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Schwann cells promote the survival of rat retinal ganglion cells after optic nerve section

(degeneration/transplant/nerve growth factor)

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ABSTRACT **Schwann cells (SCs) are known to play an important role for the regeneration of mammalian peripheral nerves. Their effect is likely due to the production of neuronotrophic and/or supportive factors. Here we study the effect of intraocular transplant of SCs on the survival of rat retinal ganglion cells (RGCs) after the intracranial section of the optic nerve. SCs were injected intraocularly in adult hooded rats. Surviving RGCs were retrogradely labeled with horseradish peroxidase applied to the proximal stump of the optic nerve. Results show that intraocular transplants of SCs promote the survival of a large number of RGCs for periods as long as 9 and 14 weeks after optic nerve section. In experimental retinae, surviving RGCs were 2- to 8-fold more numerous than in controls. This finding suggests that SCs are the source of factors that promote the survival of RGCs. Nerve growth factor is produced by SCs, and the intraocular injection of nerve growth factor has been previously shown to promote RGC survival. The rescuing effect of SCs on RGCs is greater than that obtained by intraocular injection of nerve growth factor. This greater effect may be due to the action of other neurotrophic factors produced by SCs or by transplanted SCs producing NGF in a sustained fashion.**

The limited capacity of central nervous system (CNS) neurons to regenerate is thought to be due in part to the presence of nonpermissive factors such as myelin-producing oligodendrocytes (1) or to the absence of neurotrophic factors, or to both (2). In the peripheral nervous system, where regeneration takes place, Schwann cells (SCs) play an important role in promoting regeneration (3). To some extent they also can promote regeneration of CNS neurons. A peripheral nerve segment grafted to the optic nerve promotes partial survival and regeneration of rat retinal ganglion cells (RGCs) (4). This effect is supposed to be due to nonneuronal cells, presumably SCs, present in the graft (4, 5). The positive effect of SCs on regeneration and survival of injured neurons is believed to be due to the production of supportive or neuronotrophic molecules, or both. For instance, there is evidence indicating that SCs produce nerve growth factor (NGF) or a NGF-like protein *in vitro* (6) and after peripheral nerve injury *in vivo* (7, 8). Recently, it has been shown that repetitive intraocular injections of NGF enhance the survival of axotomized rat RGCs (9). In the present study we test the hypothesis that intraocular transplant of SCs promotes the survival of rat RGCs after axotomy.

Our results show that SCs are dramatically effective in rescuing RGCs from degeneration. Whereas in control retinae only 5% of the total population of RGCs survive 9 weeks after optic nerve section, in experimental retinae survival of RGCs was enhanced 2- to 8-fold. In experimental retinae RGCs also display a normal soma size. Comparable effects of transplanted SCs on RGC survival were obtained also 14 weeks after optic nerve section.

MATERIALS AND METHODS

Rat Primary SC Cultures and Transplant Procedure. Schwann cells were prepared from neonatal hooded rat sciatic nerves by the method of Brockes *et al.* (10). In brief, pooled nerves were incubated three times for 15 min with phosphate-buffered saline containing 0.25% trypsin and 0.1% collagenase. The enzymatic dissociation was stopped by the addition of 2 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Cells were dissociated by trituration through a syringe with a 22-gauge needle, passed through a 20-μm Nytex sheet, centrifuged, resuspended in DMEM/10% FCS, and counted. Approximately 10 μl of a suspension containing 2×10^5 cells [90% S-100-positive cells (10) as judged after 24 hr in culture] were injected intraocularly in adult hooded rats under ether anesthesia by means of a 25-μl Hamilton syringe. The tip of the needle was inserted under microscopic guidance through the dorsal limbus of the eye. The injection was performed several days before the section of the optic nerve. In some cases two injections were made, one before and the other at the time of optic nerve section. In two experiments injections were carried out by using SCs rendered nonviable by prolonged trypsin exposure.

Animals. Experiments were performed on adult male Long–Evans hooded rats. The section of the optic nerve was performed intracranially as described (9). A total of 8 optic nerve-section retinae with no transplant or with transplant of nonviable SCs and 11 optic nerve-section retinae with transplant of viable SCs were investigated. Survival times were 9 and 14 weeks. The retina of three normal adult rats was also examined. One retina with transplanted SCs (SC retina) was used for immunocytochemical studies 14 weeks after optic nerve section.

Histology and Analysis of Surviving RGCs. Twenty-four to 36 hr after the application of horseradish peroxidase (HRP), animals were sacrificed with an i.p. injection of chloral hydrate and immediately perfused with normal saline followed by 1.5% paraformaldehyde/0.1 M phosphate buffer, pH 7.4. Retinae were dissected and whole-mounted as described by Perry and Cowey (11). The survival of RGCs optic nerve-section retinae with and without transplanted SCs was assessed by using a double-staining procedure: retrograde transport of HRP applied to the proximal stump of the optic nerve (9) and cresyl violet staining. The staining with cresyl violet was performed after processing the retina for peroxidase histochemistry as described by Perry and Linden (12). Soma size of HRP-labeled cells was taken from camera lucida drawings at ×1420 magnification. The equivalent circle diameter of cell profile was calculated from the drawings with an Ibas-1 image-analyzing system. All of the HRP-labeled cells within the field restricted

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Abbreviations: SCs, Schwann cells; RGCs, retinal ganglion cells; NGF, nerve growth factor; HRP, horseradish peroxidase.

by an eyepiece graticule (0.00958 mm^2) were drawn. Counts of the absolute number of cells that were >15 μm in diameter were made by drawing all of the apparently large cells in each retina at ×560 magnification and then measuring cell diameter by means of the Ibas-1 computer. Counts of cell densities were made with the use of the graticule at ×1420 magnification. Sample fields (equal number for all retinae) were taken along four fixed axes at 0.12-mm intervals across the nasal and temporal retina from the optic disk to the periphery. Three normal retinae were also analyzed.

The number of surviving RGCs in control retinae 9 weeks after intracranial section of the optic nerve was 107 ± 17 when assessed in HRP preparations and 220 ± 33/mm^2 when assessed in cresyl violet preparations. Several authors in the literature reported results similar to ours (5, 13, 14), whereas others using DiI (1,1-dioctadecyl-3,3,3′,3′-tetramethylin-

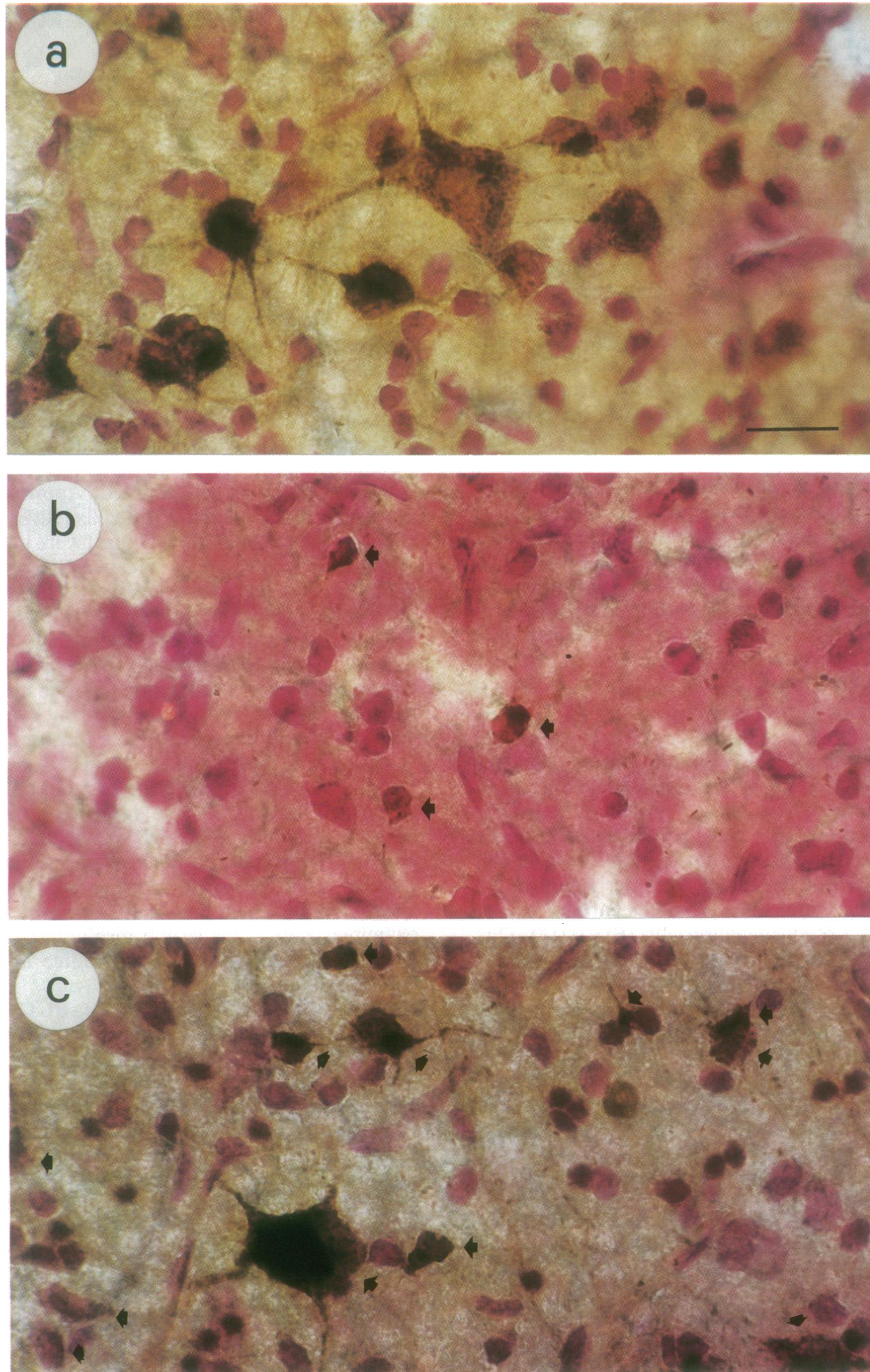

FIG. 1. Representative regions of the ganglion cell layer of a retina of an adult rat whose optic nerves were not transected (*a*) or of adult rat retinae with transplanted viable SCs (*c*) or nonviable SCs (*b*) 9 weeks after optic nerve section (*b* and *c*). Arrows indicate HRP-labeled cells. In optic nerve-section retina, the density of neurons is much lower with respect to either normal retina or retina with viable SC transplants, and HRP-labeled cells are very rare. (All photographs are at the same magnification; bar = 20 μm).

docarbocyanine perchlorate) staining recently reported higher numbers of surviving RGCs (16). It is possible that with our technique we underestimated the number of surviving RGCs for the following reasons. (*i*) The application of HRP to the degenerating stump of the optic nerve might underestimate the number of surviving RGCs because some of them may not possess an axon within the optic nerve able to transport retrograding HRP. (*ii*) In cresyl violet preparations, we considered only cells with a soma diameter > 8 μm. This was done to exclude from our counts displaced amacrine cells. However, this procedure excludes also a class of small RGCs with a soma diameter < 8 μm. (*iii*) Our criteria in counting surviving RGCs in cresyl preparations were rather conservative in that only cells with an intact membrane profile and without signs of degeneration at the level of either the cytoplasm or the nucleus were counted.

The question of the absolute number of surviving RGCs after axotomy remains an open one in that it may be bound to the methodology used to assess it. In our experiments the same staining and counting procedures were used in both control and experimental animals.

Immunocytochemistry. The cell suspension derived from sciatic nerves was immunocytochemically characterized by the S-100 antibody (Dako, Copenhagen), which specifically stains SCs (10). Immunocytochemical techniques (15) using S-100 were also used to identify the presence and location within the retina of transplanted SCs. An adult animal 14 weeks after optic nerve section and transplantation of SCs was perfused with phosphate-buffered saline followed by 4% paraformaldehyde. The eye cup was then dehydrated with a series of alcohols and embedded in Paraplast. Transverse sections (10 μm) were incubated overnight at 4°C in S-100 antibody at a dilution of 1:1000 in normal goat serum (1%). After washing, sections were incubated for 2 hr in secondary antibody at a dilution of 1:80 and for an additional 2 hr in peroxidase-antiperoxidase immunocomplex. Sections were then stained with 3,3′-diaminobenzidine tetrahydrochloride solution (0.05%) for 10 min (15).

RESULTS AND DISCUSSION

The degeneration of rat RGCs 9 weeks after section of the optic nerve is well advanced. A representative region of the ganglion cell layer of a retina 9 weeks after optic nerve section is shown in Fig. 1*b*. The density of neurons is much lower than that of the normal retina (Fig. 1*a*) and, in particular, retrograde labeled cells after application of HRP to the optic nerve are rare (arrows). The Nissl-stained cells with a small soma, which are present at this stage of degeneration, are mostly glial and amacrine cells (13). A representative region of the ganglion cell layer of an experimental retina 9 weeks after axotomy is shown in Fig. 1*c*. This retina had received the transplant of $\approx 2 \times 10^5$ SCs several days before optic nerve section. Here the density of neurons is greater than in optic nerve-section retina with transplant of nonviable SCs (Fig. 1*b*), and several HRP-labeled RGCs can be seen (arrows). Cell size distributions for the same retinae as in Fig. 1 (normal, optic nerve-section, and SC-transplanted retinae) are shown in Fig. 2. Histograms of normal and SC-transplanted retinae are quite similar, while the cell size distribution of optic nerve-section retina with transplant of nonviable SCs is drastically shifted toward small-size cells.

The localization of SCs within the retina 14 weeks after transplant was analyzed in cross sections of the retina. It reveals that SCs proliferate from the site of injection towards the papilla lying on the fiber layer. Fig. 3*a* shows SCs lying on the fiber layer in cresyl violet preparations. The immunopositivity to S-100 antibody demonstrates both the presence and viability of SCs 14 weeks after optic nerve section (Fig. 3*b*). SC proliferation into the host retina, as judged ophthalmoscopically by the presence of white plaques on the

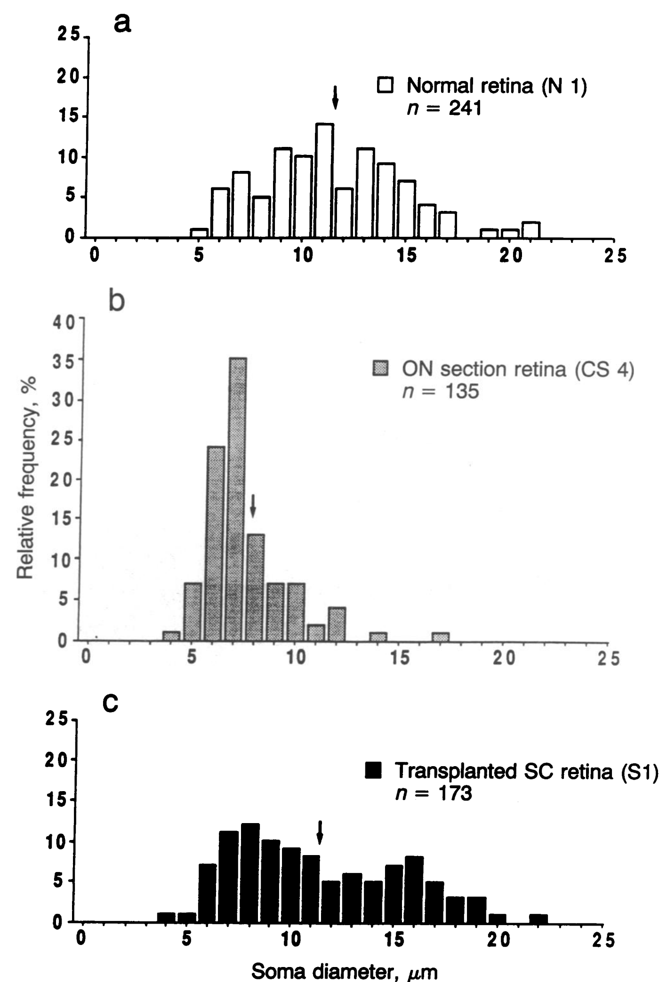

FIG. 2. Soma size distribution of HRP-labeled cells: normal retina whose optic nerve (ON) was not sectioned (*a*) or retina without (*b*) or with (*c*) transplanted viable SCs 9 weeks after optic nerve section (*b* and *c*). (Representative regions of the ganglion cell layer of these retinae are shown in Fig. 1.) With respect to normal retina, the optic nerve-section retina without transplanted viable SCs shows a strong atrophy of surviving cells and the disappearance of large cells. In the optic nerve-section retina with transplanted viable SCs, the size distribution of surviving cells is undistinguishable from that in normal retina. Arrows indicate the values of the median. Similar results were obtained in all optic nerve-section and SC-transplanted retinae. Numbers in parentheses refer to an animal code.

fundus of the eye (17), was variable from case to case and in most cases was different in different parts of the retina. The effect of transplanted SCs on the survival of RGCs is related to their localization in the retina and degree of proliferation. Sectors of the retina devoid of SCs show reduced or absent RGC survival. The figures for RGC survival reported in Table 1 refer to the number of surviving RGCs sampled over the entire retina, including the regions devoid of SCs. In one successful case, in which SC proliferation was particularly evident and uniformly diffuse throughout the retina, we counted 851 HRP-labeled RGCs per mm^2 9 weeks after optic nerve section. This value represents 44% of the total number of RGCs in the normal retinae (1933 ± 212 cells per mm^2). This high percentage of surviving RGCs is also found in the other retinae when only the retinal fields where SC proliferation is clearly present are considered. Results in detail are reported in Table 1. The number of surviving RGCs (HRP-labeled cells plus cells stained with cresyl violet) was greater than that obtained by considering only HRP-labeled cells in optic nerve-section retinae both with and without SC transplant. The total number of RGCs > 15 μm in soma diameter

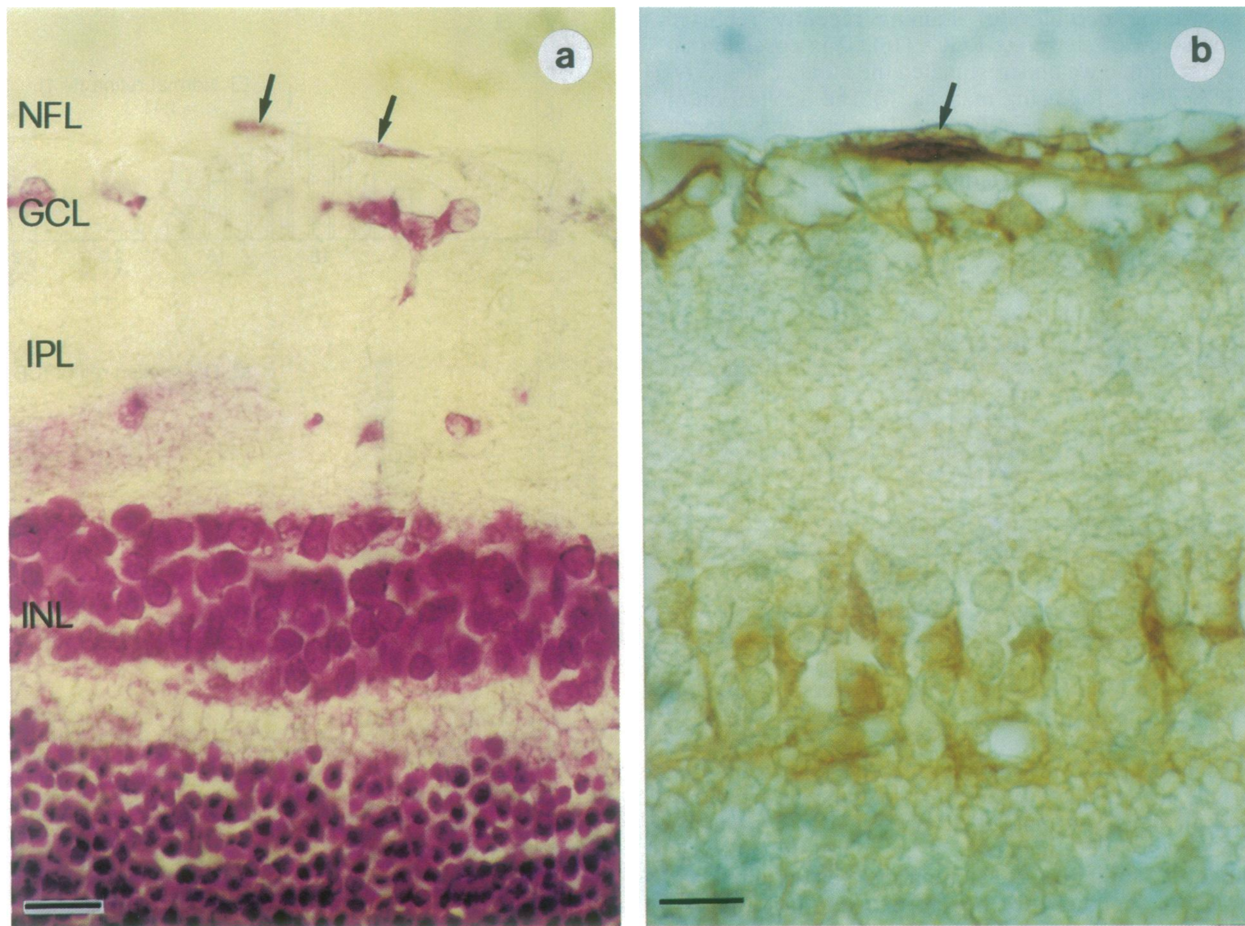

FIG. 3. Two representative transverse sections of a SC-transplanted retina 14 weeks after optic nerve section. SCs (arrows) with the characteristic spindle-shape appearance are localized on the fiber layer (NFL). Sections are stained with cresyl violet (*a*) or processed for S-100 immunocytochemistry with the peroxidase-antiperoxidase method (*b*). Note that the end-feet and soma of Müller cells are also immunopositive. (Bars = 10 μm.) GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer.

counted in each retina is also reported. Large cells are practically absent in controls, whereas they are rather numerous in experimental retinae.

Results also show that the effect of SCs on RGC survival cannot be attributed to a nonspecific response because injections of an exogenous protein, cytochrome *c* (9), or nonviable SCs (CS4 and CS5; see Table 1) do not promote RGC survival. However, the possibility that injections of live SCs can trigger an immunoresponse and therefore the production of lymphokines that are known to influence neurotrophic factors *in vivo* cannot be excluded (18).

Results are summarized in Fig. 4. The density of neurons, expressed as the number of cells per mm^2, is much higher in SC-transplanted retinae with respect to controls: 9 weeks after optic nerve section, cells labeled with HRP are 435 ± 71 and 107 ± 19, respectively. These values correspond to 22.5% and 5.5%, respectively, of the total number of HRP-labeled cells in the normal retina. We also analyzed the survival of RGCs 14 weeks after axotomy in three optic nerve-section retinae without SC transplant and three SC-transplanted retinae. In experimental retinae the mean number of Nissl-stained cells in the RGC layer with a soma diameter > 8 μm is not reduced with respect to that observed at 9 weeks (573 ± 23 vs. 601 ± 99). In control retinae this number is 192 ± 20.

The effect of SCs on optic nerve fibers at the electronmicroscopic level was also examined. Results (unpublished data) indicate that the number of myelinated axons in the proximal stump of the optic nerve is much higher in experimental animals than in control animals. The number of

Table 1. Survival of retinal ganglion cells 9 weeks after optic nerve section in adult rats

Retinae	No. of HRP-labeled cells per mm^2	No. of HRP-labeled/ Nissl-stained cells > 8 μm in diameter per mm^2	No. of cells > 15 μm in diameter per retina
Normal			
N1	1782	2034	1233
N2	2351	2547	1689
N3*	1665	—	1211
Mean ± SEM	1933 ± 212	2290 ± 256	1286 ± 185
Control			
C1	97	142	14
C2*	102	—	9
C3	58	203	12
CS4†	118	302	7
CS5†	162	232	55
Mean ± SEM	107 ± 17	220 ± 33	19 ± 9
SCs transplanted			
S1	313	425	304
S2	851	1061	255
S3	313	414	261
S4	361	471	439
S5	386	634	705
S6	449	602	689
S7*	372	—	577
Mean ± SEM	435 ± 71	601 ± 99	476 ± 75

*In these retinae, cresyl violet staining was not performed.
†Retinae with transplant of nonviable Schwann cells.

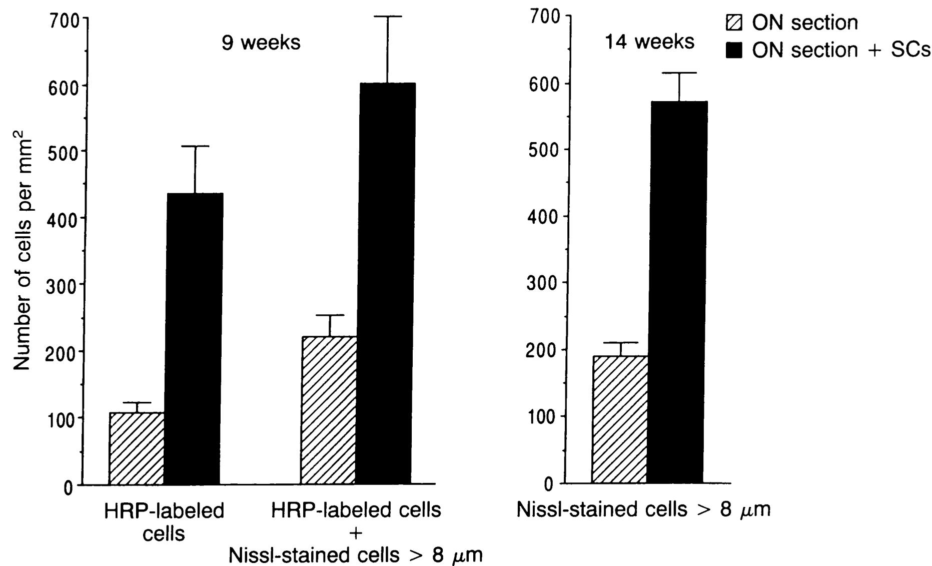

FIG. 4. Density of RGCs for retinae with and without transplanted SCs 9 and 14 weeks after optic nerve (ON) section. The rescuing effect of SC transplant on axotomized RGCs is dramatically evident when one considers both HRP-labeled cells and HRP-labeled cells plus Nissl-stained cells with a soma diameter > 8 μm ($P < 0.005$ and $P < 0.02$, respectively; Student's t test). Data from retinae 14 weeks after optic nerve section were only collected from Nissl-stained preparations ($P < 0.005$; Student's t test).

surviving fibers is quantitatively related to that of surviving RGCs when evaluated in the same preparations.

The precise mechanism by which SCs exert this effect is unclear. It is likely due to their releasing neurotrophic factors. Indeed, this hypothesis is made likely by the observation that the rescuing effects of SCs is particularly evident when their proliferation is high. SCs are known to produce NGF or an NGF-like protein *in vitro* (6) and after peripheral nerve injury *in vivo* (7, 8). The NGF receptor has been reported to be present in the rat retina during development (19). We recently found that the NGF receptor and its mRNA are also present in the retina of the adult rat following optic nerve section. The NGF receptor is localized at the level of the Müller cells and in a subpopulation of RGCs (unpublished data). This raises the possibility that the action of SCs on RGC survival could be mediated, at least in part, by NGF.

The number of surviving RGCs in SC-transplanted retinae is, however, higher than that observed after intraocular injection of NGF (9). In those experiments, an average of 19% with a maximum of 20% surviving RGCs was found 7 weeks after optic nerve section, whereas in the present experiment, an average of 22.5% with a peak of 44% surviving RGCs was determined for longer times of survival (9 weeks). We already pointed out that values between 30% and 40% surviving RGCs can be obtained in all retinae when the quantitative analysis is limited to retinal sectors where SC proliferation was present.

The more successful effect of SCs when compared with exogenously supplied NGF could be due to several factors. SCs could provide NGF in a more continuous way, while the intraocular administration of NGF was performed every other day. SCs represent *per se* (20), or via the production of extracellular matrix components (21, 22), a suitable substrate for neurite outgrowth *in vitro*. SCs are also known to produce other still-undetermined neuronotrophic factors (6), which could play a role in the survival of axotomized RGCs.

An interesting observation of the rescuing effect of SCs is that their action does not seem to decrease with time. Indeed, we observed that the number of surviving RGCs in experimental retinae was approximately the same 9 and 14 weeks after optic nerve section.

The question remains open whether RGCs in SC-transplanted retinae are still visually responsive a long time after optic nerve section. In recent experiments in our laboratory, we have clearly shown that the pattern electroretinogram in response to gratings of different spatial frequencies, which is mainly related to RGC activity (23), is present 4 months after optic nerve section in SC-transplanted retinae and absent in controls (unpublished data).

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