Slow Transport Rates of Cytoskeletal Proteins Change During Regeneration of Axotomized Retinal Neurons in Adult Rats

Lisa McKerracher, Manuel Vidal-Sanz, and Albert J. Aguayo

Center for Research in Neuroscience, Montreal General Hospital and McGill University, Montreal, Quebec, Canada H3G 1A4

To investigate cytoskeletal changes associated with axonal regrowth from damaged nerve cells in the mammalian CNS, we examined the slow transport of axonal proteins during the regeneration of adult rat retinal ganglion cell (RGC) axons. Although normally such RGC axons do not regrow after injury in the CNS, they can extend several centimeters when their nonneuronal environment is changed by replacing the optic nerve (ON) with a grafted segment of peripheral nerve (PN). Proteins transported in axons of RGCs from intact control and PN-grafted animals were labeled by an intraocular injection of ^{35}S -methionine and examined 4–60 days later by SDS PAGE. During RGC regeneration into PN grafts, the transport rate of tubulin and neurofilament increased two-fold, whereas that of actin decreased to nearly one third of its normal rate. Thus, in these regenerating RGC axons, all three major cytoskeletal proteins were largely transported within a single rate component rather than in the two separate components (SCa and SCb) normally observed in the intact ON. Furthermore, the 200 kDa neurofilament protein (NF-H) was persistently detected in Western blots during periods of active regeneration, a finding that contrasts with the late appearance of the NF-H during the developmental growth of retinal axons.

The changes in slow transport observed during RGC regeneration in adult rats may reflect growth-associated responses of mature CNS neurons during periods of active axonal extension.

Neuronal cytoskeletal proteins move along axons in the slow phase of axoplasmic transport (Lorenz and Willard, 1978; Lasek et al., 1984). Whereas in undamaged mature nerves, this transport is involved in the constant renewal of the cytoskeleton, it is not well known how axotomized neurons regulate the restoration of the cytoplasmic matrix of their damaged axons. The study of the changes in slow axoplasmic transport that accompany axonal regrowth may help define intrinsic neuronal mechanisms that influence peripheral and CNS repair.

In adult mammalian optic nerves, the transport of cytoskeletal

proteins has been subdivided into two different rate components (Lorenz and Willard, 1978; Black and Lasek, 1979; Levine and Willard, 1980). The slow component *a* (SCa) [also called rate V (Lorenz and Willard, 1978)] is composed mainly of tubulin, spectrin, and the three neurofilament subunits whose apparent molecular weights are 200 kDa (NF-H), 150 kDa (NF-M), and 68 kDa (NF-L). Slow component *b* (SCb) (rate IV) includes many different proteins, such as actin, clathrin, spectrin, and calmodulin, and also soluble enzymes (Lorenz and Willard, 1978; Black and Lasek, 1979; McQuarrie et al., 1986).

The composition and transport velocities of the SCa and SCb proteins vary with differences in age, species, and type and state of the neuron: (1) Whereas in CNS axons of the mammalian optic nerve (ON), the SCa and the SCb proteins move in two clearly separated waves, in peripheral nerves (PNs), the rate components of SCa and SCb tend to overlap (Mori et al., 1979; McQuarrie et al., 1986; Oblinger et al., 1987). (2) In peripheral nerves tubulin is found in both SCa and SCb, but in retinal ganglion cell (RGC) axons, it is transported exclusively in SCa (Tashiro et al., 1984; McQuarrie et al., 1986). The regeneration of PN axons is accompanied by an acceleration in the rate of SCb transport and an increase in the amount of tubulin migrating with the SCb proteins (Hoffman and Lasek, 1980; Hoffman et al., 1985). (3) In the developing ON of mammals, the SCa and SCb rates are not clearly separated, and the rates of both are increased over those in the adult (Willard and Simon, 1983). (4) In the ON of goldfish, tubulin is also present in the SCa and SCb (Grafstein, 1986). Furthermore, in these animals, whose RGC axons regrow spontaneously after injury, the rate of slow transport and the amount of tubulin in SCb increase during regeneration (Grafstein, 1986).

Although information has been gathered on the changes in slow transport that accompany the regeneration of CNS axons in the ON of anamniots, such as goldfish, and in the peripheral nerves of mammals, the failure of axonal regrowth after axotomy of mammalian RGCs and other CNS neurons has precluded similar studies in the CNS of adult mammals.

Recent experiments in rodents indicate that axons of CNS neurons in the retina and other regions of the brain and spinal cord are capable of extensive elongation when their central glial environment is replaced by nonneuronal components of the peripheral nervous system contained in PN grafts (David and Aguayo, 1981; So and Aguayo, 1985, reviewed by Aguayo et al., 1986). Using PN grafts as substitutes of the ON (Vidal-Sanz et al., 1987), we have now examined the slow axonal transport of proteins in regenerating RGC axons of rats to investigate cytoskeletal changes during the regrowth of severed CNS axons of adult mammals.

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Correspondence should be addressed to Dr. L. McKerracher, Center for Research in Neuroscience, Montreal General Hospital Research Institute, 1650 Cedar Ave., Montreal, Quebec, Canada H3G 1A4.

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Figure 1. Schematic illustration of the methods used to study slow transport in regenerating rat RGC axons. *A*, The left optic nerve (ON) was transected immediately behind the globe and replaced with a peripheral nerve graft. *B*, Seven days later, 500 μ Ci ³⁵S-methionine was injected into the vitreous chamber of the eye, and unlabeled methionine was applied to the graft. *C*, Seven to sixty days after isotope injection, the graft was removed and cut into consecutive 3-mm segments, and each sample was prepared for SDS PAGE.

Materials and Methods

Surgical procedures. Female Sprague-Dawley rats weighing 190–220 gm were anesthetized with chloral hydrate (0.42 mg/gm body weight, intraperitoneally). In the PN-grafted animals, the left ON was exposed and transected behind the globe, and a 3-cm segment of autologous common peroneal nerve was sutured to the ocular stump of the ON (Vidal-Sanz et al., 1987). The remaining portion of the PN graft was placed under the scalp in a bony groove drilled rostrocaudally along the skull.

Retinal ganglion cell labeling. Seven days after the operation, the animals were reanesthetized, and 500 μ Ci of ³⁵S-methionine (85% ³⁵S-methionine, 15% ³⁵S-cysteine, sp. act. 1200 Ci/mmol, ICN, Irvine, CA) in 5 μ l saline was injected into the vitreous chamber of the left, PN-grafted eye. At the same time, the PN graft was exposed, and Gelfoam (Upjohn, Don Mills, Ont.) soaked in 250 mM unlabeled methionine (ICN, Irvine, CA) was placed on the graft to reduce nonspecific labeling of nonneuronal cells (Fig. 1). From 7 to 60 d after label injection, the animals were killed by an overdose of chloral hydrate and a rapid intracardial saline perfusion. The grafts were removed, desheathed, cut into consecutive 3-mm segments (Fig. 1), and stored at -80°C . Non-operated animals were injected intraocularly with 250–500 μ Ci ³⁵S-methionine, and the optic nerves and optic tracts also were cut into 3-mm segments and stored as above.

Controls for nonspecific labeling. Because most RGC die when the ON is transected near the eye (Villegas-Pérez et al., 1988) and only a small number of axons regenerate into PN grafts anastomosed to the ocular stump (Vidal-Sanz et al., 1987), technical difficulties were anticipated in the distinction of axonally transported labeled proteins and nonspecific labeling. Thus, several experiments were carried out to differentiate proteins transported along regenerating RGC axons from proteins originating from nonneuronal components of the PN graft: (1)

Radiolabeled blood-borne proteins incorporated in the graft were investigated by injecting 500 μ Ci ³⁵S-methionine into the femoral vein of the PN-grafted animals. (2) Proteins synthesized by nonneuronal cells in the graft were examined in two ways: (a) the PN grafts were injected *in vivo* with 500 μ Ci ³⁵S-methionine, or (b) the grafts were incubated for 3 hr *in vitro* in methionine-free RPMI 1640 (Gibco Labs., Grand Island, NY) with 100 μ Ci ³⁵S-methionine. (3) The radioactive labeling of nonneuronal tissues in the PN grafts caused by direct diffusion of the label from the eye was investigated in PN segments placed loosely in the orbit and prevented from becoming innervated by RGC axons. For this purpose, the PN segments were not attached to the ocular stump of the transected ON, the eye was injected 7 d later, and the nerve segments were processed as described for those innervated by RGC axons, except that cold methionine was not applied to the graft. (4) Finally, the possibility that ON transection and PN grafting could result in a substantial leakage of the radiolabel from the eye into the circulation was investigated by comparing in the normal and the PN-grafted rats the radioactivity in the contralateral, uninjected eye to that of the injected eye. The retinæ were homogenized in 500 μ l electrophoretic sample buffer, and proteins were precipitated in trichloroacetic acid (Schaffner and Weissmann, 1973) and counted using Scintiverse (Fisher, Montreal, Canada).

SDS gel electrophoresis and densitometry. Frozen PN or ON segments were homogenized in 30 μ l of electrophoretic sample buffer per millimeter of tissue using a motorized Teflon-in-glass homogenizer. The samples were boiled, then centrifuged for 15 min at $14,000 \times g$ before electrophoretic separation (Laemmli, 1970). The gels were stained with Coomassie blue, impregnated with Enhance (NEN, Boston, MA) or 20% PPO (NEN) in glacial acetic acid, then dried and exposed to Kodak X-Omat AR film, which was preflashed to 0.2 OD (Laskey and Mills, 1975).

Autoradiographs were scanned with an LKB Ultrascan laser densitometer and analyzed using Gelscan XL software (LKB, Broma, Sweden) to determine the areas under the tubulin, actin, and serum albumin peaks.

Measurement of transport rates. For both normal and PN-grafted ONs, the distance of the front of the axonally transported proteins was plotted against time, and the transport rate was calculated from the slope (see Fig. 7). Differences between the slopes were compared by an analysis of covariance. The transport front was determined visually on the fluorographs of normal ON and tracts. With sufficient exposure time, the front was independent of the duration of exposure. During regeneration, the transport front of tubulin and actin was determined by a quantitative comparison of their levels relative to serum albumin (cf. Fig. 6) which is a labeled, nontransported protein. The gels were scanned with a densitometer and the ratios of tubulin/serum albumin and actin/serum albumin were determined for each lane. In noninnervated control animals, the average ratio was 1 (see Fig. 6A). In experimental control animals, the distance from the eye where the ratio fell to 1 was taken to be the transport front (Fig. 6, B, C). This point matched the visually detected front (Fig. 5).

Immunoblotting. The primary antibodies used were SM 31 (Sternberger-Meyer), which recognizes the phosphorylated NF-H epitope, and an antineurofilament affinity purified polyclonal antibody, which reacts mainly with NF-M and slightly with NF-H. The secondary antibody was an alkaline phosphatase-labeled antimouse (Promega, Madison, WI) for SM31 and ¹²⁵I protein A (NEN) for the antineurofilament polyclonal antibody. Following electrophoretic separation, proteins were transferred to nitrocellulose at 450 mA for 3 hr in 12.5 mM Tris, 96 glycine pH 8.1, with 0.01% SDS. The blots were then processed as described (Schoenfeld et al., 1989). Intermediate filaments used as neurofilament standards were prepared according to Chiu and Norton (1982).

Results

Slow axonal transport in normal adult rats

In normal adult rat ON, the main proteins migrating in the two components of slow transport have been identified and characterized previously (McQuarrie et al., 1986). In agreement with such observations, we have found that tubulin and the three neurofilament subunits are the most abundantly labeled SCA proteins. Furthermore, in the intact ON, these proteins are transported exclusively within the SCA (Fig. 2). Although actin is considered primarily a SCb protein (McQuarrie et al., 1986),

Figure 2. Fluorographs of ^{35}S -methionine-labeled protein from normal animals. Consecutive 3-mm segments of the optic nerve and optic tract from animals examined 8 d (*A*), 21 d (*B*), and 46 d (*C*) after isotope injection. A photograph of Coomassie blue staining (*C*) of the first segment of the 8-d animal is shown in *A*, and it is typical of the Coomassie blue staining of all of the samples from normal animals. The SCb proteins, including actin (open triangles) and spectrin (open squares), are most heavily labeled at 9 mm from the eye at 8 d. The gradual progress of the SCA proteins, identified by tubulin (closed circles) and the neurofilament polypeptides (closed triangles), is seen at increasing times after injection. The positions of the M_r standards (200, 116, 92, 66, 45, 30, kDa) are indicated by the bars on the right side of each gel. The acrylamide concentration of *A* was 9%. *B* and *C* were 5–14% gradient gels. Exposure times were 0.5, 1, and 4 d, respectively.

some actin also was identified in the SCA (Fig. 2). Spectrin was found in both SCA and SCb.

The front of SCA moved at a rate of 0.5 mm/d (correlation coefficient 0.9), and the front of SCb migrated at 2.7 mm/d (correlation coefficient 0.8; see Fig. 7). Most of the proteins that comprised SCb were only evident at early postinjection times because they travelled along the ON and tract without leaving a trail of label (Fig. 2). However, 46 (Fig. 2*C*) and 60 days (not shown) after labeling, the longest time points examined in this study, there were still labeled SCA proteins in the most proximal segments of the ON (cf. Fig. 2*C* for a 46 d time point).

Sources of nonspecific labeling of PN grafts

Previous studies in this laboratory have estimated that an average of 3600 RGC axons (range 949–12,385) regrow into PN grafts joined to the cut ON. This population of regenerated RGC axons represents approximately 3% of the RGC axons present in the intact ON of rats (Vidal-Sanz et al., 1987). Such reduction in the number of RGC axons made it necessary to carefully discriminate the axonally transported proteins from spurious labeling of graft components (see Materials and Methods).

It was found that blood-borne radioactivity resulted in the labeling of a protein of M_r 66 kDa contained within the graft (Fig. 3). This protein was the major Coomassie blue-stained

protein in all the PN grafts. Although the level of labeling of this polypeptide differed in each animal, the labeling was uniform along a given graft (Fig. 3*B*). This nonspecifically labeled protein was probably serum albumin, and we refer to it as such in this paper.

The non-neuronal cells of the graft synthesized a large number of proteins, including tubulin and actin. Neurofilaments are not present in non-neuronal cells (see Fig. 4), and there were no proteins synthesized by the non-neuronal which comigrated with NF-M cells (Fig. 3*A*).

Diffusion of the label after intraocular injection resulted in the labeling of serum albumin, tubulin, and actin in the PN grafts that were prevented from innervation by RGC axons (Fig. 3*B*). Again, proteins with the same apparent molecular weight as NF-M were not observed under such conditions.

Transection of the ON near the globe did not affect the amount of label that diffused from the eye. In grafted animals, the labeling of the contralateral retina was 7% ($n = 15$ animals) of that in the retina of the injected eye. A similar labeling index (6%) was found in the intact rats used as controls ($n = 8$ animals) following the labeling of one eye.

Thus, although there was nonspecific labeling of non-neuronal tissues, we could distinguish such spurious labeling from that of the proteins transported in the relatively small population of RGC axons that had regenerated along the PN grafts.

Figure 4. Immunoblot analysis of neurofilament reactivity in PN grafts. The proteins were electrophoretically separated and transferred to nitrocellulose before reacting with monoclonal SM 31, which labels phosphorylated neurofilaments (*A*) or a polyclonal antineurofilament antibody (*B*). *A*, Lane 1, SM 31 reactivity of spinal cord neurofilament proteins. Lane 2, An 18-d PN graft innervated with regenerating RGC axons. Lane 3, A noninnervated 18-d PN graft. In lanes 2 and 3, 50 μg protein were loaded. *B*, Lane 1, The antineurofilament reactivity to an 18-d PN graft. Lane 2, Normal optic nerve.

Identification of transported neurofilament proteins in peripheral nerve grafts

The regenerating RGCs were labeled by an intraocular injection given 7 d after grafting, a time when previous anatomical studies have shown that the RGC axons have begun to extend into the PN grafts (unpublished observations). Because the neurofilament proteins are not present in the non-neuronal cells of the PN graft, they were used as indicators of axonally transported proteins (Fig. 4). To identify the three neurofilament subunits transported along RGC axons in the PN grafts, we (1) compared the electrophoretic mobility of transported proteins in the PN grafts with neurofilament proteins transported into the intact ON (Fig. 3*A*) or with purified NF (not shown) and (2) used immunoblotting. Although the identification of NF-H in the fluorographs obtained from the grafts was obscured by the numerous bands found in the 200 kD region, some of which corresponded to non-neuronal proteins (Fig. 3), the presence of NF-H was detected by immunoblotting.

When noninnervated PN grafts were examined, no NF immunoreactivity was observed (Fig. 4). On the other hand, NF-H immunoreactivity was detected in the PN grafts innervated by regenerated RGC axons 17 d and 28 d after grafting (Fig. 4). These findings confirmed that NF-H expression correlated with RGC growth into the PN grafts. Although NF-H was detected in Western blots by SM 31, an antibody that recognizes a phosphorylated epitope, the proteins in the 200 kDa region of the fluorographs were very light, suggesting that the amount of NF-H is reduced relative to NF-M in regenerating RGC.

NF-M was the most clearly labeled NF protein in the fluorographs. This NF was identified in both PN grafts and ONs 11

A. 11 days **B. 21 days** **C. 46 days**

Figure 5. Fluorographs of labeled proteins from grafted animals. The animals were labeled as illustrated in Figure 1 and examined 11 (*A*), 21 (*B*), and 46 (*C*) d later. Symbols label identified proteins as described in the legend to Figure 3. Lane *C* in *A* shows Coomassie blue staining of a lane from the gel shown in *A*. The neurofilaments were identified by their comigration with purified neurofilaments. The acrylamide concentrations used were 7.5%, 7.5%, and 5–14%, and exposure times were 1, 3, and 3 months, respectively.

d after the intraocular injection of the tracer (Fig. 3*A*). The presence of NF-M in the PN grafts that were innervated by the regenerating RGC axons was confirmed also by immunoblots (Fig. 4).

In contrast to the normal ON and tract, the NF-L was not clearly visible on the fluorographs of reinnervated PN grafts. Although the nonradioactive serum albumin distorted the bands in this region of the gel (Fig. 5), it would appear that in regenerating RGC axons, the amount of NF-L may be reduced in relation to that of NF-M.

Tubulin and actin transport during RGC regeneration

Although the total amount of tubulin and actin visualized by Coomassie blue staining was constant along the length of the PN graft, that of radioactive tubulin and actin was much greater in the proximal segments of the PN graft (Fig. 5). To identify

the axonally transported tubulin and actin, we used densitometry to compare these proteins in PN grafts containing regenerating RGC axons (Fig. 5) with those in the noninnervated grafts (Fig. 3*B*). In the RGC-innervated PN grafts, the amounts of tubulin and actin were compared with that of a nonaxonally transported protein, serum albumin (see above). In the control noninnervated PN grafts, the tubulin/serum albumin and actin/serum albumin ratios were similar along the entire length of the PN graft (Fig. 6*A*). Their values ranged from 0.5 to 1.5, with an average of 1.0 ($n = 3$ animals). In contrast, in the PN-grafted animals, the ratios of tubulin/serum albumin and actin/serum albumin were high proximally, near the eye (an average high value of 3.2 for tubulin/serum albumin and 2.8 for actin/serum albumin) ($n = 28$ animals) and decreased distally to control levels (Fig. 6, *B–D*). Ratios greater than 1.0 were interpreted as an indication of the specific labeling of tubulin and actin. There-

Figure 6. Quantitative comparison of tubulin and actin labeling relative to a nontransported protein, serum albumin. The ratios of actin/serum albumin (*squares*) and tubulin/serum albumin (*circles*) were determined from densitometric scans of the fluorographs and representative samples are shown. *A*, Tubulin and actin labeling from a control animal similar to that of Figure 3*B*. The labeling is similar along the entire length of the graft and represents nonspecific labeling. *B–D*, PN-grafted animals examined 11 (*B*), 21 (*C*), and 46 (*D*) d after isotope injection. The ratios are high proximally and fall to control levels at early time points, but by 46 d after labeling, the ratios do not fall to background levels.

fore, the fronts of labeled protein transported along the PN graft were estimated from the sites where the ratios dropped below 1 (Fig. 7).

Rate of slow transport during RGC regeneration

The migration of tubulin, actin, and neurofilament proteins along the PN graft proceeded further distally with longer postinjection times (Fig. 5). At 7 d there was only proximal labeling (2 animals), at 11 d (*n* = 6 animals) the labeling had progressed to the middle of the PN graft, and by 21 d (*n* = 14 animals) it had neared the end of the PN graft. Labeled proteins were detected at the distal end of the PN graft by 30 d in 2 of 4 animals, by 46 d in 3 of 4 animals, and by 60 d in 1 of 2 rats. At longer time points, the proximal labeling remained high, as in the normal ON and tract (Fig. 5), but the labeling at the distal end did not decrease to the average background level (Fig. 6). No-

Table 1. Transport rates^a in normal and regenerating RGCs

Cytoskeletal protein	Optic nerve and tract	Regenerating RGC
Tubulin	0.5	1.0
Neurofilaments	0.5	1.0
Actin	2.7	1.0

^a Rates were taken from the slope of distance-on-time graphs (Fig. 7) and are expressed as millimeters per day. Correlation coefficients are given in the text.

Figure 7. Regressions of distance-on-time for slow transport in normal RGC axons and regenerating RGC axons. The fronts of SCa (*open circles*) and SCb (*open triangles*) from normal animals were determined from the furthest distance from the eye where tubulin and neurofilament protein, and actin, respectively, were located. In regenerating RGC (*closed circles*), quantitative analysis of the specific tubulin and actin labeling relative to serum albumin (see Fig. 6) was used to determine the transport front, using 1.0 as the cutoff for specific labeling. The regression line for points from regenerating animals was calculated omitting time points after 21 d because the graph was not linear after that point. The regression lines were not constrained to pass through the origin. The three population regression lines have significant differences between slopes, *p* ≤ 0.05.

tably, the tubulin, actin, and neurofilament labeling appeared to follow a very similar time course in all animals (Figs. 5, 6).

The slow transport fronts were calculated for each animal from graphs similar to those in Figure 6. The average transport rate was determined from regressions of distance-on-time (Fig. 7), and the results are shown in Table 1. The rate of slow transport during regeneration was 1.0 mm/day (correlation coefficient 0.8), a value that falls between the SCa and SCb rates in normal nerves (Fig. 7). When the rate of slow transport was calculated for the regenerating RGC axons of individual rats, the rates ranged from 0.5 to 1.6 mm/d (*n* = 18 animals).

Discussion

Detection of slow transport in RGC axons regenerating into PN grafts

The rat ON was selected for this study of changes in the transport of cytoskeletal proteins during axonal regrowth from the CNS of adult mammals because both the normal slow axonal transport in the ON (Black and Lasek, 1979; Levine and Willard,

1980; Nixon and Logvinenko, 1986) and the anatomical regeneration of RGC axons (So and Aguayo, 1985; Vidal-Sanz et al., 1987; Villegas-Pérez et al., 1988) have been investigated previously. The study of the proteins transported in RGC axons regenerating along PN grafts that replace ONs is, however, hampered by (1) the relatively small population of RGC axons that regenerate into the grafts (Vidal-Sanz et al., 1987), (2) the need for a long exposure time of the fluorographs, and (3) the labeling of nonneuronal proteins residing within the graft itself.

Nonetheless, we were able to distinguish the transported proteins from spurious labeling by several control experiments, including a comparison of innervated and noninnervated grafts (Fig. 3). We found that the transport characteristics of the cytoskeletal proteins change when RGC axons regenerate.

Neurofilament transport during regeneration

The rate of neurofilament transport in rat injured RGC axons increased approximately 2-fold during regeneration. This change in NF transport resembles that described previously in the newborn rabbit ON and in other developing neuronal projections in mammals (Hoffman et al., 1983; Willard and Simon, 1983). The rate of NF transport is also known to increase in regenerating axons of the adult rat sciatic nerve (Hoffman and Lasek, 1980; Hoffman et al., 1985; McQuarrie and Lasek, 1989). Since the function of neurofilament transport in axons is unclear, we do not know why their rate of transport increases. A possible explanation is that this change reflects the increase in the rate of tubulin transport. Because NFs are crosslinked to microtubules (Hirokawa et al., 1984), NFs could be passively cotransported with the microtubules (see below).

The presence of NF-H in the regenerating RGC axons of these rats is also of interest because, in the developing ON of the rat and other mammals, the NF-H is not detected at early postnatal times (Willard and Simon, 1983; Pachter and Liem, 1984) when axons are elongating and the neurofilament transport rate is increased (Willard and Simon, 1983). In such animals, the NF-H is first detected at a time when the rate of NF transport decreases toward adult values. Although transport rates have not been estimated in newborn rats, the increased rates observed during RGC regeneration may correspond to the intermediate values of postnatal development when the amount of NF-H expressed is small compared to the amount in adult axons. Since NF-H has been implicated in the formation of a stable stationary matrix in axons (Lewis and Nixon, 1988), it may be that the proportion of moving and stationary cytoskeletal components differs between developing nerve and regenerating nerves.

Tubulin transport during regeneration

The increased rate of tubulin transport documented here during RGC regeneration in adult rats has been observed also when mammalian peripheral nerves (Hoffman and Lasek, 1980; Hoffman et al., 1985) or goldfish ON regenerate following axotomy (Grafstein and Murray, 1969; Grafstein, 1986). In these examples, the proportion of tubulin transported in SCb also increases. These changes may all reflect complex rearrangements of the cytoskeleton that occur during axonal regrowth.

Experimental evidence suggests that although the SCa and SCb tubulin are present as microtubule polymers (Keith, 1987), the tubulin in SCa is different (Denoulet et al., 1989) and more stable than SCb tubulin (Tashiro et al., 1984). In the developing nervous system, the posttranslationally regulated nonstable forms of tubulin predominate (Cumming et al., 1984). Thus, it may

be that in rat RGC a modification of tubulin to the less stable SCb form of tubulin accompanies axonal regrowth. In addition, recent experiments have shown that there is an increased expression of a developmentally regulated tubulin gene when peripheral nerves regenerate (Miller et al., 1989). It is tempting to speculate that a newly expressed form of tubulin is less likely to be deposited in the stationary cytoplasmic matrix (Nixon and Logvinenko, 1986; reviewed by Hollenbeck, 1989), allowing a rapid mobilization of the microtubules to the growing end of the axon.

A single slow transport rate during regeneration?

In normal ON and tract, tubulin and neurofilaments are transported in SCa, whereas most of the actin moves at a faster rate in SCb (Black and Lasek, 1979; McQuarrie et al., 1986). During the regeneration of rat RGC axons into PN grafts, all of the detectable transported actin migrated at the same rate as tubulin and neurofilaments. Although our experiments did not permit a very sensitive detection of transported proteins, our results strongly suggest that most of the tubulin, neurofilament proteins, and actin migrate at the same rate for the following reasons.

First, there is an increase in the synthesis of actin during the regeneration of peripheral nerves (Hoffman and Lasek, 1980; Sinicropi and McIlwain, 1983; Tetzlaff et al., 1988), suggesting that our failure to detect a separate SCb actin component is not due to its decreased synthesis. Second, at the earliest time points we examined (14 and 17 d after PN grafting), the axons have not yet reached the distal end of the approximately 3-cm grafts (Trecarten et al., 1986; unpublished observations). Therefore, an actin front moving ahead of that of the tubulin and neurofilament proteins would have been detected as distal accumulation of actin. Finally, a transported 170 kDa protein was visible in many of our fluorographs (e.g., Figs. 3, 4B) that may correspond to clathrin, a protein found exclusively in SCb (Garner and Lasek, 1981). Thus, it seems likely that there is a coordinated transport of SCa and SCb proteins when RGC axons regenerate. These findings from the regenerating rat RGC are in agreement with the suggestion that the comigration of the SCa and SCb polypeptides is a hallmark of neurons that regenerate axons (Cancalon et al., 1988).

The decrease in the rate of actin transport suggests that the microtubule–NF matrix is required for actin transport. It is thought that the rate-limiting process to regeneration is the velocity of SCb because a correspondence between the rate of SCb and the axonal growth rate has been observed in regenerating goldfish ONs (Grafstein, 1986) and mammalian peripheral nerves (Hoffman and Lasek, 1980; Wujek and Lasek, 1983; McQuarrie and Lasek, 1989). We also observe a similarity between the rate of slow transport during RGC regeneration, which we found to average 1.0 mm/d, and the rate of elongation of RGC axons previously reported as 1.0 mm/d (Trecarten et al., 1986) to 2.0 mm/d (Cho and So, 1987). It may be that the rate-limiting step to regeneration is not the rate of SCb, which is a very heterogeneous group of proteins (Lasek et al., 1984), but the overall rate of transport of the cytoskeleton.

Conclusions

We have reported that the rate of microtubule and neurofilament transport increases during RGC regeneration while that of actin decreases. These rate changes result in the comigration of these three major cytoskeletal proteins, suggesting that they are coordinately transported. Although during RGC regeneration the

characteristics of slow transport show similarities to those during development (Willard and Simon, 1983), there are differences as well. In particular, NF-H continues to be expressed. Our observations suggest that changes in the structure of the cytoskeleton may be a prerequisite for axonal regrowth after injury because these changes in RGCs are not elicited by injury in the absence of axonal regeneration (McKerracher et al., 1989).

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