrophages upregulates the expression of NGF mRNA by Schwann cells (Lindholm et al., 1987). Furthermore, activated microglia secrete NGF *in vitro* (Mallat et al., 1989). Finally, injury to the ON induces transient NGF gene expression in the nerve during the first six postoperative days (Lu et al., 1991). Although it has not been determined if there is BDNF mRNA expression in the normal or injured ON, it has been reported that there is increased expression of BDNF in the distal stump of lesioned peripheral nerves (Meyer et al., 1990). This growth factor may be more relevant for the survival of RGCs (Johnson et al., 1986; Thanos et al., 1989; Hofer et al., 1990; see below).

Recent experiments have suggested that RGC death after ON interruption may not ensue immediately after the injury and that many RGCs continue to express mRNA

for several cytoskeletal proteins for a period of several days after axotomy (McKerracher et al., 1991). These observations suggest that the initiation of the early decreases of RGC densities may not be immediate, as expected for example after a traumatic injury to a cell. Other axotomized central neurons, the rubrospinal neurons have also been reported to continue the synthesis of the medium and light molecular weight neurofilament proteins and to increase the synthesis of actin and tubulin shortly after axotomy (Tetzlaff et al., 1991). However, increased synthesis was not maintained 14 days or more after axotomy, a time when the rubrospinal neurons have been documented to atrophy (Tetzlaff et al., 1991). All these facts suggest that protein synthesis is maintained in axotomized central neurons and that the events that lead to cell atrophy or death may not occur during the first days after axotomy. Thus, RGC death after axotomy may be a delayed event, perhaps similar to that observed in cultures of sympathetic neurons after trophic factor removal (Martin et al., 1988), after ischemic injury to the brain (reviewed by: Choi 1990; Choi and Rothman, 1990) or after neuronal exposure to AMPA (Garthwaite and Garthwaite, 1991a,b). Furthermore, because RGCs continue protein synthesis after axotomy, it is possible that these cells could die because of the expression of "killer" genes, as it has been documented for other types of neurons during development (Driscoll and Chalfie, 1991), after trophic factor deprivation (Martin et al., 1989) or after a ischemic injury to the brain (Goto et al., 1990; Shigeno et al., 1990).

Although the morphology of RGC death has not been examined in the present study, Barron et al. (1986) analyzed ultrastructurally the ganglion cell layer of the adult rat retina after intraorbital ON crush and reported that RGC nuclei showed chromatin

condensation. The form of cell death known as apoptosis is characterized by condensation of the nucleus and the cytoplasm. Furthermore, apoptosis has been reported to be the form of cell death during the development of the rodent retina (García-Porrero and Ojeda, 1979; Young, 1984; Penfold and Provis, 1986; Harvey et al., 1990), and sometimes in the delayed form of cell death observed after trophic factor deprivation (Martin et al., 1989) or after excitotoxicity (Garthwaite and Garthwaite, 1991). Cell death caused by apoptosis requires mRNA and protein synthesis (Walker et al., 1988; Bursch et al., 1990). However, whether RGC death after axotomy takes the form of apoptosis remains to be investigated.

4.1.2. LATER, MORE PROTRACTED RGC LOSS

The rate of neuronal loss during the more protracted decline of RGC densities, as judged by the comparison of the slopes of the regression lines obtained for the period between 3 and 15 months after ON interruption, was similar for all groups and apparently independent both of the distance of axotomy and the type of lesion applied to the nerve. Because the common characteristic of all experimental groups is the disconnection of RGCs from their main targets, it is tempting to speculate that this later, more protracted cell loss is due to the lack of putative influences that could arise from the contact with their targets.

The dependency of adult RGCs on their targets for survival was suggested in experiments in which the removal of the RGC targets or of the striate cortex causes the retrograde transneuronal degeneration of RGCs. Such retrograde transneuronal degeneration of RGC after visual cortex removal (reviewed by: Cowan, 1970) has

been documented in various mammals: humans (Van Buren, 1963a), primates (Van Buren, 1963b; Weller et al., 1979), and cats (Pearson et al., 1981; Tong et al., 1982; Kalil, 1990). However, the removal of the visual cortex, and even the SC (target of more than 95% of rat RGCs, Linden and Perry, 1983) in the adult rat does not cause appreciable loss of RGCs (Perry and Cowey, 1979). In contrast, the removal of these territories together or separately, as well as the injection of kainic acid in the SC, causes marked RGC death in neonatal rats (Perry and Cowey, 1979; Carpenter et al., 1986). It is possible that rat RGCs depend for their survival on their targets more strictly during development than in adulthood, as it has been reported for other neurons (e.g. DRG neurons; Lindsay, 1988). Alternatively it is possible that these cells, injured near their terminals and deprived of their target neurons, may establish contact with other neuronal populations (Campbell and Frost, 1988; Zwimpfer, 1989, 1990).

Studies of RGC survival and regeneration using peripheral nerve grafts transplanted to the adult rat ON have suggested a dependency of adult rat RGCs on their targets for their long-term survival. For instance, when the proximal end of the graft was attached to the eye and the other end of the graft was left unconnected, there was a decline with time in the populations of adult rat RGCs that regenerated their axons along the grafts, as judged by the numbers of axons recorded electrophysiologically from these PN-grafts (Keirstead et al., 1985) and in the numbers of dil-labelled RGCs that survived in these retinas (Villegas-Pérez et al., 1988). However, when these peripheral nerve segments were placed as "bridges" between the retina and the SC, RGCs regenerated their axons along the PN graft, arborized and established synaptic

contacts within the SC (Vidal-Sanz et al., 1987; Carter et al., 1991), and there was no indication for a decline in the number of terminal arborizations or synaptic contacts during long-term studies (up to 10 months in hamsters; Carter, 1991; and up to 18 months in rats; Vidal-Sanz et al., 1991).

Identification of the molecule(s) present in the RGC targets whose loss causes these neurons to die at the same rate regardless of the level of ON interruption requires further studies. The SC, target of more than 95% of RGCs, contains mRNA for BDNF (Hofer et al., 1990) and for bFGF (Enfors et al., 1990c). In addition, RGCs have been shown to be responsive to BDNF (see below) but also to various other factors. Neonatal rat RGCs survive in greater quantities when co-cultivated with their target territories (McCaffery et al., 1982; Armson and Bennett, 1983) or when BDNF is added to the culture (Johnson et al., 1986). The survival of adult rat RGCs *in vitro* is enhanced when these cells are cocultivated with activated Schwann cells (Baehr and Bunge, 1989) or in the presence of an extract from lesioned peripheral nerve, or BDNF (Thanos et al., 1989) or bFGF (Bähr et al., 1989). Furthermore, it has been reported that *in vivo* adult rat RGCs can survive in greater amounts after axotomy when: i) their embryonic targets are transplanted to the sectioned ON (Sievers et al., 1989); ii) suspensions of Schwann cells are injected into the vitreous chamber of the eye (Maffei et al., 1990); iii) peripheral nerve grafts are placed in contact with their lesioned axons (Madison et al., 1984; Berry et al., 1986; Turner et al., 1987; Villegas-Pérez et al., 1988); iv) acidic or basic FGF are applied to the sectioned ON (Sievers et al., 1987), or; v) NGF is injected into the vitreous chamber (Carmignoto et al., 1989).

At present the mechanism(s) involved in the rescuing effect of the above mentioned experiments is not known. Basic FGF has been shown to be internalized and anterogradely transported by rat RGCs (Ferguson et al., 1990), but the effects of FGFs on RGC survival (Sievers et al., 1987) may be mediated through other non-neuronal cells (e.g. FGFs have been reported to induce NGF secretion by cultured astrocytes; Yoshida and Gage, 1991). BDNF and NT-3 at high concentrations can compete with NGF for the low-affinity NGF receptor (Rodríguez-Tébar et al., 1990; Enfors et al., 1990b). However, these neurotrophins (NGF and related molecules) may require for their actions of the presence of high affinity receptors in the neurons (Sonnenfeld and Ishii, 1985; Green et al., 1986). The high affinity receptor for NGF (Hempstead et al., 1991), BDNF and NT-3 (Squinto et al., 1991; Soppet et al., 1991) have been recently identified, but whether these receptors are expressed by developing, adult or injured rat RGCs it is still unknown.

4.2. A SMALL PROPORTION OF NEURONS RESISTANT TO AXOTOMY

Six to 20 months after ON section 0.5 mm from the eye, 3 to 4% of the total RGC population remains without further apparent losses. Small numbers of RGCs that survive for long periods of time after ON section have been documented in the retina of the rat and other mammals (James, 1933; Leinfelder, 1938; Eayrs, 1952; Polyak, 1958; Richardson et al., 1982; Misantone et al., 1984). It is tempting to speculate that these neurons represent a small subpopulation of RGCs that have different requirements for survival. It is also possible that these RGCs possess or establish

connections within the retina (Dacey, 1985) that protect them from the effects of axotomy.

4.3. IMPLICATIONS OF THIS MODEL FOR OTHER NEURONAL POPULATIONS

The later slow decreases of RGC densities after ON interruption may be similar to those observed for neurons whose axons have been lesioned in the peripheral nervous system when regeneration in the distal stump is prevented by resection of a segment of the nerve and ligation of the proximal stump. In this situation, the course of neuronal loss in the dorsal motor vagal nucleus of the rat (Laiwand et al., 1987), in the dorsal root ganglia of the rat (Devor et al., 1985), and in the spinal cord motoneurons of humans (Kawamura and Dyck, 1981) has been reported to proceed at a low speed. In the dorsal motor vagal nucleus, where linear regression analysis of the data in a semilogarithmic scale was similarly used, it has been calculated that the half life of the neurons is 8.6 months (Laiwand et al., 1987), only slightly greater than that observed here for RGCs. The slow course of neuronal loss in these situations might also be due to the loss of trophic influences present in the distal stump of the peripheral nerve and in the neuronal targets. Peripheral nerves are a source of neurotrophic factors, such as BDNF (Barker et al., 1990, Meyer, 1990), NGF (Heumann et al., 1987a,b), and CNTF (Manthorpe et al., 1986; Stöckli et al., 1989) that could influence, at least temporarily, the survival of adult neurons after axonal injury, as it has been documented for dorsal root ganglion cells (Otto et al., 1987; Rich et al., 1987). NGF is also present in the target territories of NGF responsive neurons during development and in adulthood (Korsching and Thoenen, 1983; Heumann et al.,

1984; Shelton and Reichardt, 1984; Bandtlow et al., 1987; Davies et al., 1987) and there is increased expression of the NGF gene in the target organs of sympathetic neurons when their terminals are destroyed with 6-hydroxydopamine or when axonal transport is blocked with colchicine (Korsching and Thoenen, 1985).

4.4. IMPLICATIONS OF THIS MODEL OF CELL DEATH AFTER AXOTOMY

Two main goals that need to be acomplished for the reconstruction of damaged neuronal circuitries are: i) to promote the survival of neurons after axotomy; and ii) to stimulate axonal growth and connectivity. The amount of RGC loss observed early after ON interruption in this study is related to the distance of axotomy to the RGC perikarya. Thus, to avoid the early severe cell losses, an important strategy might be to avoid axotomies close to the neuronal somata. However, it will also be important to fully understand why axonal regrowth (Aguayo, 1985; Richardson and Verge, 1986) and the expression of molecular correlates related to neuronal regeneration (Lozano, 1988; Tetzlaff et al., 1990; Doster et al., 1991) appears to be triggered only by axotomies close to the cell somata.

The documentation that the early loss of RGCs after ON section can be modified by various factors discussed above, indicates the plasticity of the dynamics of neuronal loss after injury. However, future studies aimed at increasing RGC survival after ON lesions may have to take into account the later, more protracted decrease of RGC densities documented in the present study that proceeds at a determined rate independently of the distance of axotomy. This later loss of RGCs may be caused by the disconnection of RGC from their targets and thus reflect a general model of

neuronal degeneration observed in situations such as loss of target neurons or axotomy. Axotomies distal to the neuronal somata may not cause a great amount of cell loss shortly after the lesion, but the induction of this protracted phase that, when allowed to act for long periods of time, can also result in substantial cell loss.

4.5. CONCLUSIONS

The experiments presented in this thesis analyze RGC survival on the basis of their labelling with dil between 2 weeks and 12-20 months after ON interruption at one of four different distances (approximately 0.5, 3, 8, and 10 mm) from the eye in adult rats. The results of this study support the following general conclusions:

1) Although the lesions at each of the four levels along the ON permanently disconnected all RGCs from their targets, the patterns of RGC loss varied with the lesion site, type of lesion, and time after axotomy.

2) After all levels of ON interruption, there was an early rapid and a later, more protracted decrease in cell densities.

3) The initial decreases of RGC densities varied with the location and/or type of lesion. With ON section near the eye, the initial decreases of RGC densities were greater and lasted longer than with the intracranial crush injuries.

4) The initial RGC losses were followed by later, more protracted decreases of RGC densities that continued for the period studied (12 to 15 months) in all groups. For the animals with axotomy at 3, 8, and 10 mm from the eye, the one-half survival time during this period of slow RGC loss ranged from 6.2 - 7.6 months.

5) For the animals with ON lesions closest to the eye (0.5 mm), no further loss of RGCs was detected between 6 and 20 months after axotomy, suggesting that there may be a small population (3-4% of control) of RGCs that are resistant to the effects of permanent disconnection from their targets.

6) It remains to be determined if one or several variables are responsible for the different patterns of RGC loss after these ON lesions. The persistent disconnection of these neurons from their targets, presumably an important source of trophic support, may be responsible for the later slower decreases of RGC densities. The early, more rapid decrease, which is influenced by the proximity of the lesion to the neuronal perikarya and presumably by the type of lesion applied, might involve additional variables such as the extension of an acute injury effect to the cell body or the greater loss of trophic support from the shorter ON stump. Alternatively, the different patterns of RGC loss could be due to a single mechanism that involves complicated relationships among time, type of lesion, and distance of the axotomy from the cell body.

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APPENDIX I

Villegas-Pérez, M.P., M. Vidal-Sanz, G.M. Bray, and A.J. Aguayo (1988) Influences of peripheral nerve grafts on the survival and regrowth of axotomized retinal ganglion cells in adult rats. J. Neurosci. 8: 265-280.

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Influences of Peripheral Nerve Grafts on the Survival and Regrowth of Axotomized Retinal Ganglion Cells in Adult Rats

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To investigate the role of extrinsic influences on the survival and growth of axotomized retinal ganglion cells (RGCs) in the mature mammalian CNS, both optic nerves (ONs) of adult rats were transected intraorbitally and, on one side, replaced by an autologous segment of peripheral nerve (PN) that had been left unconnected distally. The survival of RGCs and the regrowth of their cut axons into the PN grafts were assessed using morphometric techniques, neuroanatomic tracers, and immunologic cell markers to identify and count RGCs at times ranging from 15 d to 9 months.

It was observed that (1) in the absence of a PN graft, more than 90% of the RGCs died by 1 month after axotomy; (2) between 1 and 3 months after axotomy, survival of RGCs in the PN-grafted retinas was enhanced 2–4-fold; (3) nearly 20% of the surviving RGCs regrew lengthy axons into the grafts; and (4) although the density of surviving RGCs in PN-grafted retinas decreased significantly between 1 and 3 months after axotomy, the densities of RGCs with axons extending into the graft remained relatively stable.

These results confirm that in the adult rat retina, neuronal death is a major effect of axotomy near the cell soma. Although such lesions lead to the degeneration of many RGCs, we show that extrinsic influences introduced by the apposition of a PN segment at the time of severing the ON can rescue a substantial number of these neurons. Because the enhanced survival of many axotomized RGCs in the PN-grafted retinas appears to be limited to the first few weeks after injury, while those of RGCs that regenerate axons into the grafts do not show a parallel decline, it is possible that, in these experiments, neuronal viability depends on a spectrum of differently timed influences that may include the early diffusion of critical molecules arising from the graft and the subsequent establishment of more complex interactions with graft components.

The injury of optic nerves (ONs) in adult rodents is followed by a loss of retinal ganglion cells (RGCs) (Eayrs, 1952; Muchnick-Miller and Oberdorfer, 1981; Grafstein and Ingoglia, 1982; Allcutt et al., 1984; Misantone et al., 1984) and a failure of the surviving neurons to regrow their injured axons. Although the precise molecular determinants of the responses to axotomy of these and other neurons of the CNS and PNS are unknown, there is evidence from both *in vitro* and *in vivo* experiments that extrinsic conditions in the damaged tissues that surround their axons can exert important influences (Varon, 1983–1984; Cotman and Nieto-Sampedro, 1984; Aguayo, 1985). The role played by these extrinsic conditions in the regrowth of CNS axons is illustrated by the demonstration in adult rats that cut retinal axons regenerate when the CNS glial milieu of the ON is replaced experimentally by the non-neuronal components of the peripheral nerve (PN). Indeed, when PN segments were grafted to the orbital stump of the ON and used as "bridges" to link the eye and the superior colliculus (SC), some of the retinal axons extended along the PN grafts and made synaptic contacts with nerve cells in the superficial layers of the SC (Vidal-Sanz et al., 1986, 1987).

To further explore the responsiveness of RGCs to epigenetic influences that may result in a persistent enhancement of the number of injured RGCs that express these regenerative capacities, we have investigated the effects of PN grafts on the short- and long-term survival of ganglion cells in the retinas of adult rats and estimated the incidence of axonal regrowth among the RGCs that survive injury. By combining conventional histologic methods with retrogradely transported labels and immunohistochemical techniques, we show that the PN grafts not only stimulate axonal regeneration from the retina, but also enhance significantly the early survival of axotomized RGCs. It also appears that, at later intervals after PN grafting, there is a gradual decline in the population of surviving RGCs, suggesting that other interactions, presumably with target tissues, may be required to ensure persistent survival of these regenerating neurons.

Brief accounts of this work have been presented in abstract form (Villegas-Pérez et al., 1986) and in a short communication (Aguayo et al., 1987). For all procedures, animals were anesthetized with chloral hydrate (Vidal-Sanz et al., 1987).

Materials and Methods

The effects of axotomy and PN grafts on the survival and axonal regrowth of neurons in the ganglion cell layer of the retina were investigated using 2 different approaches: quantitative studies of neurons in methylene blue-stained retinas (Group I), and combinations of immunocytochemistry and retrogradely transported fluorescent tracers that identify retinal ganglion cells (Group II) (Fig. 1).

Group I experiments

Animals. Thirty-six female Sprague-Dawley rats weighing 200–300 gm were used for this study. In 12 of these animals, the right ON was cut at the level of the optic disc. In 13 animals, the left ON was transected

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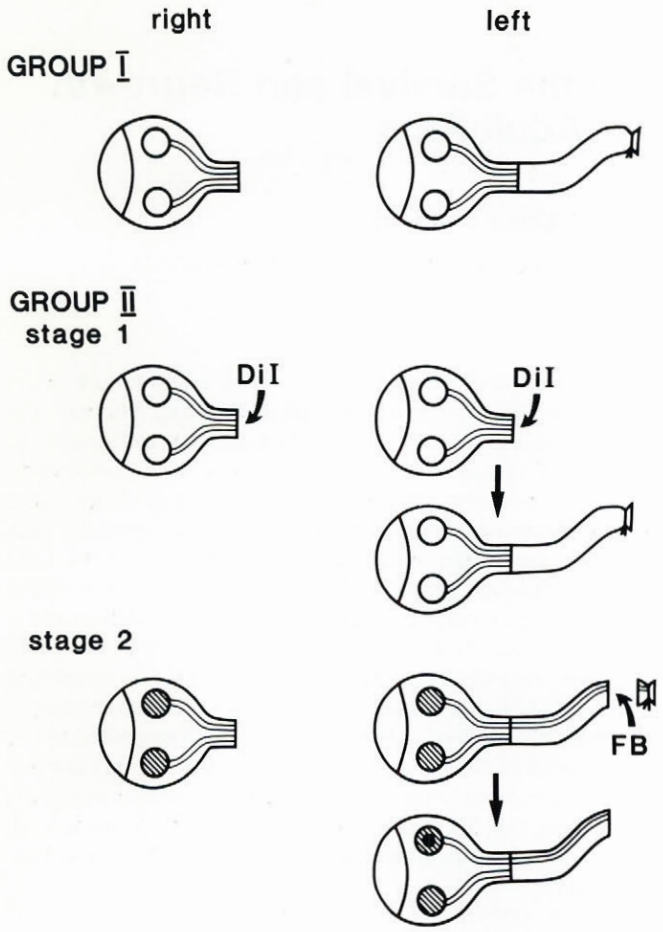

Figure 1. Diagram of the methods used to study the effects of axotomy and peripheral nerve (PN) grafting on cells in the retinal ganglion cell (RGC) layer. *Group I,* One or both optic nerves (ONs) were transected near the eye and an autologous segment of PN was sutured to the ocular stump of the left ON. After intervals of 15 d to 9 months, the retinas of these animals were stained with methylene blue and examined by light microscopy. *Group II, stage 1,* Both ONs were transected and the fluorescent tracer, diI, was applied to the ocular stumps. A PN graft was then attached to the ocular stump of the left ON. In *stage 2,* after intervals of 15 d and 1, 2, and 3 months, the distal end of the graft was trimmed and a second fluorescent tracer, fast blue (*FB*), was applied to the end of the graft. Two days later, the retinas from these animals were examined by fluorescence microscopy. In both the right (nongrafted) and left (PN-grafted) retinas, a population of RGCs that had survived axotomy was labeled with diI (*hatched*). In the left retinas, RGCs whose axons had grown to the end of the graft were also labeled with FB (*small solid circle*).

at the same level and a 3 cm segment of autologous tibial nerve was grafted to the ocular stump of the ON. In 7 animals, both ONs were severed and a PN graft was attached to the ocular stump of the left ON. Four additional animals were used to estimate the normal population of neurons in the ganglion cell layer of the rat retina. From these groups of animals, we analyzed 43 retinas: 19 in which the ON was transected without grafting; 20 in which the ON was transected and replaced by a PN graft; and 4 control retinas from animals in which both ONs were intact.

Grafting procedure. A 3 cm segment of the tibial nerve was removed and one end was sutured to the ocular stump of the intraorbitally transected ON, as previously reported (Vidal-Sanz et al., 1985, 1987). The remaining portion of the graft was placed between the skull and the scalp, with its free end over the occipital bone. To determine the proportions of retinal neurons that survive axotomy, groups of 3 or 4 retinas from these animals were analyzed 15 d and 1, 3, 6, and 9 months after ON transection with or without PN grafting.

Tissue processing. Animals were perfused through the heart with 0.9% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The retinas were dissected, and flattened whole-mounts prepared by making 4 radially oriented cuts; the retinal orientation was identified by making the deepest cut at the superior pole. These flattened retinas were post-fixed for 1 hr in the fixative solution, rinsed in 0.1 M phosphate buffer for several hours, placed on gelatin-coated slides, air-dried, stained for 10 min in 0.02% methylene blue followed by a 5 min exposure to 5% ammonium molybdate (Stone, 1965), dehydrated in graded solutions of ethanol, cleared in xylene, and mounted in DPX (Harleco Diagnostics).

Cell counts. The densities of neurons in the ganglion cell layer of the methylene blue-stained retinas were determined by photographing 3 standard rectangular areas, each measuring 0.33 × 0.22 mm at distances of 1, 2, and 3 mm from the optic disc in the central regions of each retinal quadrant (superonasal, superotemporal, inferonasal, and inferotemporal). With the aid of an IBAS-I image analyzer, the perikaryal areas of all neurons in these photographs, taken at 100× and printed at a final magnification of 700×, were measured and size–frequency histograms prepared. The densities of all neurons in these photographs were also calculated and expressed as the number of cells/mm^2; these data were not corrected for possible retinal shrinkage. Since the areas photographed were all within the central region of the retina, the data obtained from each of the 12 photographs were grouped to obtain mean densities for each retina.

In previously reported studies, estimates of the density of RGCs in the rat retina were 5000–6000/mm^2 for the central area (Fukuda, 1977; Schober and Gruschka, 1977; Dreher et al., 1984), and 2500/mm^2 for the central region (Perry, 1981). The variability among the results of these different studies may be due to the different animal strains, as well as the methods, used for histological processing and for the identification of RGCs within the retinal ganglion cell layer. In the present study, we also calculated the densities and proportions of large neurons (somata area >80 μm^2), in addition to determining the densities of all neurons in the ganglion cell layer. Because approximately 50% of neurons in the ganglion cell layer of the rat retina are believed to be amacrine cells that are smaller than most RGCs (Cowey and Perry, 1979; Perry, 1981), such size criteria have been used to estimate the densities of RGCs. However, such estimates are somewhat imprecise because there is overlap between large amacrine cells and small RGCs (Perry, 1981).

For statistical analysis, data from grafted and nongrafted retinas at different time intervals were compared using Student's *t*-test.

Group II experiments

Animals. Thirty-three Sprague-Dawley rats weighing 200–300 gm were studied to determine the survival of RGCs in grafted and nongrafted animals and to estimate the proportion of these cells that had regenerated along the PN grafts. In 22 rats, the left ON was transected and an autologous PN graft implanted, using the surgical procedure described for the Group I experiments; in 21 of these animals, the right ON was also transected, but no graft was implanted. Thirteen additional animals served as controls.

Retrograde labeling of RGCs. In previous studies in which PN grafts were attached to the ocular stumps of transected ONs in adult rats (Vidal-Sanz et al., 1985, 1987; Berry et al., 1986), HRP was applied to the distal end of the grafts to label the cell bodies of retinal neurons that had regrown their axons. For the present experiments, it was necessary to use a retrogradely transported label that could be applied to the optic nerve stumps at the time of axotomy and that would persist throughout the period of study to mark the surviving RGCs. Therefore, in 18 animals, at the time of ON transection, and immediately before attaching the PN graft, small crystals of the fluorescent carbocyanine marker, diI (Catsicas et al., 1986; Honig and Hume, 1986), were applied to the ocular stumps of both transected ONs (Fig. 1) to label RGCs by retrograde axonal transport. diI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes, Junction City, OR) is a lipid-soluble substance whose characteristics as a retrograde (Honig and Hume, 1986) or anterograde (Catsicas et al., 1986; Thanos and Bonhoeffer, 1987) marker have been studied in embryonic chicks. This intensely fluorescent substance persists for periods of several weeks without apparent fading or leakage and has been reported not to interfere with the function of the labeled cells (Honig and Hume, 1986).

After intervals of 15 d (n = 2), 1 (n = 6), 2 (n = 4), and 3 months (n = 6), a second fluorescent marker, 3% fast blue (Dr. Illing GmbH Co., FRG) was applied to the distal end of the grafts (Fig. 1) to label by

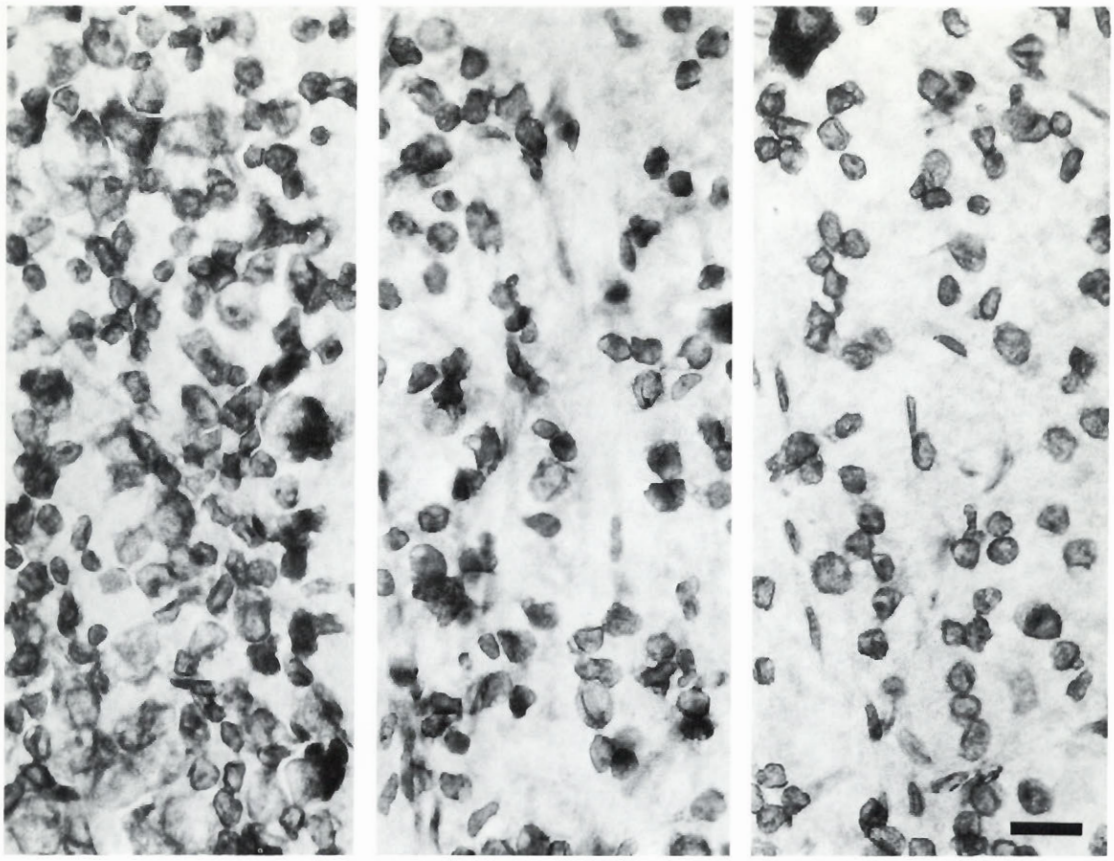

Figure 2. Portions of the ganglion cell layer photographed 1 mm from the optic disc in the superotemporal quadrant of flat-mounted, methylene blue-stained retinas. *Left,* Control (not axotomized). *Middle,* Axotomized for 1 month. *Right,* Axotomized and PN-grafted for 1 month. Bar, 20 μm.

retrograde transport (Dann et al., 1971; Skirboll et al., 1984) the retinal neurons that had elongated their axons along these grafts.

To establish the population of RGCs that would incorporate dil after ON transection in the orbit, the ONs of 7 animals were transected at the level of the optic disc and dil applied to the ocular stump. In 4 other animals, fast blue (3%) was injected into both superior colliculi to estimate the population of RGCs in normal retinas.

Tissue processing. Forty-eight hours after application of the second tracer (fast blue), the animals were perfused and the retinas dissected, postfixed, and rinsed as described for the Group I experiments. The retinas were then mounted on gelatin-coated slides in a solution of 50% glycerol in 0.1 M sodium carbonate buffer (pH 9.0) containing 0.04% *p*-phenylenediamine (Dodd et al., 1984), and observed by fluorescence microscopy with different filters to visualize neurons labeled with fast blue (excitation, 335–425 nm; suppression, 460 nm) and/or dil (excitation, 530–560 nm; suppression, 580 nm). Photographs were taken of the same standard retinal areas as described above to calculate the densities of labeled RGCs.

Neurofilament immunocytochemistry. The retinas that had been photographed for dil and fast blue fluorescence, as well as the retinas from Group II animals that had survived for 6 ($n = 2$) and 9 ($n = 2$) months without dil labeling, plus the retinas from 2 control animals, were incubated with RT 97 (Anderton et al., 1982), a monoclonal antibody that recognizes phosphorylated 200 kDa neurofilaments or a closely associated protein (N. Nukina and D. J. Selcoe, personal communication), to visualize by fluorescence microscopy the intraretinal course of the RGC axons that had survived axotomy (Vidal-Sanz et al., 1987). After rinsing for 1–2 hr in 0.01 M PBS containing 2% Triton X-100 (New England Nuclear, Boston, MA), the retinas were incubated at room temperature with RT 97 diluted 1/1000 with 0.01 M PBS containing 3% ovalbumin (Sigma Chemical Co., St. Louis, MO) and 2% Triton X-100. Twelve to 16 hr later, the retinas were rinsed with 0.01 M PBS for 1 hr and incubated for 1 hr at room temperature with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Behring Diagnostics, La Jolla, CA) diluted 1/100 with 0.01 M PBS containing 3% ovalbumin and 2% Triton X-100. The retinas were rinsed and remounted on gelatin-coated slides as described above. Each flattened retina was examined by fluorescence microscopy (excitation, 450–490 nm; suppression, 525 nm) and photographed with the aid of an automatic condenser–scanner device (Martensson and Björklund, 1984).

This immunocytochemical technique permits qualitative assessments of RGC axons in whole retinas. However, because cell somata do not normally immunoreact with this antibody, and individual axons cannot be distinguished within the large axonal bundles that extend towards the optic disc (Drager et al., 1984), the method does not allow quantitative comparisons between the effects of axotomy and of PN grafting on RGCs.

Results

Group I: number and size of cells in the ganglion cell layer of methylene blue-stained retinas

Effects of axotomy on cell numbers in the ganglion cell layer

By 15 d after axotomy, the earliest time that these animals were examined, the mean density of neurons of all sizes in the ganglion cell layer (Fig. 2, left, middle) was reduced to 3151 ± 321 (Table 1) from 5601/mm² (Table 1) in control (nonaxotomized) retinas. This represents a decrease to 56% of the normal nerve cell density in this layer. There were even greater reductions in the densities of populations of neurons larger than 80 μm²; by 15 d after axotomy, the densities of these large neurons were reduced to 450 ± 27, or 17.7% of control (Table 1), a loss of more than 80%.

The marked reductions in both the total numbers of neurons and in the population of large neurons (>80 μm²) in the ganglion

Table 1. Effects of axotomy and peripheral nerve grafting on the density of neurons in the ganglion cell layer of adult rat retinas[a]

	Number of cells/mm²				
	Total		Cells >80 μm²		
Control animals					
	5218		1656		
	4777		2044		
	6048		3520		
	6362		2608		
Mean ± SEM	5601 ± 365		2547 ± 404		
	Right retina	Left retina	Right retina	Left retina	Left/ right
Experimental animals					
15 d	3970	3851	519	622	
	2636	2906	449	563	
	3358	—	384	—	
	2643	—	446	—	
	—	3444	—	405	
	—	3262	—	531	
Mean ± SEM	3151 ± 321	3365 ± 196	450 ± 27	530 ± 45	1.2
Percent of control	56.3	60.1	17.7	20.8	
1 Month	3693	3658	560	856	
	3274	3547	267	499	
	2547	2496	286	414	
	3162	—	540	—	
	—	2742	—	786	
Mean ± SEM	3176 ± 230	3111 ± 289	413 ± 79	639 ± 107	1.6
Percent of control	56.7	55.5	16.2	25.1	
3 Months[a]	—	2348	—	441	
	—	2863	—	429	
	—	3024	—	470	
	2530	2719	355	528	
	2918	—	125	—	
	3149	—	234	—	
	2024	—	436	—	
Mean ± SEM	2656 ± 246	2748 ± 136	287 ± 68	467 ± 22	1.6
Percent of control	47.4	49.1	11.3	18.3	
6 Months	—	2822	—	600	
	—	2772	—	121	
	—	3349	—	291	
	2877	2947	456	590	
	2451	—	218	—	
	2576	—	550	—	
Mean ± SEM	2635 ± 126	2973 ± 130	408 ± 98	400 ± 117	0.98
Percent of control	47	53.1	16	15.7	
9 Months	—	2829	—	148	
	—	3754	—	283	
	—	3119	—	274	
	—	2315	—	444	
	2935	—	226	—	
	3118	—	224	—	
	2988	—	296	—	
	2620	—	216	—	
Mean ± SEM	2915 ± 105	3005 ± 300	241 ± 18	287 ± 60	1.2
Percent of control	52	53.7	9.5	11.3	

Right retinas, optic nerve transected. Left retinas, optic nerve transected and PN graft attached.

[a] At 3 months, there was a statistically significant difference between the densities of cells greater than 80 μm² in the axotomized (right) and PN-grafted (left) retinas. (Student's t test; $p < 0.05$.)

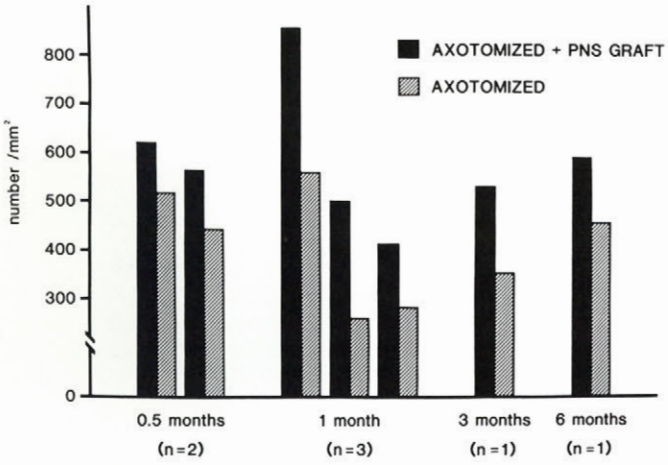

Figure 3. Densities of neurons greater than 80 μm^2 in the ganglion cell layer of methylene blue-stained retinas from adult rats in which both ONs were transected and a PN graft attached to the left ON stump. Each pair of *bars* depicts the data from one animal. The density of these large neurons is greater in the retinas with PN grafts attached (*solid bars*) than in the retinas with axotomy alone (*hatched bars*). Using Student's t test for paired data, the differences were statistically significant at 15 d ($p < 0.02$) and 1 month ($p < 0.03$).

cell layer of the retina during the first 15 d after axotomy were followed by further, less dramatic declines in the densities of these cells over the subsequent 9 months (Table 1). The decrease in the densities of large cells between 15 d and 9 months after axotomy was statistically significant ($p < 0.001$).

Effect of PN grafting on the number of cells in the ganglion cell layer

Between 15 d and 3 months after axotomy, there was a consistent trend for the densities of the large neurons to be greater in the retinas from animals with axotomy plus PN grafts than in the retinas with axotomy alone (Table 1). However, these differences were only statistically significant ($p < 0.05$) at 3 months, when the mean density of neurons with cell somata greater than 80 μm^2 was 467 ± 22 (18.3% of control) in the PN-grafted retinas, compared to 287 ± 68 (11.3% of control) in the nongrafted retinas. The trend towards an enhancement of the survival of the population of large neurons in the RGC layer was more apparent when the effects of axotomy with or without PN grafting were compared in the same animals (Table 1, Fig. 3). In these animals, the density of cells in the RGC layer was always greater in the retinas with PN grafts than in the retinas with axotomy but no grafts, differences that were statistically significant (Student's t test for paired data; $p < 0.02$ at 15 d, $p < 0.03$ at 1 month).

Effects of axotomy and PN grafts on neuron size

The areas of all neurons in the photographs of the ganglion cell layer of each control, axotomized, and PN-grafted retina were measured and the effects of axotomy and PN grafts on neuron size investigated by comparing the median areas as well as the proportions of neurons greater than 80 μm^2.

Medians. Although the ranges of neuron sizes in the ganglion cell layer of the experimental retinas (Table 2, Fig. 4) indicated that some large neurons persisted after axotomy, the median perikaryal areas of the neurons in the ganglion cell layer decreased from 75.5 ± 7.5 μm^2 in the control retinas to approximately 55 μm^2 by 15 d and 1 month after axotomy. Between 15 d and 9 months after axotomy, there was a further, less marked, but statistically significant ($p < 0.05$) decrease in the median areas of cell somata in the ganglion cell layer of both the PN-grafted and nongrafted experimental retinas to approximately 50 μm^2 (Table 2).

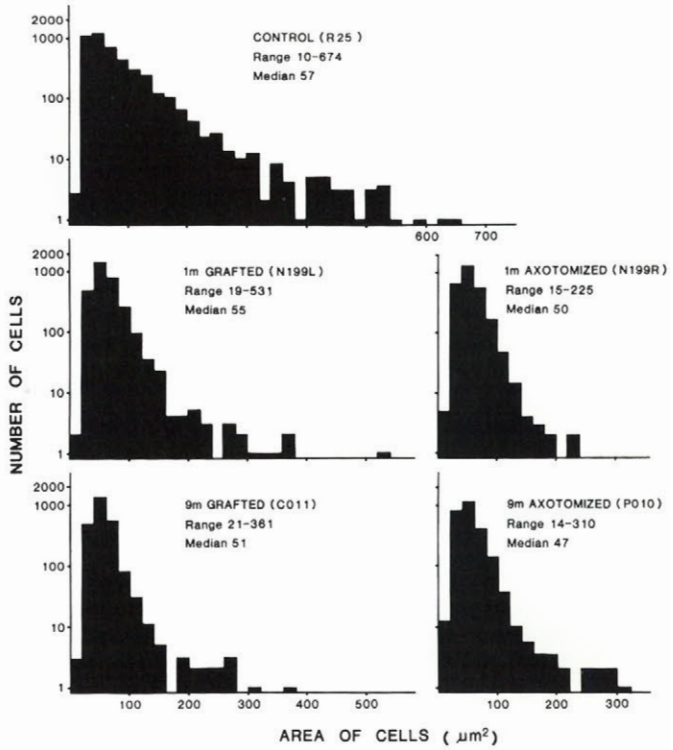

Figure 4. Representative size–frequency histograms for neurons in the ganglion cell layer of individual control and experimental retinas. Perikaryal sizes of all neurons in photographs of representative areas of each retinal quadrant were processed with an IBAS-I image analysis system. Number of neurons in each category are represented on a logarithmic scale. *Top,* Control (nonaxotomized) retina. *Middle,* One month after axotomy plus grafting (*left*) or axotomy alone (*right*). *Bottom,* Nine months after axotomy plus grafting (*left*) or axotomy alone (*right*).

Proportions of large neurons. In the 4 control retinas, 46.4% of the total population of neurons had perikaryal areas greater than 80 μm^2 (Table 2). In the experimental retinas without PN grafts, the proportions of these large neurons fell to 14.6% by 15 d and reached 8.3% by 9 months (Table 2); this difference between 15 d and 9 months was statistically significant ($p < 0.01$).

When cell sizes were compared in the PN-grafted and nongrafted retinas (Table 2), there was a trend for the medians and proportions of large neurons toward being greater in the PN-grafted retinas, but this difference was not statistically significant.

Group II: studies of RGC survival using retrogradely transported tracers and neurofilament immunocytochemistry

Although examination of the methylene blue-stained retinas from the Group I animals indicated a tendency for enhanced survival of neurons in the ganglion cell layer of the PN-grafted retinas, differences in the survival of the RGCs in PN-grafted and nongrafted retinas could not be established with certainty on the basis of these conventional histological techniques. To

Table 2. Effect of axotomy and peripheral nerve grafting on the size of neurons in the ganglion cell layer of adult rat retinas

	Area (μm^2)		
	Median	Range	% >80 μm^2
Control animals			
	57.11	10.3–674.7	31.7
	85.03	16.1–579.3	54.8
	90.13	21.8–472.0	58.0
	69.71	17.1–499.7	41.0
Mean ± SEM	75.5 ± 7.5		46.4

	Area (μm^2): Right retina			Area (μm^2): Left retina		
	Median	Range	% > 80 μm^2	Median	Range	% > 80 μm^2
Experimental animals						
15 d	53.6	20.1–464.0	13.0	56.9	20.0–394.5	16.1
	55.6	20.4–385.6	17.0	58.2	21.7–446.3	19.3
	54.3	19.1–479.8	11.4			
	55.7	22.9–237.9	16.8			
				50.2	14.8–545.3	11.7
				56.3	22.3–585.8	16.2
Mean ± SEM	54.9 ± 0.5		14.6	55.4 ± 1.8		15.9
1 Month	57.1	19.0–297.4	15.1	61.9	23.9–650.5	23.4
	50.3	15.6–225.8	8.1	54.9	19.5–531.0	14.0
	51.9	18.0–309.6	11.4	55.3	22.8–484.5	15.4
	57.6	16.6–454.2	16.5			
				64.3	26.4–557.3	28.6
Mean ± SEM	54.2 ± 1.8		12.8	59.1 ± 2.3		20.4
3 Months				57.1	14.6–312.0	18.5
				56.3	20.8–334.1	14.9
				57.8	19.3–471.2	15.5
	50.7	19.2–327.9	12.6	55.6	21.5–283.8	19.4
	42.5	18.0–479.8	4.2			
	50.2	17.0–447.6	7.4			
	60.5	18.2–179.9	21.6			
Mean ± SEM	51 ± 3.6		11.5	56.7 ± 0.4		17.1
6 Months				64.0	24.1–582.9	22.8
				39.8	15.0–339.3	4.3
				46.8	15.7–451.6	8.7
	56.2	20.4–323.4	15.8	52.2	22.6–440.4	20.0
	51.6	18.0–201.0	8.9			
	58.0	19.6–294.1	21.3			
Mean ± SEM	53.9 ± 2.0		15.4	51.7 ± 5.3		14.0
9 Months				51.1	21.8–361.1	6.0
				48.2	14.5–376.2	7.5
				45.0	15.8–408.0	8.8
				54.8	22.6–341.7	19.1
	46.7	14.5–310.9	7.7			
	46.4	18.3–543.9	7.2			
	52.7	32.4–255.5	9.9			
	53.8	24.3–304.0	8.2			
Mean ± SEM	49.9 ± 1.9		8.3	49.8 ± 2.0		10.4

Right retina, optic nerve transected. Left retina, optic nerve transected and PN graft attached.

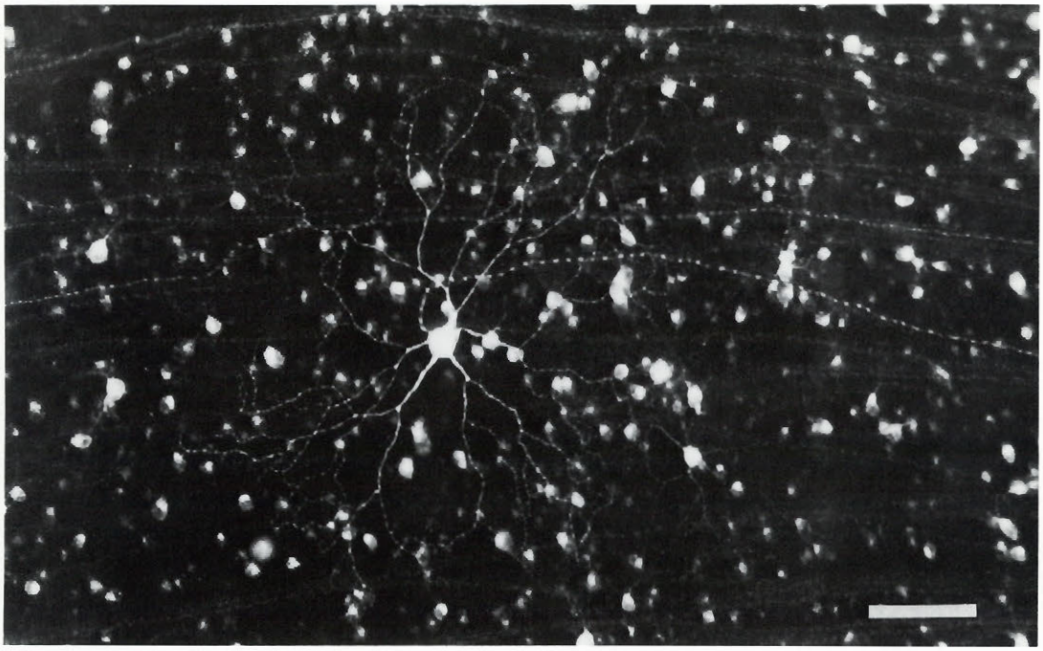

Figure 5. Neurons in the RGC layer 48 hr after the ON was transected and dil crystals were applied to the ocular stump. The perikarya and axons of many neurons are labeled with the retrogradely transported tracer. The dendrites of one neuron in this photograph are outlined by discontinuous labeling with dil fluorescence. Bar, 100 μm.

investigate RGC survival specifically, we labeled retinal neurons by applying retrogradely transported tracers to the ONs and grafts. In these experiments, both ONs were transected intraorbitally and a PN segment was grafted to the left ON to permit (1) an analysis of the PN-grafted and nongrafted retinas in the same animal; (2) the application of one fluorescence marker (dil) to both ON stumps at the time of axotomy in order to label RGCs throughout the period of study; (3) the application of a second fluorescence marker (fast blue) to the distal tip of the blind-ended PN graft prior to animal death to identify RGCs that had regenerated axons along the PN grafts (Vidal-Sanz et al., 1985, 1987); and (4) the visualization of the intraretinal axons of surviving RGCs on the basis of their immunoreactivity to RT 97.

Non-neuronal labeling by dil

In both the control and experimental retinas, a narrow ring of fluorescence surrounded the optic disc, but did not extend to the regions of the retina in which RGCs were counted. Small clumps of fluorescent material were observed in the extracellular space of the ganglion cell layer of experimental and control retinas (Fig. 7). Although not quantitated, the amount of this material was greater in the experimental retinas than in the retinas that had been axotomized for only 2 d. Presumably some of this extracellular fluorescent material was derived from RGCs that had degenerated after incorporating the label. Finally, most retinas showed labeling in a few cells that had the characteristic appearance of Muller cells; because the labeled Muller cells extended their processes through several layers of the retina, they could be readily distinguished from the RGCs.

RGC labeling with dil after intraorbital transection of the ON

In 7 animals in which dil was applied to the ocular stump of the intraorbitally transected ON 48 hr prior to tissue processing, fluorescent cells with the distinct morphology of RGCs were identified in the ganglion cell layer of all sectors of the retinas (Fig. 5). The neuronal fluorescence, which was either punctate or diffuse, was most dense in the perikaryal cytoplasm and proximal dendrites; in the most intensely fluorescent neurons (less than 1% of the labeled cells), dil fluorescence outlined axons, as well as the secondary and tertiary dendrites, permitting a Golgi-like visualization of the neuron (Fig. 5).

The density of the dil-labeled RGCs calculated for these 7 retinas varied from 604 to 1081/mm^2, with a mean value of 767 ± 60 (Table 3). The reasons for the relatively low efficiency of labeling with dil applied to the transected ON, compared to that of other tracers, such as fast blue (see below) or HRP (Perry, 1981), are unknown. However, for the purposes of the present experiments, the capacity of this substance to remain within neurons for several weeks (Honig and Hume, 1986) outweighed this disadvantage. Furthermore, the anatomical characteristics and distribution of the labeled RGCs throughout the retina did not suggest that dil labels certain classes of RGCs selectively.

Effect of axotomy on survival of dil-labeled RGCs

The RGCs labeled with dil were identified throughout the retinas of the experimental animals with ONs transected for periods of 15 d to 3 months. In the (right) retinas without PN grafts, the mean density of labeled RGCs decreased from 767 ± 60 at 2 d after axotomy to 16% (127 ± 21) of this value at 15 d, and to 6.7% by 1 month; there were only slight, nonsignificant ($p > 0.05$) further declines in the proportions of dil-labeled cells, to 6.4 and 4.7% at 2 and 3 months after axotomy, respectively (Table 3, Fig. 6).

Effects of PN grafts on axotomized RGCs

Although there was considerable variation in the number of labeled neurons in different animals, the density of dil-labeled nerve cells was consistently greater for the PN-grafted eyes when both retinas of the same animal were compared (Table 3, Fig.

Table 3. Effects of axotomy and peripheral nerve grafting on the survival and axonal regrowth of retinal ganglion cells in adult rat retinas

	RGCs/mm²					
	diI			Fast blue		
Control animals						
	1081			2313		
	769			2211		
	604			2060		
	765			1879		
	660					
	826					
	663					
Mean ± SEM	767 ± 60			2116 ± 94		
	Labeled RGCs/mm²					
	A	B	C	B/A	D (%)	E (%)
Experimental animals						
15 d	106	343	118	3.2	5.6	12.5
	149	203	90	1.3	4.3	16.0
Mean ± SEM	127 ± 21	273 ± 70	104 ± 14	2.1	4.9	14.3
Survival (%)	16	35				
1 Month*	72	282	151	3.9	7.1	19.4
	96	128	70	1.3	3.3	19.8
	51	158	77	3.0	3.6	17.6
	14	232	68	16.9	3.2	10.6
	23	230	81	10.0	3.8	12.8
		138	64		3.0	16.8
Mean ± SEM	51.3 ± 15	194.7 ± 25	85 ± 13.4	3.8	4.0	16.2
Survival (%)	6.7	25.3				
2 Months*	57	118	57	2.9	2.7	17.5
	45	180	101	4.0	4.8	20.3
	32	247	195	7.7	9.2	28.7
	65	90	43	1.3	2.0	17.3
Mean ± SEM	49.6 ± 7	158.8 ± 34	99 ± 34	3.1	4.7	20.9
Survival (%)	6.4	20.7				
3 Months*	41	112	70	2.7	3.3	22.7
	43	106	72	2.4	3.4	24.5
	21	57	32	2.6	1.5	20.2
	61	108	43	1.7	2.0	14.6
	35	114	45	3.2	2.1	14.3
	30	89	34	2.8	1.6	13.9
Mean ± SEM	35.8 ± 5.4	97.7 ± 9	49.4 ± 7	2.8	2.3	18.4
Survival (%)	4.7	12.7				

diI Controls, density of retinal ganglion cells (RGCs) retrogradely labeled 2 d after application of diI crystals to the transected optic nerve of normal rats. Fast blue controls, density of RGCs retrogradely labeled 2 d after injection of fast blue in both superior colliculi of normal rats. A, Right retina (optic nerve transected); density of RGCs retrogradedly labeled with diI. B, Left retina (optic nerve transected and PN graft attached); density of RGCs retrogradely labeled with diI. C, Left retina; density of RGCs doubly labeled with diI (at the time of optic nerve transection and PN grafting) and with fast blue (applied to the distal unconnected tip of the graft, 15 d or 1, 2, or 3 months later). D, Proportion of cells retrogradely labeled with fast blue applied to the distal end of the graft, calculated by relating the density of fast blue-labeled cells/mm² to the mean density of the fast blue controls (2116 cells/mm²). E, Incidence of axonal growth into PN grafts calculated by relating the proportions of fast blue-labeled RGCs (C/mean density for fast blue controls) to the proportion of diI-labeled RGCs (B/mean density for diI controls), where the mean densities for fast blue and diI controls were 2116 and 767, respectively.

* Statistically significant difference between groups A and B (Student's t test; 1 month, $p < 0.01$; 2 months, $p < 0.05$; 3 months, $p < 0.001$).

6); the ratios of the mean densities of diI-labeled neurons in the PN-grafted and nongrafted retinas were 2.1 at 15 d, 3.8 at 1 month, 3.1 at 2 months, and 2.8 at 3 months (Table 3). Assuming that the efficiency of diI retrograde labeling is similar at 2 d and at the later intervals after axotomy with or without PN grafting, one could calculate the survival of RGCs in the PN-grafted and nongrafted retinas by relating the mean densities of diI-labeled cells in each experimental group to the mean labeling densities in the control retinas 2 d after axotomy (767 RGCs/mm²). In the PN-grafted retinas, the calculated rates of neuronal

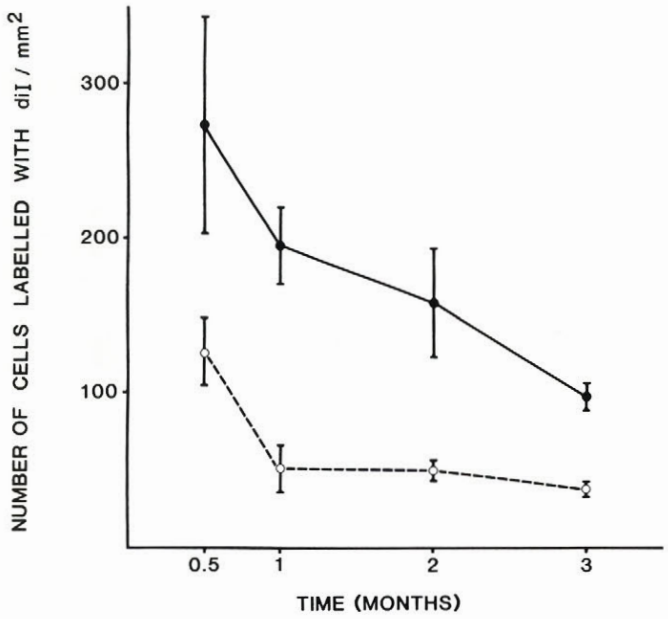

Figure 6. Densities of diI-labeled RGCs, expressed as means ± SEMs, in the retinas of adult rats following ON transection with (*filled circles*) or without (*open circles*) attachment of PN grafts. The differences between the densities of surviving RGCs in the retinas with and without grafts were statistically significant at 1, 2, and 3 months after axotomy and graft placement ($p < 0.01$, $p < 0.05$, and $p < 0.001$, respectively). The densities of diI-labeled RGCs remained relatively stable in the retinas without PN grafts, but, in the retinas with PN grafts, there was a significant decrease ($p < 0.01$) in the density of these labeled neurons between 1 and 3 months after axotomy and graft placement ($n = 2$ at 15 d, $n = 5$ at 1 month, $n = 4$ at 2 months, and $n = 6$ at 3 months).

survival—which were always greater than those in the contralateral axotomized, but nongrafted, retinas—were 35% at 15 d, 25.3% at 1 month, 20.7% at 2 months, and 12.7% at 3 months (Table 3).

The decreases in the densities of diI-labeled neurons in the PN-grafted retinas from 195 ± 25 at 1 month to 98 ± 9 at 3 months, which were statistically significant ($p < 0.01$), were presumably due to a progressive loss of the axotomized neurons. Because the mean densities of diI-labeled neurons in the nongrafted retinas remained relatively stable at 1, 2, and 3 months after axotomy, it seems unlikely that decreases in the intraneuronal concentration of the label to levels below the resolution of fluorescence microscopy were responsible for substantial decreases in the densities of the labeled retinal neurons (Fig. 6).

Axonal regrowth among surviving neurons

In the 4 animals in which fast blue was injected into both superior colliculi—the targets of more than 95% of RGCs in the rat (Linden and Perry, 1983)—the mean density of RGCs retrogradely labeled with this fluorescent tracer was 2116/mm² (Table 3). When fast blue was applied to the distal end of the PN grafts in the experimental animals, fluorescent RGCs were present throughout the entire retina (Fig. 7, lower) in a pattern of retrograde labeling similar to that observed when HRP was applied as the retrograde marker in similar experiments (Vidal-Sanz et al., 1987). In these retinas, the neurons that were labeled with fast blue were also labeled with diI (Fig. 7). Thus, although the proportion of fast blue-labeled neurons, expressed as a percentage of the mean density of fast blue-labeled neurons in the control animals, was only 4–5% for the first 2 months and decreased to 2.3% at 3 months (Table 3, Fig. 8), the incidence of axonal elongation related to the densities of surviving, diI-labeled RGCs was actually considerably higher (Table 3). On the basis of such calculations, it was possible to document the mean incidences of axonal regrowth along the PN grafts to the site of fast blue application, approximately 2 cm from the retina (Fig. 9; Table 3), as being 16, 21, and 18% at 1, 2, and 3 months, respectively.

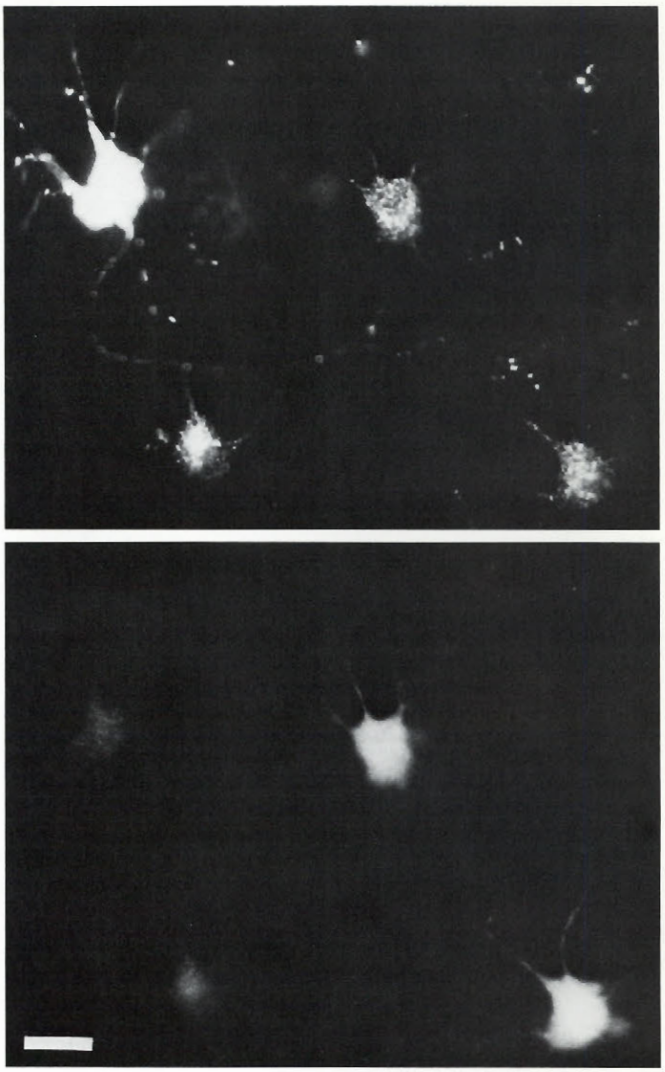

Figure 7. RGCs retrogradely labeled with diI applied to the ocular stump of the ON at the time of transection, and with fast blue applied to the end of the graft 1 month later. *Upper,* The cell bodies of 4 neurons, as well as the proximal axon of one neuron, show dense fluorescence with diI. This retina also contains small amounts of extracellular fluorescent material that may be derived from neurons that had degenerated after labeling. *Lower,* Two of the 4 diI-labeled RGC perikarya also show diffuse fast blue fluorescence, indicating that they are neurons that had been labeled with diI at the time of ON transection and that their axons had grown to the end of the graft by the time the fast blue was applied. The weak fluorescence in the other 2 neurons (on the *left* is due to the diI labeling; it did not have the characteristic color of fast blue fluorescence. Bar, 30 μm.

Effects of PN grafts on intraretinal RGC axons

In flattened whole-mounts of normal retinas, RT 97 immunoreactivity outlined thick bundles of RGC axons converging toward the optic disc (Fig. 10). In the retinas from the experimental

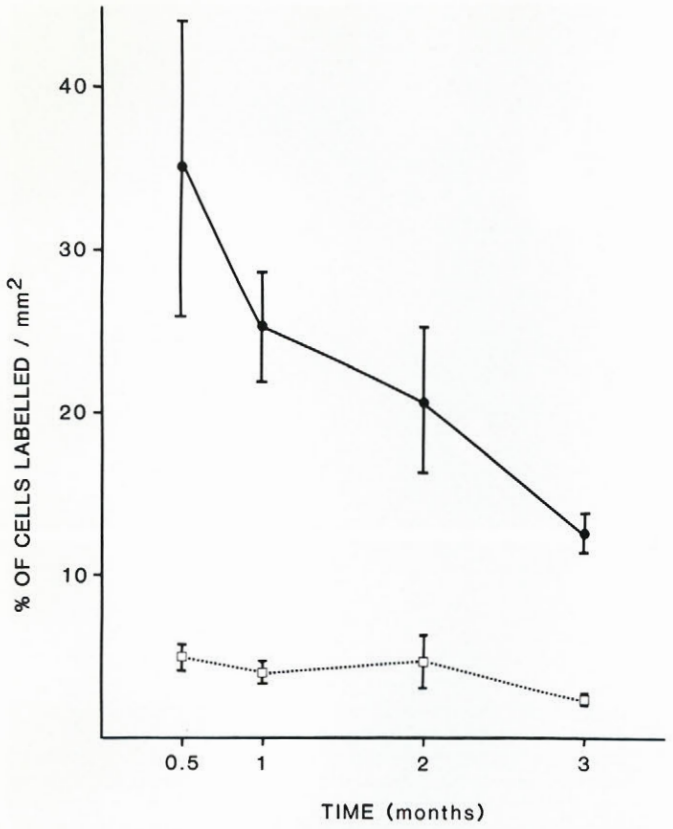

Figure 8. Densities of dil-labeled (*filled circles*) and fast blue-labeled (*open squares*) RGCs, expressed as proportions of the respective labeling of control retinas with these tracer substances in the retinas of adult rats following ON transection and attachment of PN grafts. Although the densities of surviving RGCs (dil-labeled) decreased significantly ($p < 0.01$) between 1 and 3 months after axotomy and PN graft attachment, the densities of these neurons with axons regenerating to the end of the graft (fast blue-labeled) remained relatively stable ($n = 2$ at 15 d, $n = 6$ at 1 month, $n = 4$ at 2 months, and $n = 6$ at 3 months).

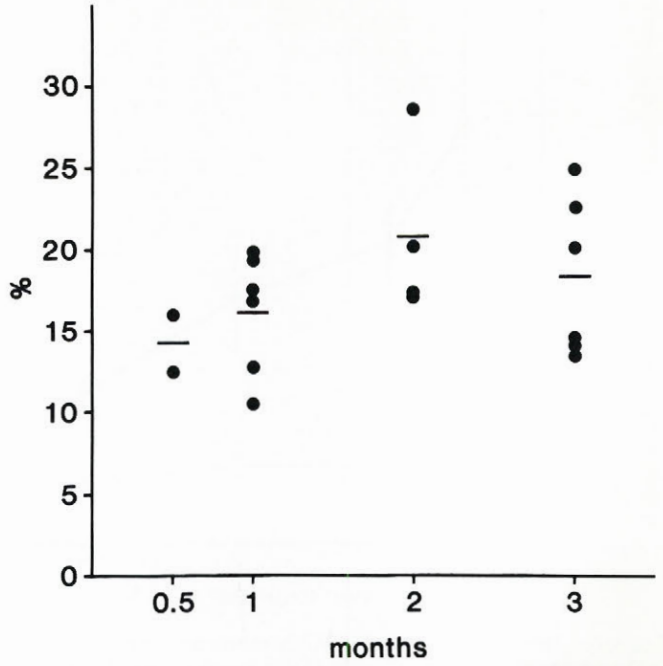

Figure 9. Incidence of axonal regeneration among surviving retinal ganglion cells (RGCs). The *data points* for individual animals represent the proportions of dil-labeled cells that were also labeled with fast blue. The *horizontal bars* indicate the mean for each time interval. Approximately 20% of the dil-labeled cells had grown along the PN grafts.

animals, there were reduced numbers of RGC axons and many individual axons could be identified (Fig. 11). However, when the PN-grafted and nongrafted retinas were compared, there were consistently greater numbers of RGC axons in the retinas that had PN grafts attached to the transected ocular stump of their optic nerves (Fig. 11). This effect of the PN grafts on the survival of RGCs was not only apparent 1, 2, and 3 months after axotomy, thereby confirming the quantitative data from the dil labeling experiments, but was also present in the retinas examined 6 and 9 months after axotomy with or without PN grafting (Fig. 11). Thus, on the basis of this qualitative assessment of the survival of the proximal segments of the injured RGCs, it was also possible to establish that the PN graft-related enhancement of RGC survival persisted for several months.

Discussion

The severing of axons leads to a spectrum of retrograde neuronal responses that can culminate in the death of the axotomized cells (for review, see Lieberman, 1974). In mammals, neuronal degeneration due to axotomy is prominent in the CNS (Lieberman, 1974; Barron, 1983), but also follows injury to peripheral nerves (Aldskogius et al., 1980; Aldskogius and Risling, 1982; Tessler et al., 1985). In the present study, we have used different anatomic techniques to investigate the early and long-term effects of axotomy on the RGCs of adult rats, and have documented that, although many of these cells die soon after injury close to their perikarya, RGC survival is enhanced by the presence of peripheral nerve grafts anastomosed to the tran-

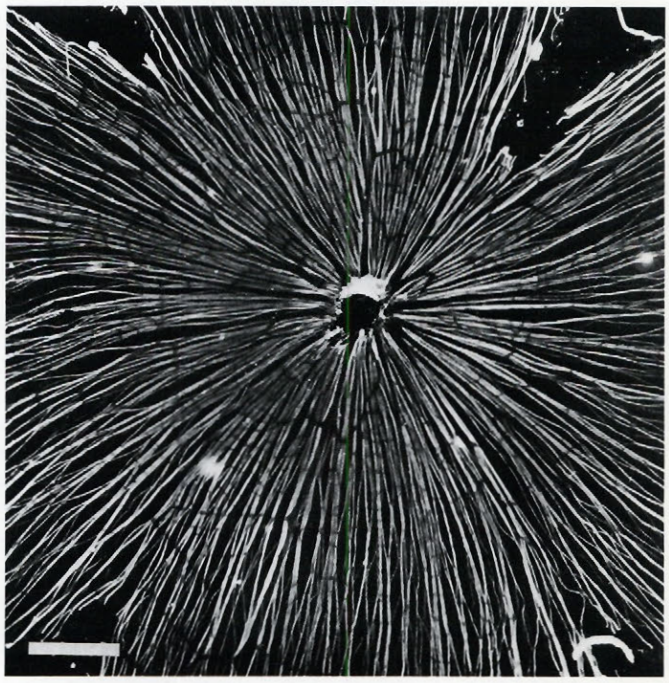

Figure 10. Intraretinal axons of RGCs in a control retina, delineated by their immunoreactivity to RT 97, a monoclonal antibody that reacts with phosphorylated 200 kDa neurofilament subunit, as visualized with FITC-labeled secondary antibodies. Thick bundles of axons course towards the optic disc. Bar, 500 μm.

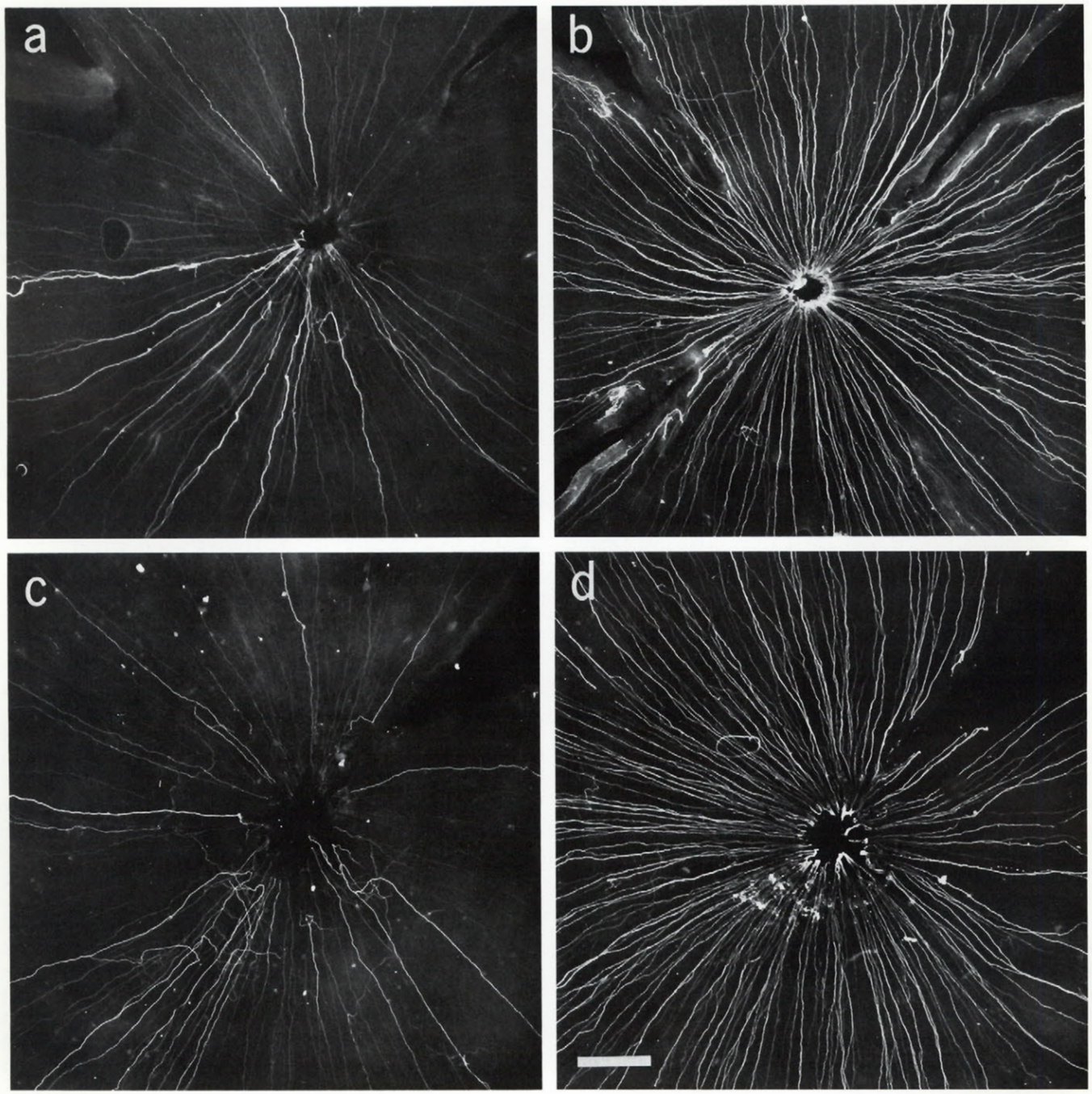

Figure 11. Appearance of the intraretinal axons of the RGCs immunoreacted with RT 97 (see Fig. 10) in the experimental retinas 6 (*a, b*) and 9 (*c, d*) months after axotomy with or without PN grafting. The density of axons is reduced and many individual axons can be identified. There are more immunoreactive axons in the retinas with PN grafts (*b, d*) than in the corresponding contralateral retinas without PN grafts (*a, c*). (Fluorescence micrograph prepared with the aid of an automatic condenser–scanner device; see Martensson and Björklund, 1984.) Bar, 500 μm.

sected optic nerve of these animals. Moreover, axons from approximately one-fifth of the surviving RGCs regenerate extensively along the grafts.

Retrograde effects of axotomy on retinal neurons

The effects of ON transection on retinal neurons vary with the animal studied, its age, and the proximity of the lesion to the cell body. In goldfish, most, if not all, RGCs survive axotomy (Murray et al., 1982; Grafstein, 1986). In *Rana pipiens,* on the other hand, only about one-third of the RGCs are present 6 months after ON crush (Scalia et al., 1985; Stelzner and Strauss, 1986); marked reductions of RGC numbers also occur in other amphibians (Humphrey and Beazley, 1985). In newborn mammals and birds, there is nearly a complete depletion of RGCs following the surgical interruption of the ON (Muchnick and Hibbard, 1980; Muchnick-Miller and Oberdorfer, 1981; Allcutt et al., 1984). Injury to the ON of adult mammals also leads to a marked loss of retinal neurons (James, 1933; Leinfelder, 1938; Mantz and Klein, 1951; Eayrs, 1952; Polyak, 1958; Grafstein and Ingoglia, 1982; Allcutt et al., 1984; Misantone et al., 1984; Berry, 1986). The results of our present experiments and those of other studies in adult rodents (Grafstein and Ingoglia, 1982;

Allcutt et al., 1984; Misantone et al., 1984) are in general agreement in finding that approximately one-half of the neurons in the ganglion cell layer of the retina die after axotomy.

Assessments of nerve cell loss based on cell counts and measurements of the surviving neurons in the ganglion cell layer, however, cannot distinguish with certainty between actual ganglion cells and the displaced amacrine cells that may account for nearly one-half of the neurons in this layer (Cowey and Perry, 1979; Perry, 1981). Because the axonless amacrine cells would not be damaged directly by cutting the ON, counts of all neurons in the ganglion cell layer undoubtedly underestimate the effects of axotomy on RGCs, and estimates based on size criteria can be inaccurate because of the overlap between large amacrine cells and small ganglion cells (Perry, 1981). Moreover, estimates of the effects of axotomy based on ratios between RGCs and the total number of neurons in the ganglion cell layer may also be distorted by shrinkage of the RGC perikarya after injury and by possible indirect effects on the amacrine cells due to transneuronal degeneration or subtle retinal trauma or ischemia resulting from the surgical procedures used in the experiments. Although there are specific markers that identify subpopulations of retinal neurons (Barnstable, 1982), RGCs in rodents can only be preferentially labeled with Thy-1 (Barnstable and Drager, 1984; Perry et al., 1984), a technique that does not lend itself to quantitative studies of RGC populations in retinal wholemounts. Thus, in the present experiments, we used retrograde labeling with diI, a fluorescent marker that remains in neurons for several weeks (Honig and Hume, 1986), to demonstrate that axotomy near the eye causes a loss of more than 90% of RGCs by 1 month after injury.

The early and abrupt loss of RGCs demonstrated in these experiments is different from the temporal pattern described when retinal axons are interrupted farther away from the eye. Indeed, it has been reported that when the adult rat ON was interrupted intracranially, the retrograde loss of most retinal neurons was delayed for several weeks (Misantone et al., 1984). These different results are in keeping with previous observations (Lieberman, 1974; Barron, 1983), which indicated that the distance between the cell somata and the site of injury modified the severity and timing of cell damage. The observation that such distances affect these neuronal responses becomes even more puzzling in view of the marked differences in axonal regrowth from RGCs observed when PN grafts are inserted near or far from their cell somata; there was extensive axonal regeneration into peripheral nerve segments inserted into the retina (So and Aguayo, 1985) or attached to the ON intraorbitally (Vidal-Sanz et al., 1985, 1987), but not when grafted to the ON intracranially (Richardson et al., 1982). Furthermore, it has also been observed in the rat that immunoreactivity in RGCs to the growth-associated protein, GAP-43 (Skene and Willard, 1981), is only expressed when these neurons are axotomized near their cell bodies (Lozano et al., 1987). The mechanisms responsible for these different effects are unknown.

In the methylene blue-stained retinas (Group I animals) of the present study, we also investigated changes in the size of neuronal somata and found that (1) the median perikaryal areas of neurons in the ganglion cell layer of the retina decreased soon after axotomy by approximately 25%; (2) there was a disproportionate loss of the large and medium sizes in the histograms of neuronal somata (Fig. 4); and (3) there was no recovery of soma size throughout the period of study. However, because there were substantial decreases in the total numbers of neurons, it was not possible to establish the extent to which the observed size changes were due to axotomy-induced atrophy or to the selective loss of the larger nerve cells. The effect of axotomy on the size of neurons in the retina was also investigated by Misantone et al. (1984), who observed a decrease in their mean size by approximately 50% during the first month after intracranial transection of the ON, although there appeared to be no marked loss of these neurons until approximately 3 months after the lesion. The different effects of axotomy on the size of nerve cells in the ganglion cell layer between our studies and those of Misantone et al. (1984) may be another puzzling manifestation of the effects of distance between the site of axonal injury and the perikarya. Alternatively, the differences may be related to the rat strains or experimental methods used in each of these studies.

In the present experiments, we measured the changes in the sizes of the methylene blue-stained neurons that survived in the ganglion cell layer of the retina. In previous studies, using retrograde labeling with HRP to identify regenerating RGCs specifically, it was demonstrated that RGCs of a size spectrum that resembled that seen in normal retinas had contributed to the innervation of the PN grafts (So and Aguayo, 1985; Vidal-Sanz et al., 1987). Thus, the extension of RGC axons into the PN grafts appears to have prevented perikaryal atrophy or protected the larger neurons from undergoing degeneration following axotomy.

Effects of PN grafts on axotomy-induced neuronal loss

The results of the present experiments indicate that PN grafts have both *early* and *late* effects on the survival of axotomized neurons.

Early effects. A consistent result of the present study was the documentation of an early enhancement of the survival of ganglion cells in the retinas with PN grafts. Such a rescuing effect of the grafted PN segments on RGC survival was suggested by neuronal counts in the methylene blue-stained retinas (Group I animals). Using indirect histological methods to estimate RGC survival, Berry and colleagues (1986) have also reported that the survival of neurons in the ganglion cell layer of the retina was enhanced in rats with PN grafts implanted into the ON for 1 month. In the present experiments, a more definite indication that PN grafts prevented the degeneration of some of the axotomized RGCs came from the Group II animals, in which the retrogradely transported tracer diI was used to compare the densities of RGCs in the grafted and nongrafted retinas for longer periods of time. As applied in the present experiments, this fluorescent cell marker only labeled approximately one-third of the RGCs in control retinas (axotomized for 2 d) and therefore did not provide a full estimate of the entire population of RGCs. Nevertheless, there were statistically significant differences between the RGC population densities in the grafted and nongrafted retinas from 1 to 3 months after injury, and the ratios of the densities of labeled RGCs in grafted/nongrafted eyes ranged from 2.1 to 3.8 throughout the 3 month period studied.

Although greater densities of diI-labeled RGCs survived in the PN-grafted retinas, there was a further, statistically significant decrease in the population of surviving RGCs in these retinas between 1 and 3 months after grafting (Fig. 6). Since the densities of fast blue-labeled neurons did not show a significant decrease throughout this same period (Table 3, Fig. 8), it is possible that the continued decrease in neuronal densities in the grafted retinas was due to the loss of neurons that had not grown

into the graft. It is interesting to speculate that the early and late influences on neuronal survival are related to different trophic modes of action by the PN grafts. For example, the early enhancement of survival might be due to diffusible substances, while the later effects might also depend on more complex interactions with Schwann cells or other graft components.

Late responses. Quantitative estimates of the effects of PN grafts on the long-term survival of RGCs were not obtained beyond 3 months after axotomy. Although there were no differences between the densities of large neurons in the PN-grafted and nongrafted retinas of the Group I animals at 6 and 9 months, the usefulness of such size criteria to estimate RGC populations is limited both by the normal overlap in sizes between RGCs and amacrine cells and by the possibility that the reduction in the proportions of larger neurons could be due to their atrophy as well as to their selective loss. Although we did not use retrograde labeling with diI to identify surviving RGCs for time periods longer than 3 months, striking differences in the population of RGCs, estimated on the basis of their axonal RT 97 immunoreactivity in the PN-grafted and nongrafted retinas, provided convincing qualitative evidence that an effect of the PN grafts on RGC survival persisted for as long as 9 months after axotomy.

In contrast to this morphological evidence for greater long-term survival of axotomized RGCs in the PN-grafted retinas, there is evidence from other experiments to suggest that the structural and functional integrity of regenerating neurons that are prevented from forming terminal connections may not be maintained indefinitely by the interactions of their axons with the grafted peripheral nerve segments. Neurons regenerating into PN grafts from the retina (Keirstead et al., 1985) or the brain stem of adult rats (Gauthier and Rasminsky, 1987) showed apparently normal electrophysiological properties during the first 5 months, but fewer responses were detectable 9–12 months after PN grafting. Additional indications for a finite capacity of the PN environment to sustain neurons come from experiments in which nerve cells from the fetal CNS were transplanted and isolated within peripheral nerves of adult rats (Doering and Aguayo, 1987). In such grafts, nerve cells differentiated well for approximately 5 months, but subsequently showed progressive changes in cytoskeletal components. In these different experiments, one of many possible explanations for the late decline in the functional and structural integrity of neurons is that critical influences provided by the blind-ended PN grafts may not continue to be expressed after they are reinnervated; evidence for a decline in the expression of certain molecules within peripheral nerves after regeneration has been described (Muller et al., 1986). Thus, certain trophic influences along these and other pathways may be transiently present and ultimately conditioned to the establishment of terminal contacts with target tissues (Doering et al., 1987). Although a gradual loss of nerve cells has also been reported when axotomized cells do not make terminal connections with the periphery (Lieberman, 1974; Kawamura and Dyck, 1981; Tessler et al., 1985), it has not been determined whether, in PN-grafted retinas, the formation of synaptic contacts with the SC (Vidal-Sanz et al., 1987) prevents the anatomical and electrophysiological changes observed in the axotomized RGCs.

Our results not only indicate that mature RGCs are capable of mounting the metabolic responses to axotomy that permit them to survive injury and regrow lengthy axons, but also imply that early interactions between the RGCs and the PN grafts mitigate the retrograde effects of axotomy on the survival and atrophy of some of these neurons. The mechanisms whereby the PN grafts exert these effects are unknown. Although the differences in neuronal survival between the grafted and nongrafted retinas are well-established by 1 month after injury, when RGC axons are known to have grown into the grafts (Trecarten et al., 1986), the enhanced early survival of RGCs may not depend directly on the extension of their axons into the PN grafts; surface interactions with cellular or matrix elements at the ON–PN graft junction or the diffusion of molecules released by the graft might also play a role in these early effects on the axotomized RGCs. It is also unclear why some RGCs survive after axotomy in the rats without PN grafts; it is possible that there are subpopulations of RGCs with different requirements for survival, that some retinal neurons might be sustained by intraretinal collaterals (Dacey, 1985), or that there are sources of trophic molecules available in the retina, as have been shown in other regions of the injured CNS (Nieto-Sampedro and Cotman, 1985; Manthorpe et al., 1986).

Proportion of surviving RGCs that grow lengthy axons into PN grafts

In previous experiments in which retrograde labeling with HRP was used to identify the RGCs that had grown to the end of PN grafts after 8–10 weeks (Vidal-Sanz et al., 1987), the number of HRP-labeled RGCs per retina ranged from 949 to 12,385 (mean, 3610 ± 633; $n = 20$). Assuming that the population of RGCs in the normal rat retina is 110,000 (Perry, 1981), these rates of axonal regrowth into the PN grafts represent 0.8–11.2% (mean, 3.3%) of the normal RGC population. Similar rates of RGC axonal growth into the PN grafts were obtained for the Group II animals of the present experiments. Using the retrograde transport of fast blue to identify RGCs that had grown to the ends of the PN grafts by 2 months, the numbers of fast blue-labeled cells ranged from 2.0 to 9.2% of control values, with a mean of 4.7%. Thus, with regard to the populations of RGCs in normal rat retinas, the results of these 2 different sets of experiments both indicate similar amounts of axonal regrowth.

When the incidence of RGCs that regrow axons into the PN grafts was expressed as a percentage of the RGCs that survive in these retinas by relating the proportions of RGCs retrogradely labeled with diI at the time of axotomy to the proportions of these neurons labeled with fast blue applied to the distal, blind-ended tips of the grafts (Table 3, E, Fig. 9), the mean axonal elongation among surviving neurons was calculated to be 16, 21, and 18% after 1, 2, and 3 months, respectively. In other words, approximately one-fifth of the RGCs that survived axotomy regenerated lengthy axons into the PN grafts. Because shorter axons that had not grown to the site of application of the tracer would not be labeled by these techniques, it is possible that the actual proportion of the surviving RGCs that regenerate axons into the grafts is even higher.

All RGCs labeled with fast blue applied to the end of the graft were also labeled with diI. Although alternative explanations are possible, this observation suggests that the regrowing RGCs labeled with the diI may constitute the main source for axonal growth.

Survival and regrowth: general comments

The precise components of the PN grafts that are responsible for the enhanced survival and regrowth of axotomized RGCs have not been identified, but there is mounting evidence that

injured or excised segments of peripheral nerve contain NGF and other neurotrophic factors (Riopelle et al., 1981; Richardson and Ebendal, 1982; Varon et al., 1983–1984; Abrahamson et al., 1986; Heumann et al., 1987), as well as molecules whose role in regeneration is under study (Skene and Shooter, 1983; Muller et al., 1986; Ignatius et al., 1987). Because it has not been shown that the RGCs of adult rodents are either responsive to NGF or express NGF receptors, it seems possible that other growth factors released by the PN graft, perhaps acting together with cellular and matrix components (Matthew and Patterson, 1983; Liesi et al., 1984; Carbonetto et al., 1987) of the transplanted PN segments, influence both the survival and growth of the axotomized RGCs exposed to PN grafts. Critical molecular components similar to those present in the degenerating nerve segments used in these experiments may also be expressed in the CNS of developing embryos and in the transplanted fetal neural tissues used by others (Björklund and Stenevi, 1984) to influence neuronal survival and axonal growth. The implantation of grafts of fetal CNS has been reported to protect nerve cells in the injured CNS of neonatal rodents (Cunningham and Haun, 1974; Haun and Cunningham, 1974; Bregman and Reier, 1986).

The administration of specific molecules such as NGF (Hendry and Campbell, 1976; Nja and Purves, 1978; Hefti, 1986; Williams et al., 1986; Kromer, 1987), gangliosides (Cuello et al., 1986), or other substances (for review, see Manthorpe et al., 1986) also reduces the retrograde effects of damage to axons in the PNS or CNS. With reference to the survival of RGCs, it has been demonstrated that the viability of these neurons *in vitro* is enhanced by coculturing with their target tissues (Nurcombe and Bennett, 1981; McCaffery et al., 1982; Armson and Bennett, 1983; Sarthy et al., 1983). Furthermore, the concentration of brain-derived neurotrophic factor (BDNF), which also supports the survival of neonatal RGCs *in vitro* (Barde et al., 1982; Johnson et al., 1986), is apparently enhanced in the superior colliculus (Johnson et al., 1986). The identification and use of specific molecules that influence the responses of injured neurons may eventually lead to a greater enhancement of neuronal survival than that observed with PN grafts alone.

The transplantation of PN segments into several regions of the adult rat CNS has helped document the intrinsic capabilities of different central neurons to regrow lengthy axons and to generate and conduct apparently normal electrical impulses (Keirstead et al., 1985; Munz et al., 1985; Salame and Dum, 1985; Gauthier and Rasminsky, 1987). Moreover, it has been shown in the visual system that some of the regenerated axons make terminal synaptic contacts in the SC when guided to the tectum along PN "bridges" that span the eye and the SC (Vidal-Sanz et al., 1987). However, one of the circumstances that has limited further studies of the appropriateness, persistence, and function of these synaptic contacts is that only few of the regenerated fibers penetrate the SC (Keirstead et al., 1987; Vidal-Sanz et al., 1987). Although conditions at the interface between the PN graft and the CNS may be partly responsible for the small number of retinal axons that re-enter the SC, the present studies indicate a need to develop strategies aimed specifically at increasing the pool of surviving retinal neurons as a potential source of regrowth into the target regions of the CNS. Because most of the RGC loss occurred soon after axotomy near the soma, the enhancement of cell survival would more likely be effective if critical agents were introduced soon after injury.

It is also apparent that, under usual circumstances, the CNS environment that surrounds injured axons offers little support either for axonal regrowth or for RGC survival. As shown in the present experiments using retrograde labeling with diI, more than 90% of the axotomized RGCs died after axotomy near the eye; a similar loss is observed in the nucleus basalis magnocellularis of the adult rat after interruption of its projections in the fimbria-fornix (Hefti, 1986; Williams et al., 1986; Kromer, 1987). However, although intrinsic conditions within the CNS milieu did not appear to modify substantially the retrograde neuronal degeneration that followed axotomy in either of these examples, both these groups of neurons in the adult rat brain have proven capable of overcoming some of the effects of injury when they were provided with critical molecules or with tissues that are presumably the source of these or similar molecules. The development of new quantitative techniques to estimate RGC populations should help document further the extent and persistence of the effects of experimental manipulations aimed at increasing neuronal survival and axonal growth by modifying the responses of injured nerve cells through epigenetic influences that arise from the axonal environment and the target tissues of retinal projections.

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APPENDIX II

Vidal-Sanz, M., M.P. Villegas-Pérez, G.M. Bray, and A.J. Aguayo (1988) Persistent retrograde labelling of adult rat retinal ganglion cells with the carbocyanine dye, dil. Exp. Neurol. 102: 92-101.

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Persistent Retrograde Labeling of Adult Rat Retinal Ganglion Cells with the Carbocyanine Dye dil

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To study the retrograde labeling of intact and axotomized retinal ganglion cells (RGCs) over long periods of time, we applied the carbocyanine dye diI to the superior colliculi (SC) and dorsal lateral geniculate nuclei (dLGN) in adult albino rats and examined the retinas by fluorescence microscopy after different periods of survival. Retrogradely labeled RGCs, which were observed in the retinas as early as 3 days after application of the dye, gradually increased in density so that by 7 days more than 80% of the RGCs were labeled and by 30 days diI-labeled cell densities were similar to those observed after short applications of other tracers. Using short-term retrograde labeling with fast blue (FB) as an independent marker of RGCs, it was determined that these neurons remained labeled with diI for periods of up to 9 months without apparent leakage of the tracer to other retinal cells. In addition, diI labeling persisted in the somata of more than 80% of axotomized RGCs whose contact with the source of label had been interrupted for 3 months. Thus, we propose that retrogradely transported diI is a useful label for quantitative studies of neuronal populations, even after axotomy.

INTRODUCTION

Quantitative studies of the survival of axotomized retinal ganglion cells (RGCs) are difficult because conventional histologic techniques do not completely distinguish RGCs from other retinal neurons (31), particularly the "displaced" amacrine cells that account for approximately 50% of the cells in the ganglion cell layer of the rat (6, 19). Among the neuronal markers presently available, the axonally transported carbocyanine dye diI is a potentially useful label for accurate quantitative assessments of neuronal survival.

DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) is a lipid-soluble substance that has been used to measure lipid mobility in cell membranes, to assess cell fusion (for review, see (10)), and to study growth cone metabolism (11), and that is retrogradely (4, 5, 10, 31, 32) and orthogradely (28) transported in neurons, both *in vivo* and *in vitro* (9–11). The potential advantages of diI as a neuronal tracer substance are its brightness, slow fading during examination, and persistence in neurons without apparent leakage (10). In a previous study in which diI crystals were applied to the transected optic nerves (ONs) of adult rats (31), diI fluorescence was observed in RGCs for up to 3 months but, with this method of application, only one-third of the RGC population was labeled. For the present study, we partially dissolved diI and applied it to the main target nuclei of the RGCs in adult rats to determine if (i) most of the RGCs that project to these nuclei can be retrogradely labeled with diI, (ii) the fluorescent label will persist in the RGCs for longer periods of time without diffusion to other cells, and (iii) axotomized RGCs will also retain the diI label.

METHODS

DiI Application

Adult Sprague–Dawley rats weighing 200–225 g were anesthetized with intraperitoneal cloral hydrate (0.42 mg/g body weight). Both superior colliculi (SC) and dorsal surfaces of the thalami were exposed, the pia overlying the SC and the dorsal lateral geniculate nuclei (dLGN) was carefully removed, and a small piece of Gelfoam (Upjohn, Kalamazoo, MI) soaked in 5 μl of a diI suspension was applied to the surfaces of these structures, which are the main targets of RGCs (14, 16).

The diI suspension was prepared by mixing 3 mg of diI (Molecular Probes, Junction City, OR) in 1 ml of saline containing 1–3% Triton X-100 (New England Nuclear Corp.). Sonication and repeated agitation (10) produced a mixture of dissolved diI and small diI crystals in suspension.

To determine the earliest time at which the retrogradely transported diI would label most of the rat RGCs that project to the SC and dLGN, the dye was applied to both the SC and dLGN of adult rats, and the retinas were examined 3 ($n = 4$), 5 ($n = 4$), 7 ($n = 8$), 20 ($n = 4$), and 30 ($n = 8$) days later. Retinas from other rats were examined at 2 ($n = 2$) and 7 ($n = 2$) months after diI application to assess whether the dye would be retained within the labeled cells for long periods of time without apparent leakage to other neurons of the retina.

Fast Blue Application

To identify the RGC population with a different tracer substance, we applied the retrogradely transported

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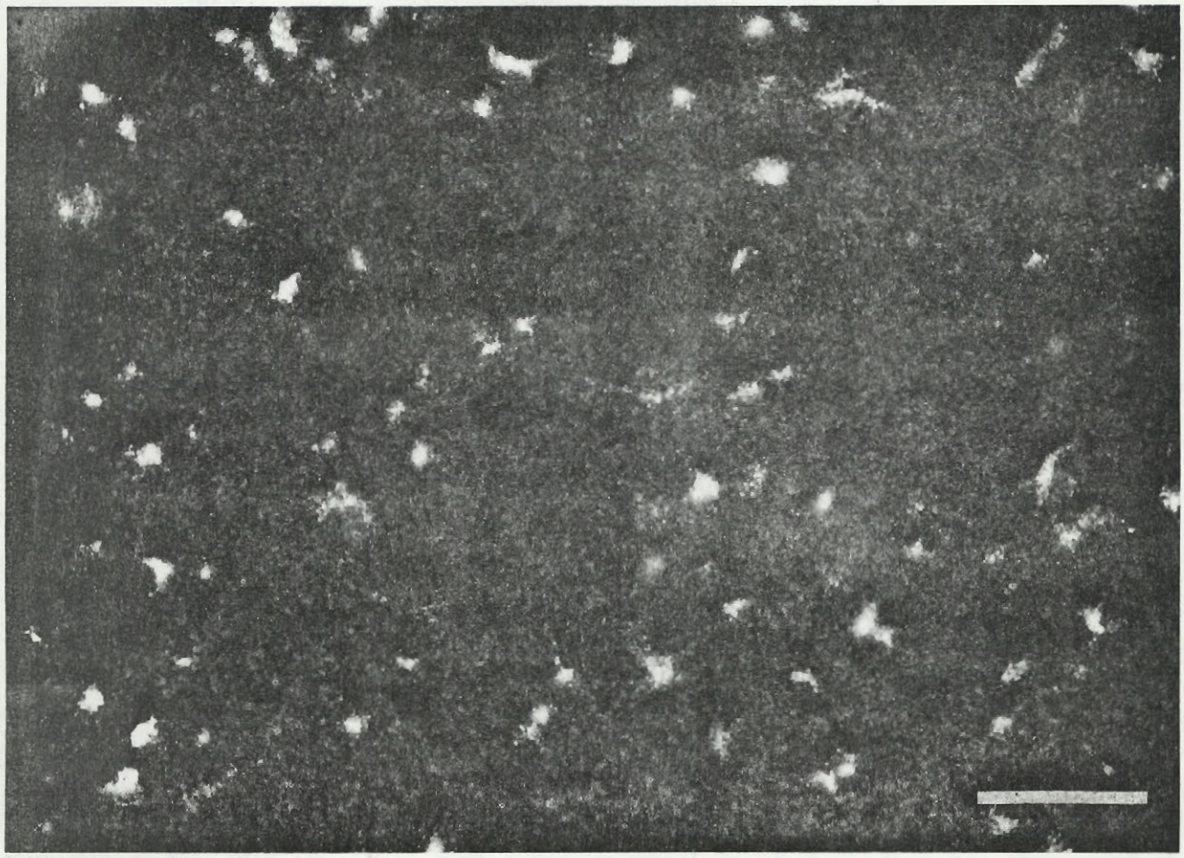


FIG. 1. Seven days after diI application to the SC and the dLGN, cell bodies and some dendrites of neurons in the ganglion cell layer of the superior nasal quadrant of the retina are outlined by punctate diI fluorescence. Fluorescence micrograph. Bar = 50 μm.

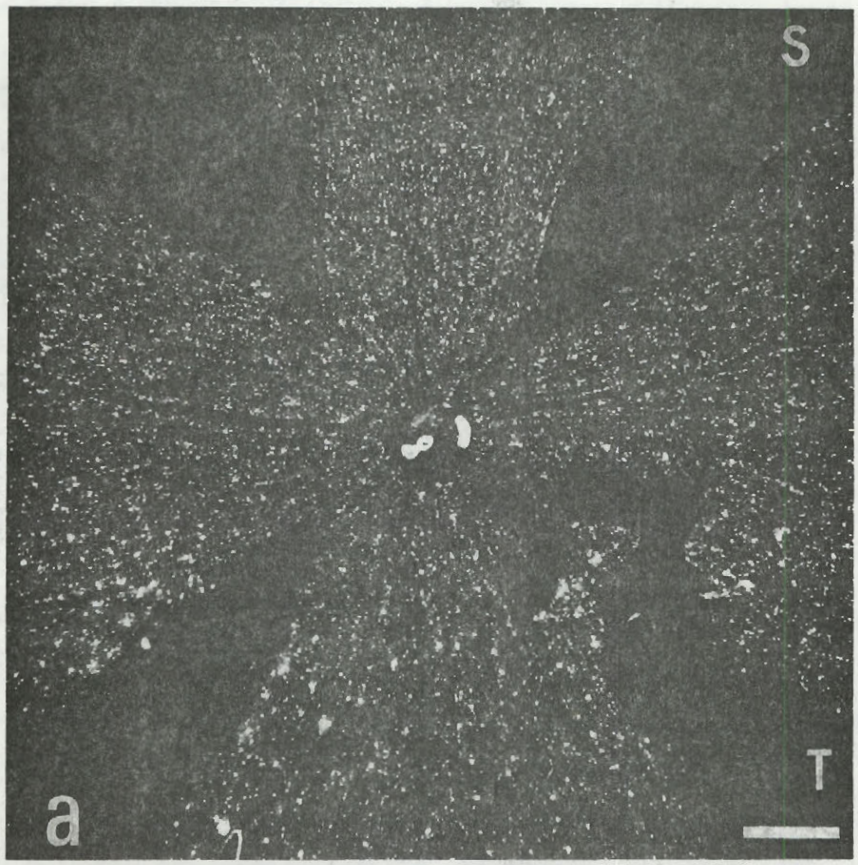
tracer substance fast blue (FB) (27) to the optic nerve (ON) in some of the experimental rats. At various intervals after diI application, the intraorbital portion of the ON was exposed, the dural sheath was opened longitudinally, and the ON was transected 3 mm from the posterior pole of the eye, taking care not to damage the retinal blood supply (30). A small piece of Gelfoam soaked in 3% FB (Dr. Illing GmbH Co., KG, Makromoleculare und Pharmazeutische Chemie, 6114 GroB-Umstadt, FRG) dissolved in 0.9% NaCl was applied to the ocular stump of the ON and left in place until the animal was sacrificed 36 to 48 h later.

Experimental Manipulations

Removal of source of diI labeling. To determine if the continuous uptake of diI by the RGC terminals was necessary for the long-term labeling of retinal neurons, the source of labeling was removed in one animal. Seven days after diI application, the SC and dLGN were reexposed, the piece of Gelfoam and the adjacent brain tissue were removed, and the wound was thoroughly washed with 0.9% NaCl. One month later, both retinas from this animal were analyzed and the number of diI-labeled neurons was estimated.

Intraorbital optic nerve transection. In rats in which diI had been applied to both SC and dLGN for 1 (n = 1) or 9 (n = 2) months, the ON was transected intraorbitally and FB was applied to the ocular stump 36 to 48 h prior to processing (see below) to determine if (i) the diI was labeling most of the RGCs, and (ii) the dye was diffusing to other retinal neurons. When applied as crystals, diI is known for its low diffusion properties (4, 5, 10, 28, 31). In the present experiments, the dye was partially dissolved in an attempt to label the majority of the RGC population that projects to the midbrain. Thus, it was important to assess whether or not the dye would remain within the labeled RGCs over long periods of time without leakage to other nonprojecting retinal neurons. A close correspondence between the ratios of RGCs doubly labeled with DiI and FB would suggest that diI was labeling most of the projecting RGCs and that the dye was not leaking from RGCs to other retinal neurons, even after long periods of time.

Intracranial optic nerve crush. Using repeated crushes with jeweller's forceps to interrupt ON fibers without disrupting the continuity of the dural sheath, the left (n = 3 rats) or both (n = 2 rats) ONs were crushed midway between the optic foramen and the op-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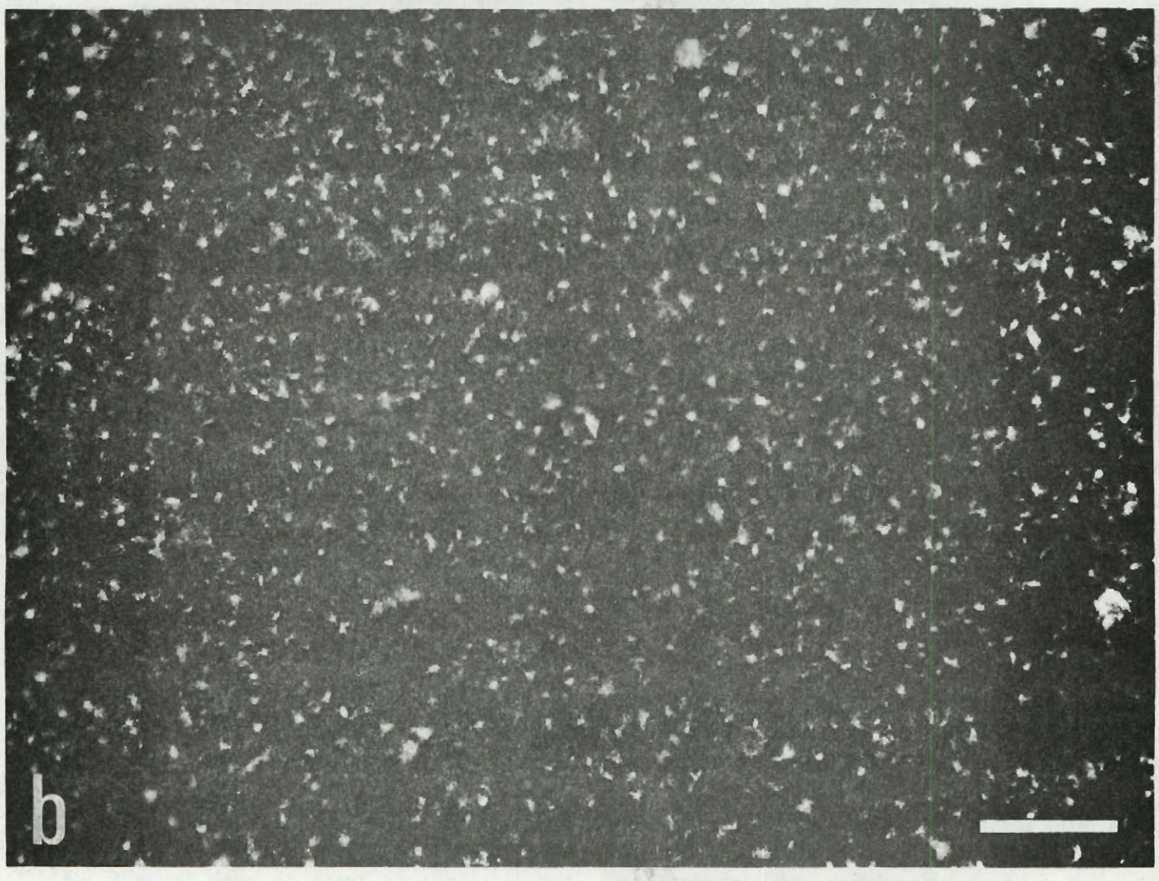
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TABLE 1

Densities of dil-Labeled Cells in the Retina

Interval after dil application	dil-labeled cells/mm²	Mean ± SEM	Statistical significance[a]
5 days	1856		
	1791		
	2259		
	2020	1981 ± 104	
7 days	2107		
	2112		
	2010		*b*
	2004		
	2305		
	1965		
	1943		
	2218	2083 ± 45	
20 days	2158		
	2466		$P < 0.02$
	2232		
	2298	2288 ± 66	
1 month	2261		
	2505		
	2280		
	2523		$P < 0.05$
	2512		
	2616		
	2390		
	2398	2436 ± 44	
2 months	2163		$P < 0.01$
	2104	2133 ± 29	
7 months	2532		
	2415	2473 ± 58[c]	

[a] Student's *t* test.
[b] Not significant.
[c] Not significantly different from one month.

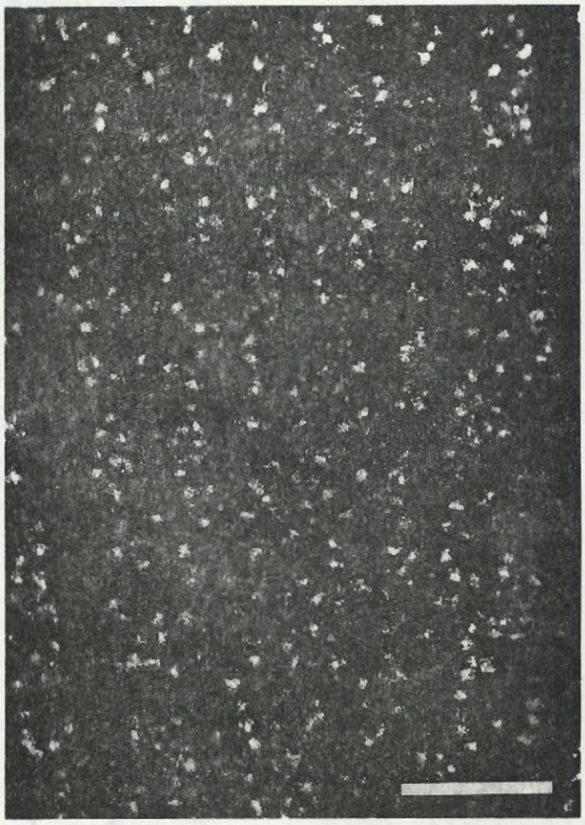

FIG. 3. Seven months after dil application to the SC and dLGN, retrogradely labeled RGCs in the inferior nasal quadrant of the retina still show dense fluorescence. The labeled neurons tend to be aligned in columns by the retinal blood vessels and bundles of axons. Bar = 100 μm.

tic chiasm (i) immediately after dil application with examination of the retinas 7 days later to prove that the labeling of RGCs was due to retrograde transport of the partially dissolved dye, and (ii) seven days after dil application with examination of the retinas 30 or 90 days later to determine if the retrogradely transported label would persist even after axotomy. Two days prior to processing these retinas, the ONs were transected intraorbitally and FB was applied to the ocular stumps to identify RGCs with axons in the ON. Double-labeling of cells in the retina with FB and dil would indicate that they were RGCs that had survived axotomy and retained the dil in their somas.

Retinal Processing

The animals were processed according to previously described methods (30, 31). In brief, the rats were anesthetized with an overdose of chloral hydrate and then perfused through the heart with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer (PB). The retinas were dissected and prepared as flattened whole-mounts, with four radially oriented cuts in each retina, the deepest one indicating the superior pole of the retina. The retinal whole-mounts were postfixed for 1 h, washed for 30 min in 0.1 *M* PB, and mounted on gelatin-coated slides in a 2:3 solution of glycerol in 0.1 *M* sodium carbonate buffer (pH 9.0) containing 0.04% *p*-phenylenediamine (7). The retinas were observed and photographed within 2–3 h by fluorescence microscopy.

Cells labeled with dil can be viewed through the fluorescence microscope with rhodamine filters with maximal absorption at 540 nm and maximal emission at 556 nm (26). Observed through rhodamine filters, dil-labeled cells present a bright orange-red fluorescence; these cells can still be viewed, although much less in-

FIG. 2. DiI fluorescence in retinas 1 month after application of the tracer to the SC and dLGN. (a) Fluorescence micrograph, prepared with the aid of an automatic condenser–scanner device (15), of the central portion of a flattened, whole-mounted retina. The dil-labeled cells are distributed throughout all quadrants of the retina. S, superior; T, temporal. Bar = 500 μm. (b) At a higher magnification, the dil labeling is seen in RGCs of various sizes and in smaller profiles that could only be identified as axons, dendrites, or cell bodies out of the plane of focus by examining the retinas at the magnifications illustrated in Fig. 1. Bar = 100 μm.

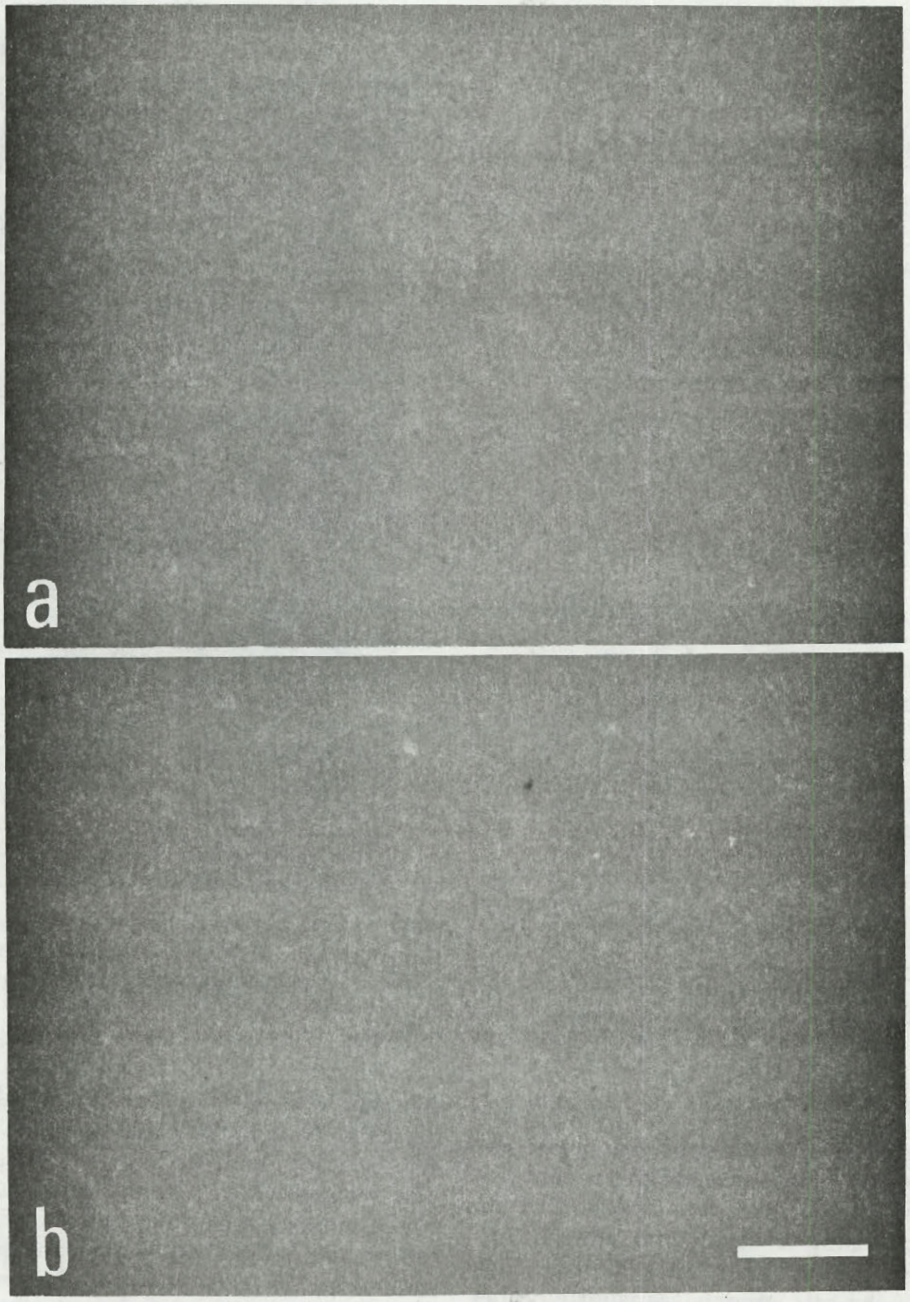

FIG. 4. Retinal neurons labeled with diI (a), applied to the SC and dLGN for 1 month, and with fast blue (b), applied to the intraorbital optic nerve 2 days prior to perfusion–fixation. The same area of the retina was photographed using different filters to visualize the two fluorescent tracers. Both large and small RGCs show the typical diI or fast blue fluorescence. Most of the diI-labeled cells also show fast blue fluorescence, indicating that the diI was confined to RGCs and had not diffused to other retinal cells. Bar = 50 μm.

tensely, with FITC filters and others with shorter wavelengths such as violet or ultraviolet.

When FB was applied, the perfusion liquids were cooled to 4°C and the retinas were maintained at this temperature until photographed to minimize extracellular diffusion of the dye. DiI was observed with an excitation filter of 530–560 nm, and a suppression filter of 580 nm. FB was observed with an excitation filter of 335–425 nm and a suppression filter of 460 nm.

Cell Counts

Three standard areas (0.22 × 0.33 mm) 1, 2, and 3 mm from the optic disc were photographed (×100) in each

TABLE 2

Comparison of diI and Fast Blue Labeling of Retinal Neurons (Labeled Cells/mm^2)

Interval after diI application	diI[a]	FB[b]	Both	A	B
1 month	1938	1866	1832	98.2	94.5
	2487	2443	2408	98.6	96.8
	2384	2157	2139	99.2	89.7
Mean ± SEM	2270 ± 168	2155 ± 167	2126 ± 166	98.7	93.7
3 months	2011	1923	1920	99.8	95.5
9 months	2069	1992	1958	98.2	94.6
	2075	2037	2018	99.1	97.2
Mean ± SEM	2072 ± 3	2014 ± 22	1988 ± 30	98.6	95.9

Note. A, percentage of fast blue-labeled cells that were also double-labeled with diI. B, percentage of diI-labeled cells that were also double-labeled with fast blue.

[a] Applied to both SC and dLGN.

[b] Applied to the intraorbital optic nerve 36–48 h prior to processing.

of the four retinal quadrants (31). These pictures were printed at a final magnification of ×700. The number of labeled cells were counted in each photograph and the densities of labeled RGCs per mm^2 were calculated by averaging the 12 counts. No corrections were made for retinal shrinkage. The Student *t* test was used for statistical analysis.

RESULTS

DiI Labeling of Retinal Neurons

Three days after diI application, a few fluorescent cells, which resembled RGCs in size and shape, were observed in the ganglion cell layer of all quadrants of the retinas. At 7 days after diI application, these RGCs were more intensely labeled than at 3 or 5 days. The retrogradely labeled cells showed a punctate cytoplasmic fluorescence that was most intense in the perikaryal cytoplasm and the proximal dendrites (Fig. 1). In some neurons, the diI fluorescence was observed in the secondary or tertiary dendrites as well as in the proximal segments of axons. Displaced RGCs ("Dogiel cells"), also labeled with diI, were occasionally seen in the inner nuclear layer.

The retrogradely labeled RGCs were distributed throughout each retina (Fig. 2), with the greatest densities in the central areas where RGCs are the most prevalent (19). Although there were variations within each group, the numbers of retrogradely labeled RGCs increased gradually from 3 days to 1 month after diI application (Table 1; Fig. 2). In the retinas of animals in which the dye had been applied for 5 days, the densities of retrogradely labeled RGCs ranged from 1791 to 2259/mm^2 (mean ± SEM, 1981 ± 104). The mean densities of retrogradely labeled RGCs were 2083 ± 45/mm^2 at 7 days, increased to 2288 ± 66/mm^2 at 20 days, and peaked at 2436 ± 44/mm^2 1 month after diI application. The differences in the densities of fluorescent retinal neurons were not significant between 5 and 7 days but the increases at 7, 20, and 30 days were significant ($P < 0.05$).

The densities of retrogradely labeled RGCs, which were maximal at 30 days, were similar 7 months (2473 ± 58/mm^2) after diI application (Fig. 3). For the two animals examined 2 months after diI application, the densities of retrogradely labeled RGCs were 2133 ± 29/mm^2 (Table 1). Possible explanations for these lower values for the 2-month animals include differences in the rate of diI uptake or application in different animals or variations in the extent to which the diI was dissolved.

To confirm that the diI fluorescence seen in the RGCs was due to retrograde axonal transport of the tracer, the left ON was crushed intracranially at the time of diI application in two animals. When examined 7 days later, the left retinas in these animals did not contain any diI-labeled cells, whereas the contralateral retinas showed RGC labeling densities similar to those of the group of retinas examined 7 days after diI application (Table 1).

Comparison of diI and FB Labeling of RGCs

In the retinas doubly labeled by retrograde transport of diI (applied to the SC and dLGN for 1 month) and FB (applied to the ONs transected intraorbitally for 2 days), the FB-labeled RGCs were identified by their intense blue, granular, and diffuse fluorescence within the cell soma and the proximal dendrites (Fig. 4b). The mean density of diI-labeled neurons in the retinas of these animals was 2270 ± 168/mm^2, while the mean density of FB-labeled neurons was 2155 ± 167/mm^2 (Table 2). Most of the FB-labeled RGCs were also labeled with diI and vice versa: 93.7% of the diI-labeled RGCs were also labeled with FB, while 98.7% of the FB-labeled RGCs were also labeled with diI (Table 2; Fig. 4).

Similar findings were observed in the retinas from animals in which diI had been applied for 3 or 9 months and in which FB was applied intraorbitally to the ON 2 days before processing (Table 2). At 9 months, for example, 95.9% of cells labeled with diI also contained FB, while 98.6% of the cells labeled with FB were also labeled with diI.

The results of these double labeling experiments suggest that diI was retained within the RGCs; if the diI had diffused to large numbers of other retinal neurons, the close correspondence between the densities of diI- and FB-labeled cells would not have been observed.

Persistence of DiI Labeling

Two experimental manipulations were carried out to determine if the long-term diI labeling of RGCs depends

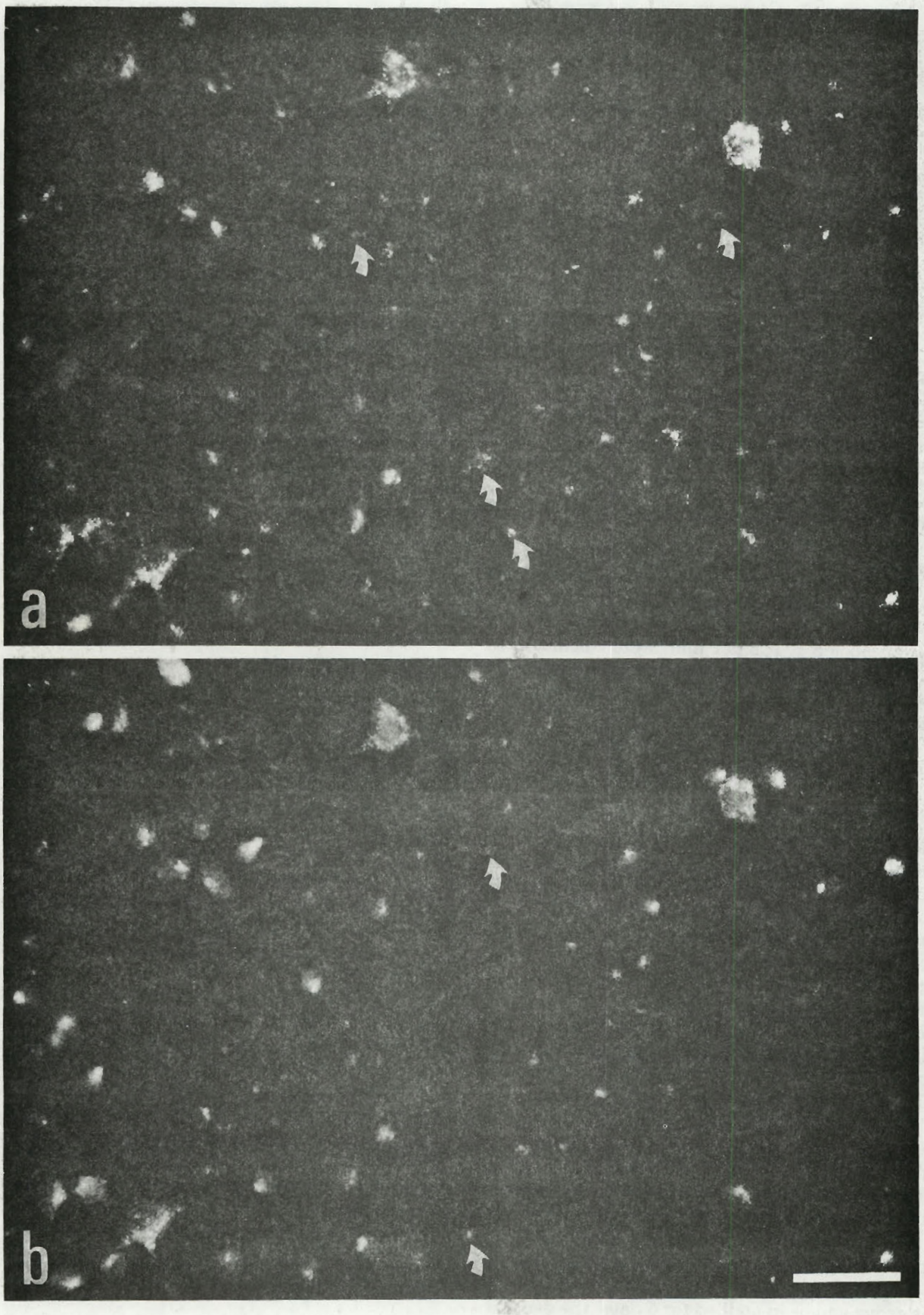
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TABLE 3

Effect of Intracranial Optic Nerve Crush on diI-Labeled Retinal Neurons (Labeled Cells/mm^2)

Interval after axotomy[a]	diI	FB[b]	Both	A	B
1 month	1560	1336	1320	98.1	69.8
	1558	1110	1088	98.8	84.6
	1537	1299	1248	96.1	81.2
Mean ± SEM	1552 ± 7*	1248 ± 70	1219 ± 69	97.7	78.6
3 months	1145	1036	1032	99.6	90.1
	1338	1263	1246	98.7	93.1
	1063	979	979	100.0	92.1
Mean ± SEM	1182 ± 82**	1093 ± 87	1086 ± 82	99.4	91.8

Note. A, percentage of fast blue-labelled cells that were also double-labeled with diI. B, percentage of DiI-labeled cells that wre also double-labeled with fast blue.

[a] Intracranial optic nerve crush 7 days after diI application.

[b] Applied to the intraorbital optic nerve 36–48 h prior to processing.

* Significantly less ($P < 0.001$) than the densities of diI labeled retinal ganglion cells, 7 days after the application of diI to the SC and dLGN (Student's t test).

** Significantly less ($P < 0.03$) than the densities of diI-labeled cells 1 month after ON crush (Student's t test).

on the continuous uptake of the tracer by their terminals of the SC and the dLGN.

DiI removal. In one animal, the source of diI was removed 7 days after its application. Examined 30 days later, the retinas of this animal had densities of diI-labeled RGCs of 2022 and 1896/mm^2. The differences between these values and those obtained in the group of animals 7 days after diI application (2083 ± 45/mm^2; Table 1) were not statistically significant.

Intracranial ON crush. Six retinas were obtained from animals whose ONs were crushed intracranially 7 days after application of diI. Examined 1 (n = 3) or 3 months (n = 3) after intracranial crush injury to interrupt axonal continuity between the retinas and the source of diI in the brain, these retinas still contained many diI-labeled neurons in all retinal quadrants (Fig. 5a). The mean densities of diI-labeled cells were 1552 ± 7/mm^2 at 1 month and 1182 ± 82/mm^2 at 3 months (Table 3). These values represented 75 and 57%, respectively, of the densities of diI-labeled neurons in retinas without axotomy (Table 1, 7 days); such decreases in the densities of diI-labeled cells in the axotomized retinas are consistent with the extent of retrograde neuronal death that follows intracranial transection of the optic nerve (18, 32).

In this group of animals with intracranial ON crush for 1 or 3 months, FB was also applied to the intraorbital ON 36 to 48 h before sacrifice. In these retinas, the mean densities of FB-labeled cells was 1248 ± 70/mm^2 and 1093 ± 87/mm^2 at 1 and 3 months, respectively (Table 3; Fig. 5b). Thus, at 1 month, 97.7% of the FB-labeled cells were also labeled with diI but only 78.6% of the DiI-labeled cells were labeled with FB; at 3 months, 99.4% of the FB-labeled cells were double-labeled with diI, while 91.8% of the diI-labeled cells also contained FB (Table 3; Fig. 5).

The results of these two sets of experiments indicate that the diI persists in most neurons even after the source of the label has been removed or its axonal transport interrupted.

DISCUSSION

In vivo assessments of the effects of trophic substances and neuron–target interactions on neuronal survival require reliable labels that permit the identification and quantitation of normal and experimentally manipulated neuronal populations for long periods of time. For such quantitative studies of RGCs, specific antibodies or retrogradely transported tracer substances are the most likely candidates. RGCs express Thy-1 immunoreactivity that can be detected in tissue culture (2, 23) and in radial or tangential sections of rat retinas (1, 21). However, there are two important limitations to the use of Thy-1 immunoreactivity for quantitative studies of RGCs: cells in the retina other than RGCs may show Thy-1 immunoreactivity and the Thy-1 reaction is less successful for studies of retinal whole-mounts. Another antibody, AB5, specifically labels RGCs in several species but not rats or monkeys (8).

In addition to diI, retrogradely transported tracers that have been used to identify RGCs include horseradish peroxidase (HRP) (3, 13), true blue (12), nuclear yellow (12), fast blue (20, 27), and rhodamine B–isothiocyanate (RITC) (29). Most of these retrogradely transported tracers do not persist in neurons for long periods of time. HRP is metabolized (17) and, after retrograde labeling with fluorescent tracers such as true blue (24) or fast blue (20, 27), there is a tendency for diffusion from neurons into the extracellular spaces and neighboring cells with prolonged survival and during the processing of the tissues.

The aim of the present experiments was to determine if the fluorescent carbocyanine dye diI could be used as a persistent retrograde marker of intact or axotomized

FIG. 5. Persistence of diI labeling in retinal neurons. DiI was applied to the SC and dLGN for 7 days and then the optic nerve was crushed intracranially. Three months later, fast blue was applied to the intraorbital optic nerve. After an additional 2 days, the perfusion-fixed retina was photographed using different filters to visualize diI (a) or fast blue (b) fluorescence. In these photographs of the same retinal areas, most of the diI-labeled cells (a) are also labeled with fast blue (b), indicating that they are neurons with axons projecting along the optic nerve that have also retained the diI. The arrows indicate cells that are not doubly-labeled. Bar = 50 μm.

neurons. For these studies, we used the adult rat visual system, in which most of the RGCs project to the contralateral SC and dLGN (14, 16, 25). In the present study, the mean optimum density of diI-labeled RGCs, obtained at 1 month, was 2436 ± 44/mm^2 for the central regions of the retina (Table 1). These results compare favorably with estimates of the densities of rat RGCs determined with other retrogradely transported tracer substances. Using HRP applied to subcortical visual centers (19) or to the SC (14), the mean densities of retrogradely labeled RGCs were approximately 2500/mm^2 for comparable regions of the retina. When FB, another well-documented retrograde tracer (27), was applied to the SC of four adult albino rats, the densities of retrogradely labeled RGCs ranged from 1879 to 2313/mm^2 with a mean ± SEM of 2116 ± 94/mm^2 (31). Furthermore, when the labeling of RGCs with FB, applied to the intraorbital ON stump, and the labeling of RGCs with diI, applied to the SC and dLGN, were compared in the present experiments (Table 2), similar results were obtained. Thus, the efficiency of long-term labeling with diI appears to be comparable to that of other tracer substances applied for shorter periods.

In addition to documenting the efficiency of RGC labeling with diI, the present experiments also addressed three other issues concerning the labeling of RGCs by diI applied to their main target regions: the evidence that the diI reached the RGC cell bodies by retrograde transport; the possibility of leakage of the dissolved diI from RGC cell bodies to other retinal cells; and the persistence of the label in neuronal cell bodies, even after axotomy.

Evidence for Transport of diI

The gradual increases in the intensity of diI labeling and the numbers of labeled cell bodies suggested that the diI was being retrogradely transported from the site of application. The observation that transection of one ON at the time of diI application prevented the diI labeling of RGCs further supports the concept that the labeling of RGCs by diI applied to the target regions of their axons is due to retrograde axonal transport rather than some other mechanism such as a hematogenous spread.

Possible Diffusion of diI from RGC Cell Bodies

Although it has been reported that diI applied in fixed tissues can diffuse orthogradely or retrogradely for distances of 10–12 mm over periods of several weeks (9), *in vivo* studies suggest that the dye, at least when applied as crystals, does not diffuse from the site of application for more than 1.5 mm (4, 5, 31). Thus, it is probable that only cell processes that are in direct contact with the diI are labeled (10). In the present experiments, in which the diI was partially dissolved, it was also important to exclude the possibility that the increase in the number of fluorescent cells in the retinas was due to passive diffusion from labeled RGCs to other retinal neurons or glia. Evidence against such diffusion comes from an analysis of the densities of doubly labeled neurons in the retinas that had been labeled first with diI for 1, 3, or 9 months and then with FB applied to intraorbitally transected ON 48 h prior to sacrifice (Table 2). In these retinas, there was a close correspondence between the densities of FB-labeled cells that were also diI labeled, indicating that FB was able to label 98% of the RGCs labeled with diI. Because approximately 95% of the diI-labeled RGCs were also labeled with FB at 1 and 9 months after application (Table 2), it is reasonable to suggest that most of the dye was retained in the RGCs to which it had been retrogradely transported and that little, if any, had diffused to other retinal neurons.

Persistence of diI Labeling

In the experiments in which the RGC axon terminals remained in contact with the source of diI, the fluorescent material seen in the neuronal cell bodies in the retina could have been due to continuous transport rather than to persistent labeling. Evidence indicating that diI persists in RGCs was provided by the experiments in which continuous labeling was prevented either by removing the source of diI or by interrupting its transport 1 week after application and examining the retinas 1 or 3 months later (Table 3). In both these circumstances, many diI-labeled cells were observed in the retinas.

In addition, when FB was applied to the ON intraorbitally 48 h prior to sacrifice, the high proportion of cells doubly labeled with diI and FB confirmed that at least 80–90% of the cells in which the diI had persisted for up to 3 months were actually RGCs (Table 3). Although we do not have a definite explanation for the approximately 10–20% of the diI-labeled cells that were not labeled with FB, it is possible that some diI leaked from the axotomized RGCs and was taken up by other retinal cells, that some of the interrupted RGC axons had retracted their axons from the site of FB application, or, as has been shown for HRP (22), that their capacity for retrograde axonal transport had been altered.

In summary, the results of these experiments have documented that the application of the fluorescent tracer diI to the SC and dLGN resulted in the retrograde labeling of RGCs whose densities at 30 days are similar to those obtained with other retrogradely transported tracers applied for shorter periods of time. Furthermore, the diI fluorescence was retained in approximately 95% of surviving RGCs for intervals of up to 9 months and persisted without detectable leakage in at least 80–90% of identifiable RGCs for as long as 3 months after the interruption of their connections with the source of labeling. Thus, we suggest that the application of diI to axon terminals may be a reliable technique for the iden-

fication of other axotomized neurons over long periods of time.

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