GAP-43 Immunoreactivity and Axon Regeneration in Retinal Ganglion Cells of the Rat

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SUMMARY

Retinal ganglion cells (RGCs) in rats were retrogradely labeled with the fluorescent tracer Fluorogold (FG) and subjected to GAP-43 and c-JUN immunocytochemistry to identify those RGCs that are capable of regenerating an axon. After optic nerve section (ONS) and simultaneous application of FG to the nerve stump (group 1 experiments), GAP-43 immunoreactive RGCs (between 2 and 21 days after ONS) always represented a subfraction of both FG-labeled (i.e., surviving) RGCs and RGCs exhibiting c-JUN. GAP-43 immunoreactive RGCs represented 22% of RGCs normally present in rat retinae and 25% of surviving RGCs at 5 days after ONS but were reduced to 2% and 1%, which is 6% and 5% of survivors at 14 and 21 days, respectively. In animals that received a peripheral nerve (PN) graft after ONS (group 2 experiments), RGCs with regenerating axons were identified by FG application to the graft at 14 and 21 days. When examined at 21 and 28 days, all FG-labeled RGCs exhibited GAP-43 immunoreactivity, and FG/GAP-43-labeled RGCs were 3% and 2% of those present in normal rat retinae. In relation to surviving RGCs GAP-43 immunoreactive RGCs represented 10% at both time points. FG-/GAP-43-labeled RGCs also exhibited c-JUN, but c-JUN immunoreactive RGCs were at both time points at least twice as numerous as FG-/GAP-43-labeled RGCs. These data suggest that regenerating axons in PN grafts derive specifically from GAP-43 reexpressing RGCs. Appearance of GAP-43 immunoreactivity may therefore identify those RGCs that are capable of axonal regeneration or sprouting. © 1994 John Wiley & Sons, Inc.

Keywords: **rat optic nerve lesion, peripheral nerve graft, axonal regeneration, GAP-43/c-JUN immunocytochemistry, retinal ganglion cell quantification**

INTRODUCTION

Several observations suggest that not only environmental factors but also intrinsic properties of neurons may contribute to the success or failure of axonal regeneration (Fawcett, 1992; Stuermer et al., 1992).

Lesioned axons in the mammalian visual system form regenerative sprouts that in the nonpermissive extraneuronal central nervous system environment, fail to elongate (Ramon y Cajal, 1968). However, some retinal ganglion cells (RGCs) succeed in regenerating long axons when they are offered a peripheral nerve (PN) graft in place of the optic nerve (Vidal-Sanz et al., 1987). The number of RGCs that regenerate axons represents less than 5% of the entire population (Villegas-Perez et al., 1988). In fact, most RGCs in the mammalian retina die after axotomy (Thanos et al., 1989; Villegas-Perez et al., 1993). The regenerating cells, referred to here as "competent," may possess specific properties distinguishing them from other RGCs (Stuermer et al., 1992).

How these competent neurons differ from others is obviously a relevant question, especially in attempts to increase the number of axon-regenerating neurons (Thanos et al., 1989; Bähr et al., 1992; Mey and Thanos, 1993). One way to identify competent neurons is to determine whether they have the ability to up-regulate specific pro-

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teins thought to be required for axonal growth and elongation. Two proteins known to be up-regulated by rat RGCs on injury, even in the absence of grafts, have been considered as potential markers for competent RGCs: the growth-associated protein GAP-43 (Skene and Willard, 1981; Jacobson et al., 1986) with a potential function for axon regrowth and sprouting (Meiri et al., 1986; Skene, 1989; Doster et al., 1991; Aigner and Caroni, 1993) and c-JUN (Herdegen et al., 1993; Hüll and Bähr, 1994), a transcription factor of the immediate early gene family (Ryseck et al., 1988). Up-regulation of these same two proteins also occurs in RGCs of fish (Benowitz and Lewis, 1983; Herdegen et al., 1993), which regenerate their injured axons spontaneously and to full recovery of function. GAP-43 immunoreactivity appears in axons in the retina on optic nerve section (ONS) (Benowitz and Lewis, 1983; Doster et al., 1991) and persists (in some RGCs) over months. A quantification of GAP-43 immunoreactive RGCs over time after ONS has, to our knowledge, not been made. Moreover, whether GAP-43 identifies competent RGCs and whether regenerating axons in PN grafts derive from GAP-43 expressing RGCs is still unknown.

c-JUN is reexpressed by most RGCs after ONS (Herdegen et al., 1993), but its expression is sustained only in a subpopulation of RGCs. It was suggested that long-lasting c-JUN expression may be indicative of axonal sprouting (Herdegen et al., 1993). Accordingly, since RGCs with axons in PN grafts represented a high percentage of c-JUN expressing RGCs, it has been proposed that the regenerative state might correlate with long-lasting expression of c-JUN (Hüll and Bähr, 1994).

In the search for markers of RGCs that do regenerate their axons in mammals, we examined whether GAP-43 or c-JUN immunoreactivity delineates a specific group of RGCs. Based on findings already cited, we would predict that regenerating axons (in the case of grafts) derive from them exclusively.

In the first group of animals, we determined the number of GAP-43 immunoreactive RGCs and their relation to surviving and to c-JUN immunoreactive RGCs between 2 and 21 days after optic nerve cut. In the second group, we examined whether regenerating axons in animals with PN grafts derive selectively from RGCs expressing GAP-43, c-JUN, or both.

As in the report by Hüll and Bähr (1994), we used Fluorogold (FG; Fluorochrome, USA) to identify RGCs by retrogradely labeling them through their axons in the optic nerve. Since FG vanishes from RGCs with time and since more and more microglial cells exhibit FG with time after lesion (Hüll and Bähr, 1994), we examined only retinae between day 2 and day 28 after dye application.

METHODS

Surgical Techniques and *In Vivo* RGC Labeling

The left optic nerves of adult female Wistar rats (200–300 g body weight) were cut intraorbitally and 16 animals received a sciatic nerve graft according to the procedure of Villegas-Perez et al., (1988). All surgery was performed under deep chloral hydrate anesthesia (420 mg/kg body weight) and in compliance with animal welfare legislation. Retinae were examined ophthalmoscopically through the lens immediately after surgery to ascertain that the blood supply was normal. Roughly 1 μl of the retrograde tracer FG 5% in phosphate buffered saline (PBS) was applied by holding a small piece of surgical cotton soaked in FG solution for 5 min against the eye-sided optic nerve stump at the time of ONS in group 1 (n = 15) and group 3 (n = 8) animals. Group 3 animals received in addition a sciatic nerve transplant to determine the influence of the graft on RGC survival at postoperative days 21 and 28. Operations of and data collection from group 3 animals were done in the course of an earlier study that determined the influence of the graft on RGC survival (Hüll and Bähr, 1994). Although counts of FG-labeled RGCs at 21 days after surgery were published earlier (Hüll and Bähr, 1994) those at 28 days were not. Since RGCs tend to loose FG gradually over time, RGCs at 28 days were more weakly labeled than those at 21 days. Consequently, the RGC cell counts at 28 days may slightly underestimate the number of surviving RGCs at this time point (see also Hüll and Bähr, 1994).

In group 2 animals (n = 8) FG was applied to the sciatic nerve graft. An incision was made in the grafted nerve, 8 mm behind its anastomosis to the optic nerve stump at 14 and 21 days after grafting to apply FG (as already described) and to label those RGCs that had regenerated an axon into the graft (Hüll and Bähr, 1994). The dye is taken up by the injured axons and retrogradely transported to the RGCs.

The labeling efficiency of FG was previously compared to that of other dyes (Hüll and Bähr, 1994) and found to correspond to that of DiI after injection into the superior colliculus. Moreover, numbers of FG-labeled RGCs at 2 days after ONS of the present study corresponded to those in Hüll and Bähr (1994) and to counts

of earlier studies that are discussed in detail in Hüll and Bähr (1994).

The animals were perfused transcardially (4% paraformaldehyde in PBS) under deep anesthesia. Those in group 1 (three specimens at each time point) were sacrificed 2, 5, 8, 14, and 21 days after axotomy. Those in group 2 (four specimens each time point) were sacrificed at 21 and 28 days to allow sufficient time (7 days) for retrograde transport of FG.

Histological Procedures

The posterior eye cup of the isolated eye was separated from the cornea and lens, postfixed for 12 h in the same fixative used for perfusion, and cryoprotected by immersion in 30% sucrose (w/v) in PBS overnight. Sagittal cryostat sections, 10 μm, were collected on polylysine-coated slides, permeabilized in methanol (5 min, −20°C), rinsed in PBS and preincubated in 2% goat serum (30 min). They were exposed to monoclonal antibody against GAP-43 (Schreyer and Skene, 1991; kindly provided by P. Skene) overnight at 4°C (dilution 1:100.000), and after rinses in PBS incubated in polyclonal anti-c-JUN antibodies (Herdegen et al., 1993; dilution 1:1000) for 36 h at 4°C. The specificity of polyclonal antibodies against c-JUN has been shown previously by immunoprecipitation and *in vivo* preabsorption experiments (Herdegen, et al., 1991; Kovary and Bravo, 1991). Secondary antibodies were fluorescein isothiocyanate-coupled goat anti-rabbit and rhodamine isothiocyanate-coupled goat anti-mouse antibodies (1:200, Dianova) applied for 2 h at room temperature. FG-labeled and c-JUN and GAP-43 immunostained RGCs were counted and photographed with 20× and 40× lenses under epifluorescence in a Zeiss Axiophot equipped with the appropriate filter sets.

RGC Counts and Statistical Analysis

Labeled RGCs were counted on every other section (n = 4) of serial sections through each retina. Only those sections that contained the optic nerve head as a standard anatomical reference were used. Counts per retina were averaged by dividing the number of RGC by the number of sections examined.

The number of FG-labeled RGCs (FG-RGCs) at 2 days after axotomy was taken as 100% and FG-RGCs at later times as well as GAP-43 and c-JUN immunoreactive RGCs were expressed as percentages (including standard deviations) of those at 2 days. In addition, GAP-43 and c-JUN immunoreactive RGCs were compared with FG-labeled (surviving RGCs) at relevant time points after ONS. Counts at different time points and of group 1, 2, and 3 experiments were subjected to statistical analysis using the two-sided Student's t test.

RESULTS

Relationship of GAP-43 Immunoreactive to FG-Labeled and c-JUN Immunoreactive RGCs after Optic Nerve Cut

Application of FG to the optic nerve stump at the time of ONS permitted identification of surviving RGCs by their content of tracer in sections between 2 and 21 days after ONS (group 1 experiments). Exposure of these sections to c-JUN and GAP-43 antibodies revealed those FG-RGCs that were reexpressing one or both proteins at selected time points during this interval.

Although nearly all FG-RGCs exhibited c-JUN immunoreactivity at 2 days after ONS, their numbers decreased with time (Hüll and Bähr, 1994). GAP-43 immunoreactive RGCs always represented a subpopulation of FG- and c-JUN-labeled RGCs. In all sections examined, all GAP-43–positive RGCs carried c-JUN immunoreactivity and FG label. This is exemplified in Figure 1 by a section of retina at 5 days after ONS. The relationship of GAP-43 immunoreactive RGCs to c-JUN and FG-labeled RGCs is demonstrated in Figure 2.

Consistent with a recent report (Hüll and Bähr, 1994), the number of RGCs labeled with FG and c-JUN decreased most dramatically between days 5 and 8 after ONS [Fig. 2(a)]. FG-labeled RGCs at 2 days were taken as 100% because the number of the labeled RGCs at this time was similar to that given previously as the total number of RGCs in rat retinae (Hüll and Bähr, 1994). Compared to FG-labeled RGCs at 2 days after ONS (100%), FG-RGCs fell to 90% (±6%) at 5 days and 43% (±5%) at 8 days. c-JUN immunoreactive RGCs whose number at 2 days was close to that of FG-RGCs fell to 64% (±4%) at 5 days and 20% (±4%) at 8 days. FG-RGCs further decreased to 27% (±4%) and 21% (±2%) at 14 and 21 days, respectively, and at both time points to 5% (±2%) in the case of c-JUN immunoreactive RGCs. The loss of RGCs with time after optic nerve lesion seen here is in the range of that reported previously (Barron et al., 1986; Bray et al., 1987; Sievers et al., 1989; Mey and Thanos, 1993; Villegas-Perez et al., 1993; Hüll and Bähr, 1994). This also applies to the decrease of c-JUN immunoreactive RGCs (Hüll and Bähr, 1994). c-JUN as well as GAP-43 (see later) immunoreactive cells were related to FG label and thus surviving RGCs are noted at the relevant time points [Fig. 2(b)]. At 2 days c-JUN immunoreactive RGCs represented 93% (±15%) of surviving

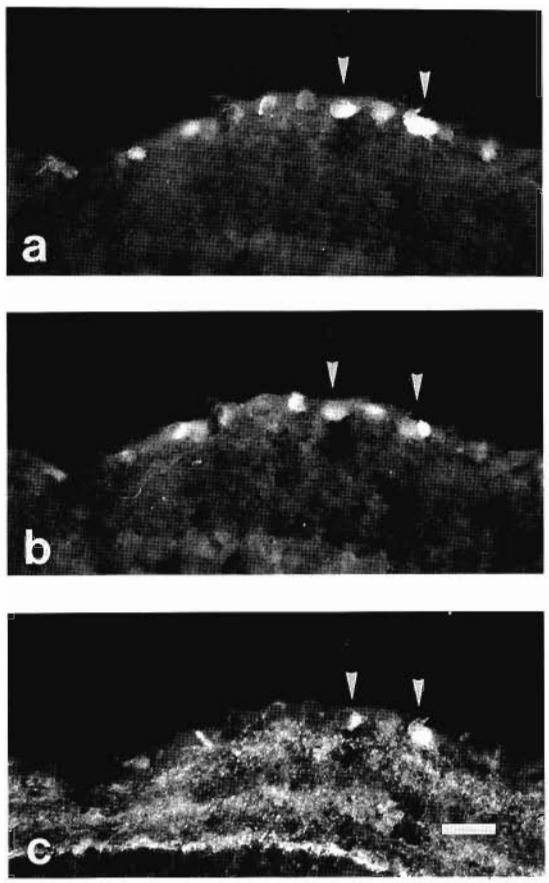

Figure 1 Section through a rat retina, 5 days after ONS and FG application, and exposed to antibodies against GAP-43 and c-JUN. (a) FG-labeled RGCs and (b) RGCs exhibiting c-JUN immunoreactivity. Arrowheads in a, b, and c, mark those FG-RGCs that exhibit in addition to c-JUN also GAP-43. (c) GAP-43 immunoreactive RGCs represent a subfraction of FG and c-JUN–positive cells. Calibration bar, 25 μm.

RGCs, and 72% (±5%), 52% (±12%), 20% (±7%), and 28% (±9%) at the subsequent time points.

In contrast to c-JUN, which at 2 days was seen in almost all FG-labeled RGCs, GAP-43 immunoreactive RGCs were infrequent (1% ±0.5%) at 2 days after ONS [Fig. 2(a,b)]. The number of RGCs reexpressing GAP-43, however, increased significantly at 5 days, a time at which RGC axons form sprouts (Campbell et al., 1992). In relation to FG-RGCs at 2 days [Fig. 2(a)] they represented 22% (±5%) and were 25% (±6%) of RGCs surviving at 5 days [Fig. 2(b)]. The number of GAP-43 immunoreactive RGCs, expressed as ratio of FG-RGCs at 2 days [Fig. 2(a)] decreased to 11% (±3%) at 8 days, and fell further to 2% (±0.5%) and 1% (±0.5%) at 14 and 21 days, respectively. This correlates with the loss of sprouts (Campbell et al., 1992). Compared to FG-RGCs at each of the 8, 14, and 21 day time points after ONS [Fig. 2(a)], GAP-43 immunoreactive RGCs represent a fraction of 30% (±8%), 6% (±1.5%), and 5% (±1.5%) of the surviving RGCs [Fig. 2(b)]. Since all or almost all GAP-43–positive RGCs were FG labeled and c-JUN immunoreactive (Fig. 1), RGCs reexpressing GAP-43 represent a subfraction of c-JUN–positive and FG labeled (i.e., surviving) RGCs. The decline in absolute numbers of RGCs with GAP-43 between 5 and 21 days after ONS [Fig. 2(a)] may indicate either that GAP-43 positive RGCs died or that they down-regulated GAP-43 protein expression. Since GAP-43–positive RGCs always also exhibited c-JUN, but not all c-JUN–positive RGCs had GAP-43, it is possible that c-JUN is necessary for GAP-43 reexpression.

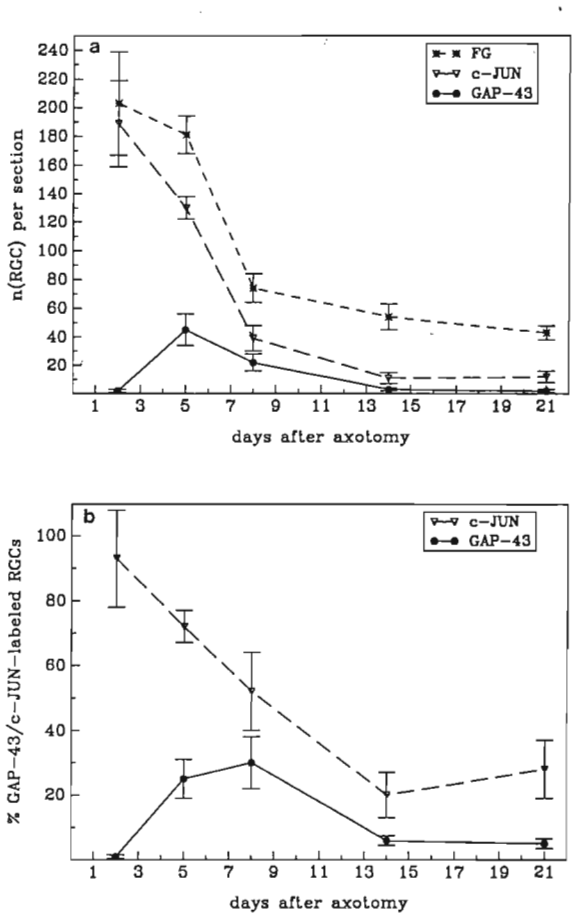

Figure 2 Graph demonstrating in (a) the number [n(RGC)] of FG-labeled, c-JUN immunoreactive and GAP-43–positive RGCs between 2 and 21 days after ONS. (b) GAP-43 and c-JUN immunoreactive RGCs are expressed as percent of FG-labeled RGCs at the corresponding time points. Bars represent standard deviation.

Expression of c-JUN and GAP-43 during RGC Axonal Regeneration

To determine which RGCs sprout or regenerate an axon into a peripheral nerve graft, FG was applied to the graft at 14 and 21 days after surgery (group 2 animals) and FG-labeled RGCs were counted at 21 and 28 days. In group 2 animals FG-labeled RGCs in sections of the corresponding retinae were therefore those that had grown an axon into the graft. All or almost all of the RGCs that were backlabeled from the graft by FG (i.e., those with regenerating axons) were GAP-43 immunoreactive [Fig. 3(a,b)] and accordingly the number of FG- and GAP-43–positive RGCs was nearly identical [Fig. 4(a)]. Compared to 1% (±0.5%) of GAP-43 immunopositive RGCs at 21 days in rats of group 1

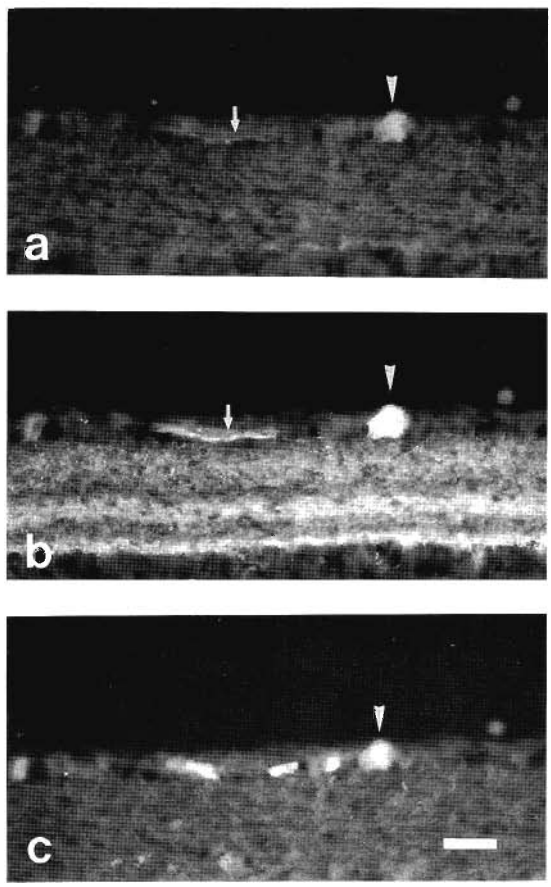

Figure 3 Section through a rat retina, 21 days after ONS and PN grafting, and 7 days after FG application to the graft. (a) All FG-labeled RGCs exhibit GAP-43 (b) and c-JUN (c) immunoreactivity (arrowheads), but the number of c-JUN immunopositive RGCs exceeds that of FG and GAP-43–labeled RGCs. The arrows in a indicate a regenerating FG-labeled axon expressing GAP-43 (b). Calibration bar, 25 μm.

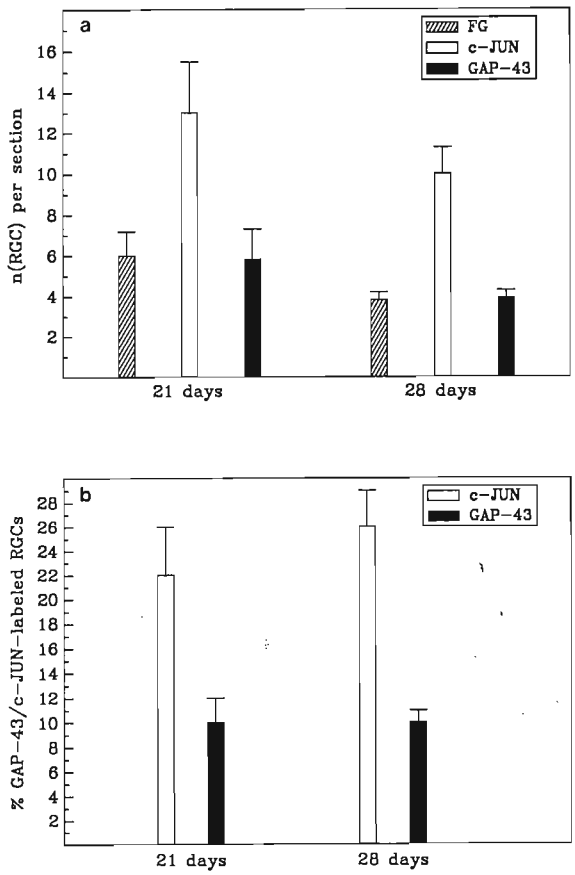

Figure 4 (a) Graphic representation of the relationship of FG-labeled and GAP-43 and c-JUN immunoreactive RGCs at 21 and 28 days after ONS and PN grafting and 7 days after application of FG to the graft [n(RGC), number of RGC]. Note that GAP-43 immunoreactive and FG-labeled RGCs are equal in number. The graph in b demonstrates the percentage of GAP-43 immunoreactive/c-JUN–positive RGCs or surviving RGCs at 21 and 28 days *surviving RGCs were determined by FG application to the optic nerve stump at the time of ONS and PN grafting in group 3 animals). Bars represent standard deviation.

experiments [Fig. 2(a)], there were significantly (p = 0.05) more GAP-43–positive RGCs at 21 days in animals with regenerating axons in PN grafts. They were 3% (±1%) at 21 days and 2% (±0.2%) at 28 days. Despite the graft, however, the number of RGCs labeled with GAP-43 and FG at 28 days decreased significantly (p = 0.01) to 66.6% of those at 21 days. This indicates that RGCs with axons in the graft are not prevented from dying (Villegas-Perez et al., 1988; Hüll and Bähr, 1994).

To compare the number of GAP-43 immunoreactive RGCs with regenerating axons in PN grafts

to the number of surviving RGCs at 21 and 28 days, the latter were determined in a separate set of animals (group 3). Group 3 animals received application of FG to the optic nerve stump at the time of PN grafting. In their retinae FG-labeled RGCs amounted to 59 (±6) and 38 (±5) at 21 and 28 days, respectively. Consistent with earlier findings (Villegas-Perez et al., 1988; Hüll and Bähr, 1994) the presence of the graft has led to an increase in the number of surviving RGCs, which was in the present study 1.4-fold at 21 days [compared to group 1, Fig. 2(a)]. GAP-43 immunoreactive RGCs, which according to the foregoing results were those RGCs that had regenerated axons in the graft, represented 10% (±2%) at 21 days and 10% (±1%) at 28 days of surviving RGCs at the respective time points [Fig. 4(b)].

All FG-labeled, GAP-43–positive RGCs of group 2 animals [Fig. 3(a,b)] also exhibited c-JUN [Fig. 3(c)], but at least twice as many cells [statistically significant, $p = 0.05$ (14 days); $p = 0.025$ (21 days)] showed c-JUN immunoreactivity alone, as there were FG-labeled RGCs and RGCs showing immunoreactivity to GAP-43 [Fig. 4(a)]. That c-JUN immunoreactive cells seen here indeed represent RGCs is derived from group 1 results (Fig. 1), where c-JUN immunoreactivity was exclusive to RGCs (also Hüll and Bähr, 1994), and the latter had been identified by their FG label. The number of c-JUN–positive RGCs in grafted animals at 21 days (6.5% of RGCs at 2 days), however, was not significantly greater than c-JUN expressing RGCs in group 1 animals at 21 days (5%) [Fig. 2(a)]. Compared to surviving RGCs at 21 and 28 days (group 3 animals), c-JUN positive RGCs represented 22% (±4%) and 26% (±3%), respectively [Fig. 4(b)], a difference that was statistically insignificant.

From these data, it appears that expression of GAP-43 may be causally related to successful axonal regeneration. Sustained c-JUN expression may also be relevant bu' this alone does not appear to be sufficient.

DISCUSSION

The foregoing experiments addressed the question whether RGCs in adult rats that are capable of axonal regeneration can be identified by specific changes in their metabolic state and thus be distinguished from those that lack this capacity.

The proteins c-JUN and GAP-43 were considered to be markers for such RGCs because both are reexpressed on injury even when neurons are left in their natural nonpermissive environment (Doster et al., 1991; Herdegen et al., 1993) where they form sprouts but fail to produce long axons. This study demonstrates that RGCs that had extended axons into PN grafts and that were backlabeled from the graft by FG were identical to RGCs exhibiting GAP-43 reexpression. At both time points examined, that is at 21 and 28 days after ONS and PN grafting, FG-RGCs backlabeled from the graft were the only RGCs exhibiting GAP-43 immunoreactivity. This finding indicates that GAP-43 reexpression and the neuron's ability to regenerate an axon are causally linked (Skene, 1989).

In animals with PN grafts as well as in those with optic nerve lesion alone, GAP-43 immunoreactive RGCs represented a subpopulation of the RGCs that exhibited c-JUN immunoreactivity. RGCs exhibiting c-JUN but no GAP-43 immunoreactivity were not backlabeled by FG-application to the graft, that is, they did not have long axons in the graft. This suggests that sustained c-JUN reexpression alone (Hüll and Bähr, 1994) is not sufficient to identify competent neurons.

In animals with or without grafts, all GAP-43–positive RGCs were always also c-JUN immunoreactive. Whether c-JUN, a transcription factor, is required for induction and maintenance of GAP-43 expression is, however, not known. If c-JUN is required for the activation of genes required for axonal regeneration, this transcription factor most likely subserves additional functions. This is because c-JUN expression is found in many more than just those RGCs with regenerating axons in grafts and elevated GAP-43 levels. On the other hand, c-JUN is not seen in all surviving RGCs at the time points considered here (Hüll and Bähr, 1994); the number of surviving RGCs exceeded that of c-JUN–positive RGCs in both sets of experiments. Therefore, c-JUN may not be necessary for the mere survival of cells. RGCs in axotomized retinae are known to form dendritic sprouts (Thanos, 1988; Mansour-Robaey et al., 1994), and these RGC responses may require sustained c-JUN expression. Whatever the function of c-JUN, its limited expression in the population of axotomized RGCs is a further indication of an inhomogeneity of the metabolic states of surviving RGCs whose functional significance remains unclear.

Our findings are based on immunocytochemical techniques for the detection of the c-JUN and GAP-43 proteins. As with all studies of this kind, results and interpretations rely on the sensitivity of

the relevant antibodies. Proteins that remain below the detection level of these antibodies unavoidably escape observation. Both antibodies, however, have been used earlier for examinations of lesion-induced neuronal responses (Schreyer and Skene, 1991; Herdegen et al., 1993). From earlier studies, it was known that GAP-43 (Doster et al., 1991) and long-lasting c-JUN expression (Herdegen et al., 1993) are confined to subpopulations of axotomized mammalian RGCs. However, a direct comparison between RGCs that regenerate axons into grafts and those reexpressing GAP-43 and c-JUN was not made in the other reports. Our present results extend the outcome of earlier studies (Doster et al., 1991) in that we have used the retrograde tracer FG to identify GAP-43 immunoreactive RGCs as those that had grown axons in the grafts.

Although GAP-43 was detected in all RGCs that had grown axons into the graft, we cannot conclude that all RGCs that reexpress GAP-43 will in fact regenerate an axon. As shown by the first group of experiments (animals without grafts), the number of RGCs reexpressing GAP-43 peaked around 5 days after ONS at 22% of all RGCs that the retina originally contained and at 25% of surviving RGCs. When grafted animals were examined at 21 days, they had only 3% GAP-43–positive RGCs, which represented 10% of those suriving at this time. Thus, the number of RGCs that reexpressed GAP-43 at 5 days was higher than the number of RGCs with axons in grafts at 21 days. This leaves open the possibility that the grafts may have contained more axons at an earlier time or that they had sprouts that were abortive (Campbell et al., 1992) or that some sprouts did not gain access to the graft. In fact, GAP-43 has been implicated in RGC axonal sprouting (Doster et al., 1991). A recent report has demonstrated that not all regenerative sprouts transform into long axons when the environment is improved (Schnell et al., 1994). In this context our results could mean that more than 20% of RGCs may have sent axons or sprouts into the graft but most of them subsequently lost their GAP-43 reexpression and perhaps died. Alternatively, not all sprouts may have succeeded in gaining access to the graft and perhaps were subsequently lost. Moreover, neither the present or previous studies have shown directly that RGC axons regenerating into grafts are recruited from the population of RGCs that spontaneously up-regulate GAP-43 on ONS in the absence of a graft. In other words it is not clear whether GAP-43 expression preceeds axonal regeneration. Although unlikely, this leaves open the possibility that axons having entered the graft contribute to the up-regulation of GAP-43 in their parent RGCs. If this were so, the hypothesis that only GAP-43–positive RGCs are competent of regeneration of an axon is inadequate.

Even RGCs in grafted animals that did express GAP-43 and regenerated an axon often die, as has been shown (Villegas-Perez et al., 1988; Hüll and Bähr, 1994) and is confirmed here. The number of RGCs that can be backlabeled from the graft diminishes significantly between day 21 and day 28, indicating that the graft is not capable of stabilizing all RGCs for longer time periods. This observation is consistent with earlier studies that have provided evidence that axon-regenerating RGCs only stabilize when axons are given the opportunity to establish contacts with a target (see review by Aguayo et al., 1991).

PN grafts have been shown to increase the number of surviving RGCs (Villegas-Perez et al., 1988; Hüll and Bähr, 1994), which is confirmed here and of RGCs exhibiting long-lasting reexpression of c-JUN (Hüll and Bähr, 1994). Our data show a significant increase of GAP-43–positive RGCs at 21 days in grafted animals over controls, although no significant increase in the number of c-JUN–positive RGCs was noted. This is surprising because one might have expected an increase of c-JUN–positive RGCs along with an increase of GAP-43–positive RGCs.

It also remains open why some RGCs were still immunoreactive to GAP-43 at 14 and 21 days and possibly even later, whereas others that had contained GAP-43 at 5 days either stopped GAP-43 reexpression or were lost through cell death. At present, questions as to why some RGCs die and why a proportion survives, remain unanswered, and likewise it is not known why a number of the survivors are capable of maintaining long-lasting expression of c-JUN and GAP-43 (Doster et al., 1991). With the previously mentioned alternate view in mind, the present data are consistent with the idea that GAP-43 reexpression identifies those RGCs that possess the potential of axonal regeneration.

In fish, there is a massive up-regulation of GAP-48 in regenerating optic nerves (Benowitz and Lewis, 1983) and reexpression of growth-associated cell surface molecules by all axotomized RGCs (reviewed by Stuermer et al., 1992). A comparison between the situation in fish and rats raises the possibility that up-regulation of proteins known in fish may also occur in rat RGCs (Schaden et al., 1993) and may correlate with GAP-43

reexpression, an idea that is currently being pursued.

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