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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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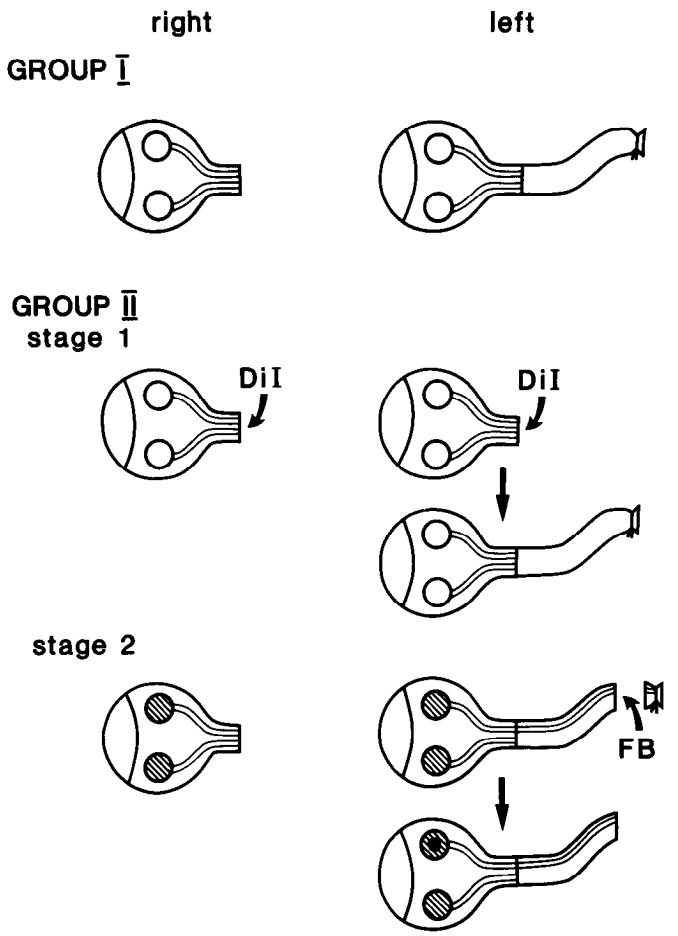

Figure 1. Diagram of the methods used to study the effects of axotomy and peripheral nerve (PN) grafting on cells in the retinal ganglion cell (RGC) layer. *Group I,* One or both optic nerves (ONs) were transected near the eye and an autologous segment of PN was sutured to the ocular stump of the left 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

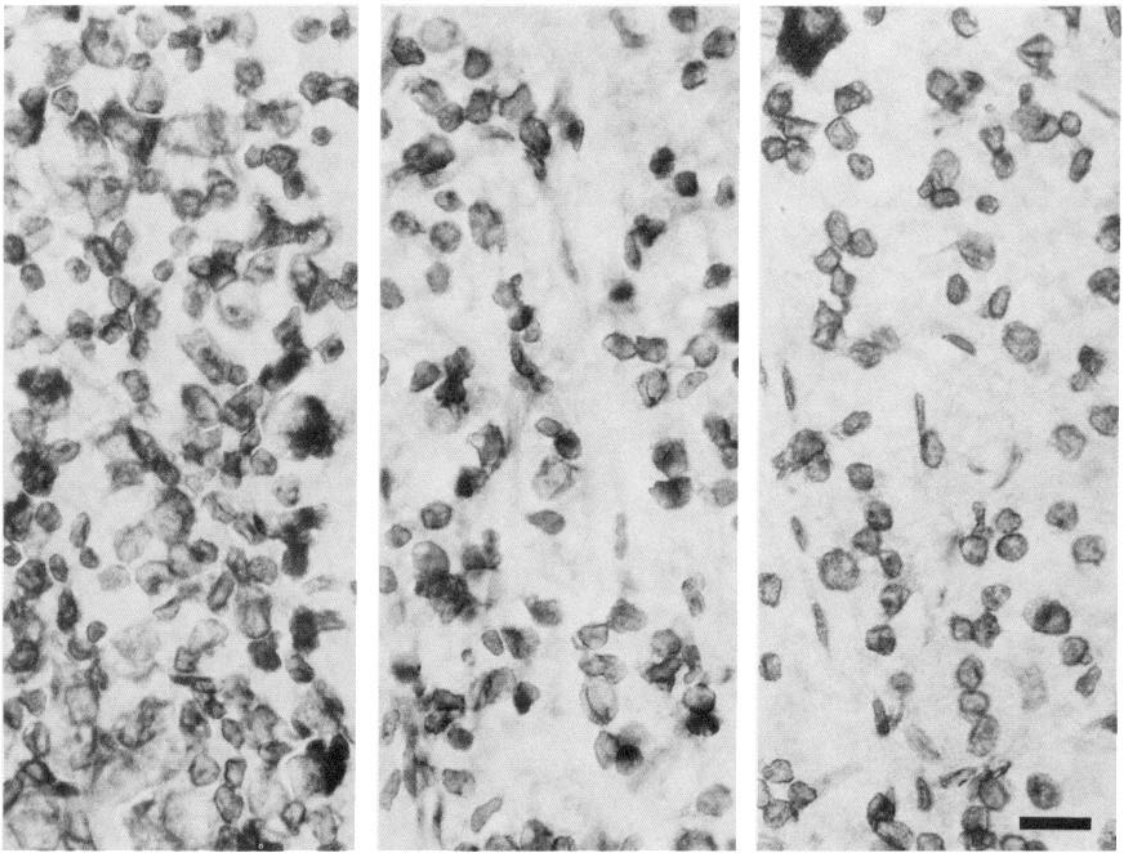

Figure 2. Portions of the ganglion cell layer photographed 1 mm from the optic disc in the superotemporal quadrant of flat-mounted, methylene blue-stained retinas. *Left,* Control (not axotomized). *Middle,* Axotomized for 1 month. *Right,* Axotomized and PN-grafted for 1 month. Bar, 20 μm.

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; $p < 0.05$.)

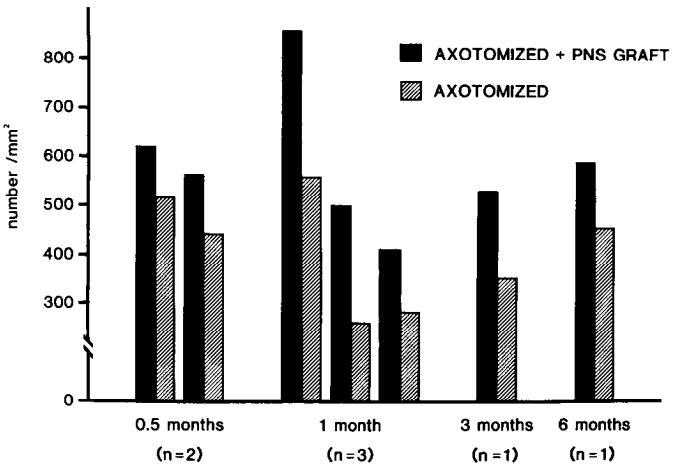

Figure 3. Densities of neurons greater than 80 μm^2 in the ganglion cell layer of methylene blue-stained retinas from adult rats in which both ONs were transected and a PN graft attached to the left ON stump. Each pair of *bars* depicts the data from one animal. The density of these large neurons is 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).

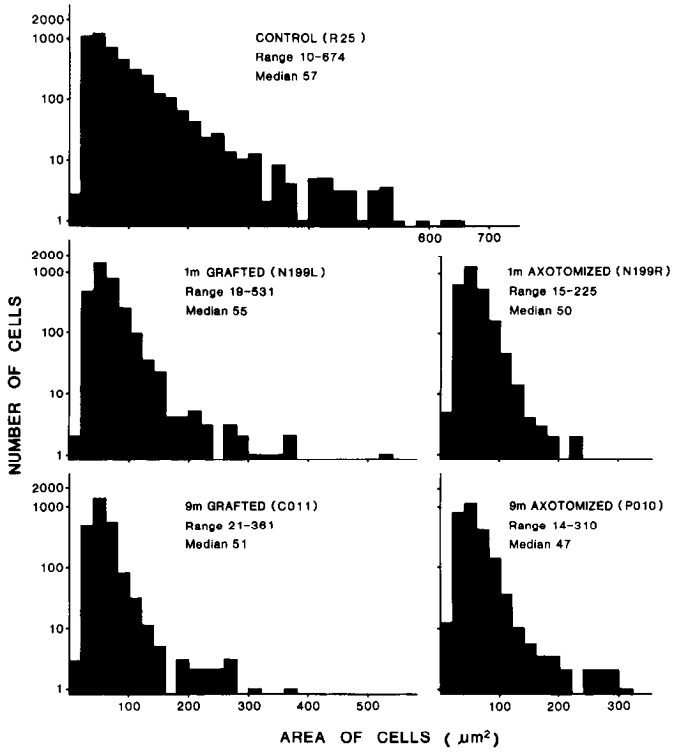

Figure 4. Representative size–frequency histograms for neurons in the ganglion cell layer of individual control and experimental retinas. Perikaryal sizes of all neurons in photographs of representative areas of each retinal quadrant were processed with an IBAS-I image analysis system. Number of neurons 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.

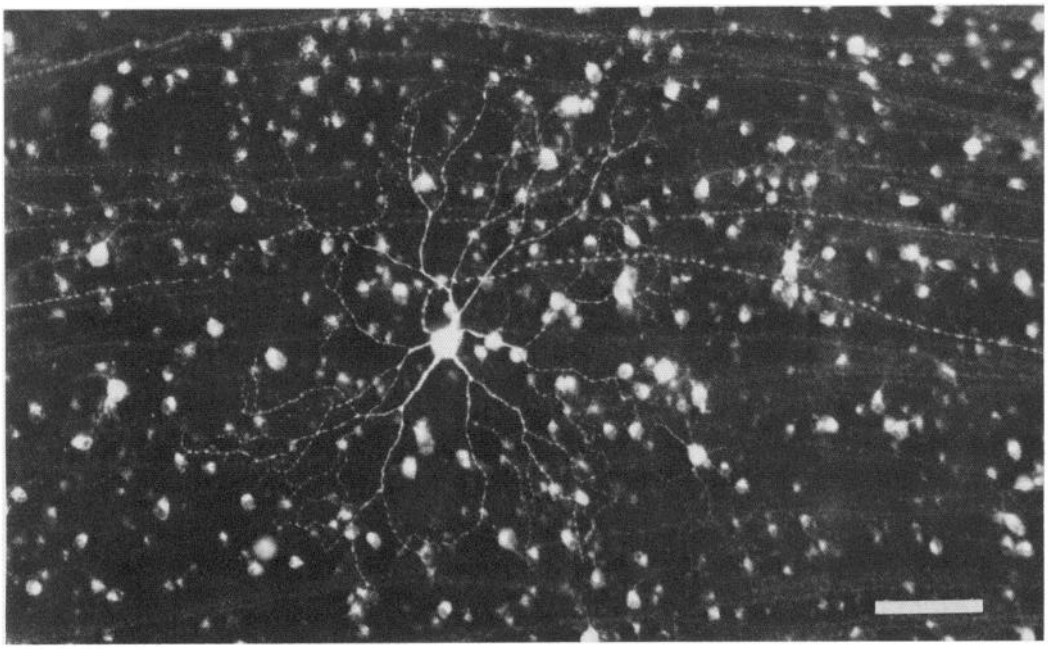

Figure 5. Neurons in the RGC layer 48 hr after the ON was transected and dil crystals were applied to the ocular stump. The perikarya and axons of many neurons are labeled with the retrogradely transported tracer. The dendrites of one neuron in this photograph are outlined by discontinuous labeling with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

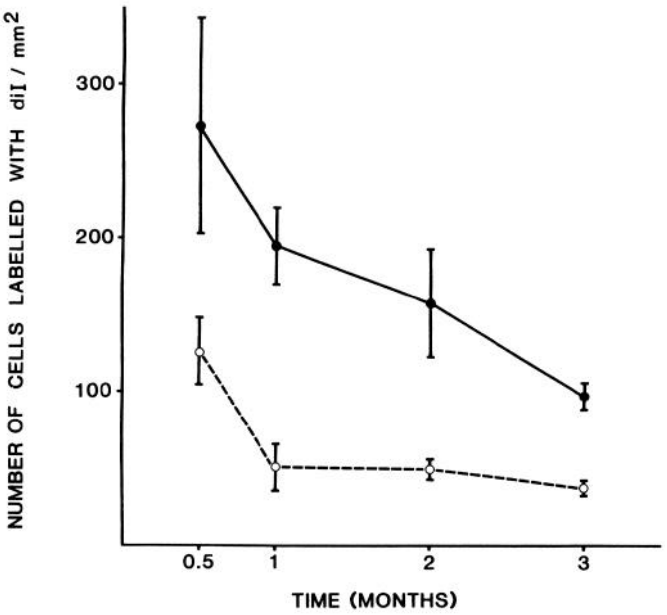

Figure 6. Densities of diI-labeled RGCs, expressed as means ± SEMs, in the retinas of adult rats following ON transection with (*filled circles*) or without (*open circles*) attachment of PN grafts. The differences between the densities of surviving RGCs in the retinas with and without grafts were statistically 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.

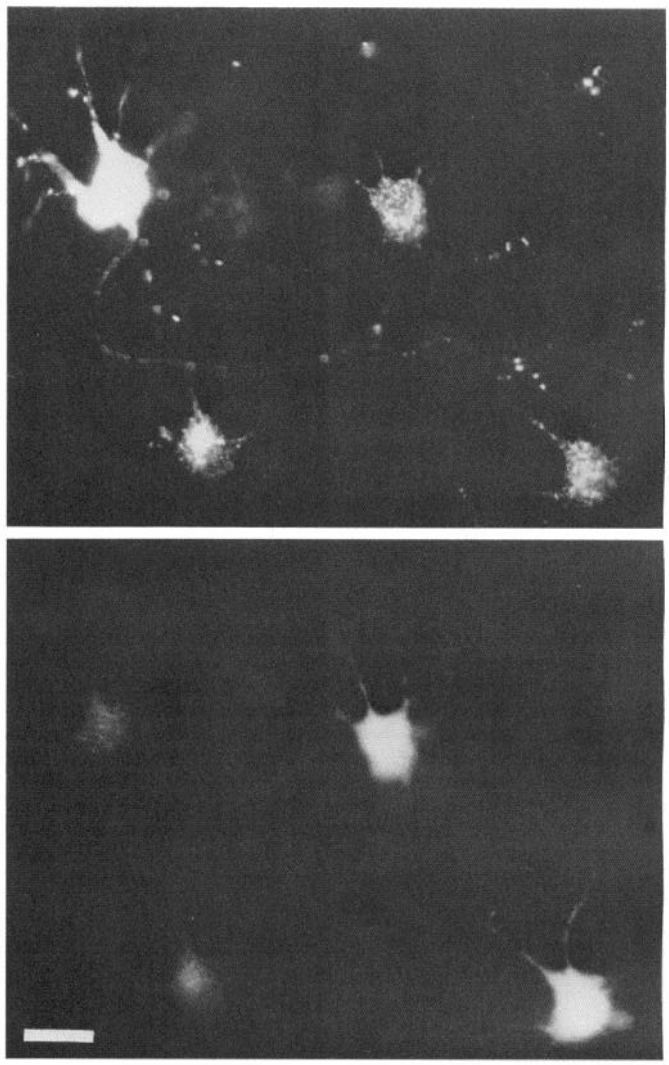

Figure 7. RGCs retrogradely labeled with diI applied to the ocular stump of the ON at the time of transection, and with fast blue applied to the end of the graft 1 month later. *Upper,* The cell bodies of 4 neurons, as well as the proximal axon of one neuron, show dense fluorescence with diI. This retina 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

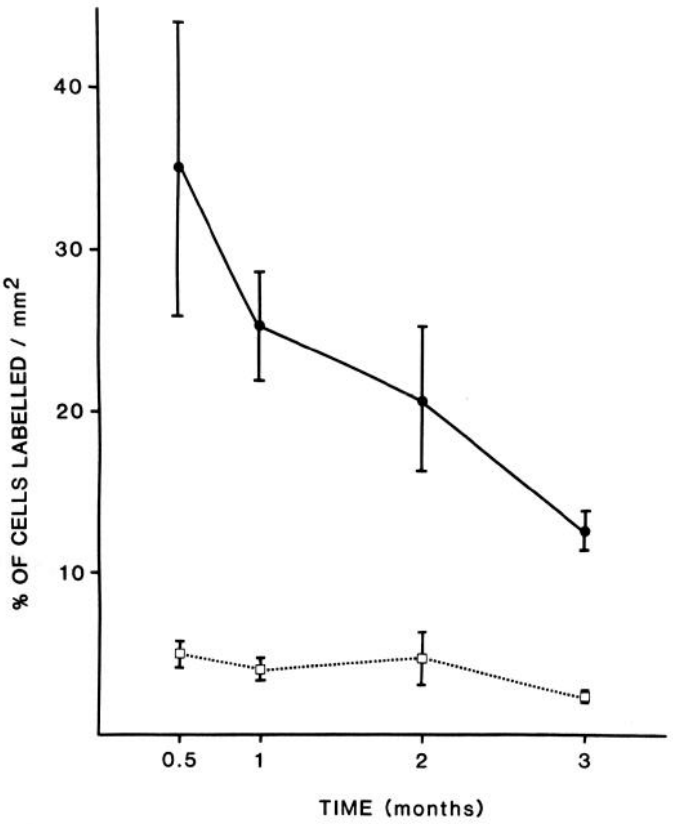

Figure 8. Densities of dil-labeled (*filled circles*) and fast blue-labeled (*open squares*) RGCs, expressed as proportions of the respective labeling of control retinas with these tracer substances in the retinas of adult rats following ON transection and attachment of PN grafts. Although the densities 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).

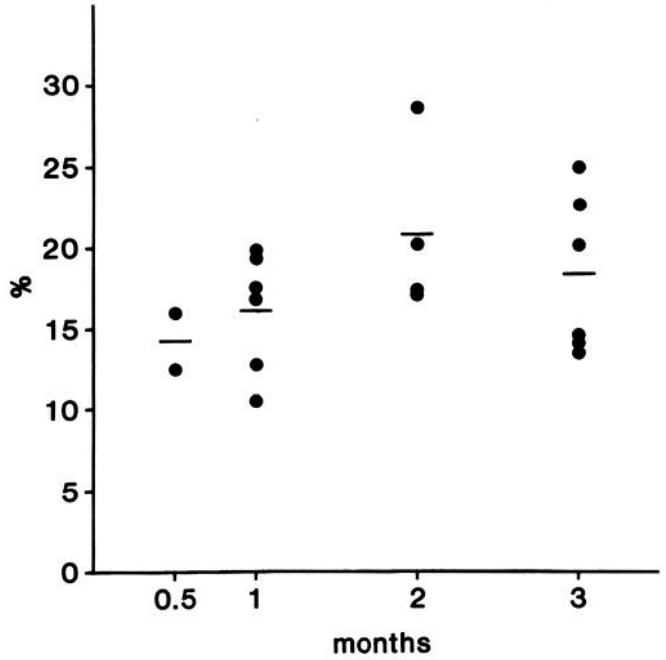

Figure 9. Incidence of axonal regeneration among surviving retinal ganglion cells (RGCs). The *data points* for individual animals represent the proportions of dil-labeled cells that were also labeled with fast blue. The *horizontal bars* indicate the mean for each time interval. Approximately 20% of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

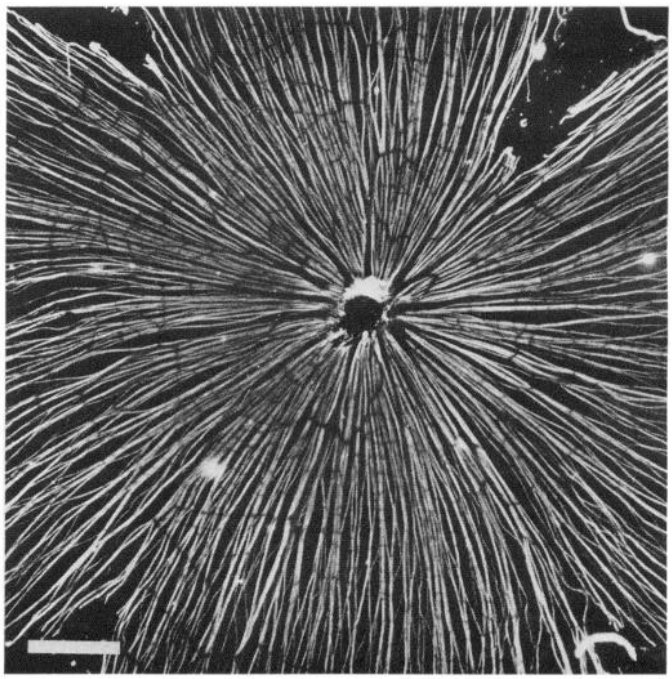

Figure 10. Intraretinal axons of RGCs in a control retina, delineated by their immunoreactivity to RT 97, a monoclonal antibody that reacts with phosphorylated 200 kDa neurofilament subunit, as visualized with FITC-labeled secondary antibodies. Thick bundles of axons course towards the optic disc. Bar,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

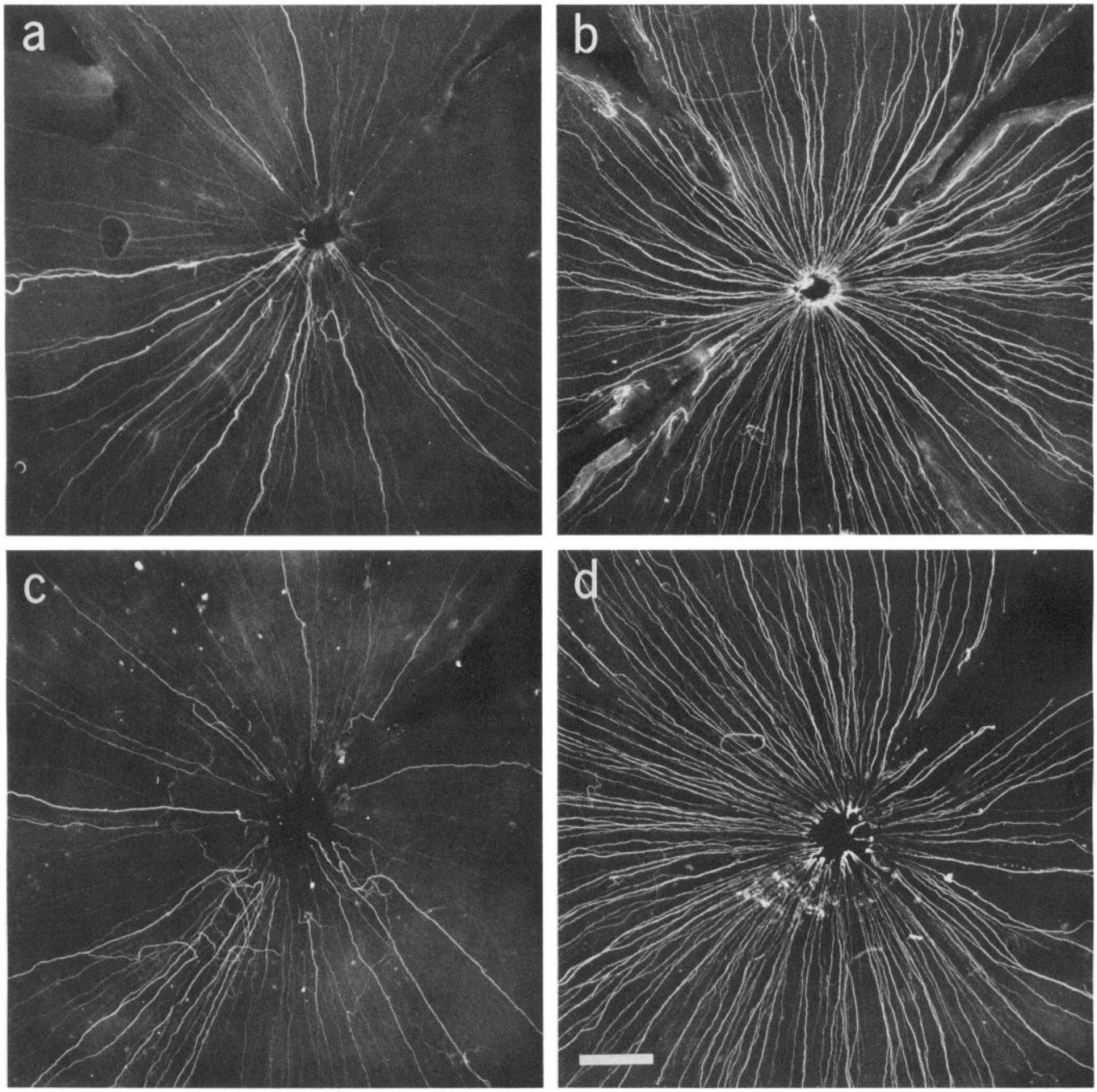

Figure 11. Appearance of the intraretinal axons of the RGCs immunoreacted with RT 97 (see Fig. 10) in the experimental retinas 6 (*a, b*) and 9 (*c, d*) months after axotomy with or without PN grafting. The density of axons is reduced and many individual axons can be identified. There are more immunoreactive 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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