Slow Components of Axonal Transport: Two Cytoskeletal Networks

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ABSTRACT We have identified two slowly moving groups of axonally transported proteins in guinea pig retinal ganglion cell axons (4). The slowest group of proteins, designated slow component *a* (SCa), has a transport rate of 0.25 mm/d and consists of tubulin and neurofilament protein. The other slowly transported group of proteins, designated slow component *b* (SCb), has a transport rate of 2–3 mm/d and consists of many polypeptides, one of which is actin (4). Our analyses of the transport kinetics of the individual polypeptides of SCa and SCb indicate that (*a*) the polypeptides of SCa are transported coherently in the optic axons, (*b*) the polypeptides of SCb are also transported coherently but completely separately from the SCa polypeptides, and (*c*) the polypeptides of SCa differ completely from those comprising SCb. We relate these results to our general hypothesis that slow axonal transport represents the movements of structural complexes of proteins. Furthermore, it is proposed that SCa corresponds to the microtubule-neurofilament network, and that SCb represents the transport of the microfilament network together with the proteins complexed with microfilaments.

Proteins are axonally transported at a relatively small number of discrete rates. The available information indicates that each rate component represents the movement of highly ordered protein complexes. For example, although the range of transport rates spans three orders of magnitude, only five rate classes have been identified (31, 38, 45, 69, 70) and, each axonally transported protein is present in only one of these (64). The movement of particles in axons (11, 18) supports this hypothesis, as do the studies of Schwartz and his colleagues (24, 25) which have clearly shown that serotonin-containing vesicles are transported in an identified serotonergic neuron of *Aplysia*. Tubulin and neurofilament protein, the subunits of microtubules and neurofilaments, respectively, are also transported in axons (29, 41). The transport kinetics of these proteins suggest that they are associated in a structural complex, a microtubule-neurofilament network, that is transported in axons. These and other considerations have led to the "central theory of axonal transport," which states that proteins move as parts of cytologically identified structures (39).

We have identified two slowly moving groups of proteins in guinea pig retinal ganglion cell axons (4). The slowest moving group, designated slow component *a* (SCa), consists of tubulin and neurofilament protein. The other group, designated slow component *b* (SCb), contains many polypeptides, one of which is actin (4, 71), the major constituent of actin microfilaments. A structural equivalent for SCb has not been fully defined. In the present study, we provide additional evidence that SCa and SCb represent the transport of structures. We reasoned that if SCa and SCb represent the transport of structures, then the transport kinetics of the polypeptides of SCa and SCb should reflect their structural associations. Accordingly, we determined the transport kinetics of the individual polypeptides comprising SCa and SCb. Our results demonstrate that tubulin and neurofilament protein are transported coordinately in retinal ganglion cell axons, and, thus, support the hypothesis that SCa reflects the movement of the microtubule-neurofilament network. Similarly, the proteins of SCb are transported coherently, but separate from those of SCa. We propose that SCb represents a structure we call the axoplasmic matrix, and we discuss the possibility that the matrix includes a network of actin microfilaments together with the proteins complexed with these microfilaments as they are transported in axons.

MATERIALS AND METHODS

Labeling Axonally Transported Proteins in Guinea Pig Retinal Ganglion Cells

We used Hartley strain guinea pigs in our studies. The axons of retinal ganglion cells project principally to the lateral geniculate nucleus and superior colliculus via the optic nerve and optic tract. Because the projection of the optic

THE JOURNAL OF CELL BIOLOGY · VOLUME 86 AUGUST 1980 616-623
 · 0021-9525/80/08/0616/08 $1.00

axons to their termination sites is almost entirely crossed (23) in these guinea pigs, we used both eyes of each animal and treated each optic nerve, its contralateral optic tract, and superior colliculus as individual samples.

Guinea pigs, 700–900 g, were anesthetized with Na pentobarbital (30 mg/kg) administered intraperitoneally. 9 μl of a tritiated amino acid solution was injected into the posterior chamber of the eye (4). In all experiments, [^{3}H]lysine (40–60 Ci/mmol; New England Nuclear, Boston, Mass.) was used. Immediately before use, an aliquot of the stock solution was brought to dryness by lyophilization and then dissolved in water at a final concentration of 20 μCi/μl.

The Distribution of Total Radioactivity in the Optic Axons

At several postinjection times between 6 and 100 d, animals were anesthetized with ether and then decapitated. Each optic nerve, together with its contralateral optic tract and contralateral superior colliculus, was carefully dissected from each animal. Because both eyes were injected, the region of the optic chiasm (~2 mm in length), where the optic axons cross the midline, was not included in these analyses. The optic nerve and contralateral optic tract were cut into consecutive 3-mm segments. Each segment and the superior colliculus were homogenized in 325 μl of a solution of 8 M urea, 1% SDS, and 5% 2-mercaptoethanol with a ground glass microhomogenizer. The homogenates were incubated at 95°C for 5 min. The samples from the optic nerve and optic tract were centrifuged at 27,000 *g* for 1 h (Sorvall SS-34 rotor, DuPont Instruments-Sorvall, DuPont Co., Newton, Conn.). The superior colliculus samples were centrifuged at 200,000 *g* (Beckman SW50.1 rotor, Beckman Instruments Inc., Fullerton, Calif.) for 1 h. Regardless of the centrifugation conditions used, >98% of the radioactivity in the tissue homogenate remained in the supernate. An aliquot (25 μl) of each supernate was used in determining total radioactivity (data were corrected for quenching by the method of external standardization). The radioactivity in each segment was then plotted against distance from the eye (Fig. 1). The remainder of each sample was analyzed by SDS-polyacrylamide gel electrophoresis.

Determining the Distribution of Labeled Polypeptides in the Optic Axons

The labeled polypeptides in each 3-mm segment of the optic axons and in the superior colliculus were analyzed in consecutive wells of slab gels (7.5% SDS-polyacrylamide slab gels, Neville buffer system [56]). 50 μl of each optic nerve and optic tract sample were applied to the gels. Gels were stained with 0.25% Coomassie Blue, and then the labeled polypeptides in each gel were visualized by fluorography (5, 42). Because the labeled polypeptides in the consecutive 3-mm segments of the optic axons were analyzed in consecutive wells of slab gels, the resulting fluorographs illustrate the distribution of labeled polypeptides in the optic axons at each postinjection time examined (Fig. 2).

After obtaining the necessary fluorographs, the amount of radioactivity within selected labeled polypeptides was determined. The fluorographs were used to locate the position of the labeled polypeptides in the gels. The appropriate region of the gel was excised and incubated in 0.5 ml of 30% H_2O_2 at 60°C for 2 d to

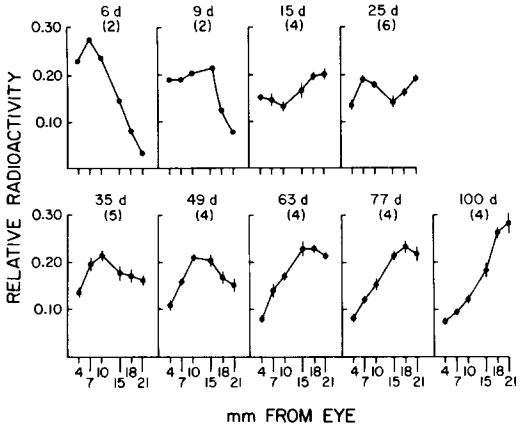

FIGURE 1 The distribution of total radioactivity in the optic axons at several postinjection times between 6 and 100 d. The amount of radioactivity in consecutive 3-mm segments of the optic nerve and contralateral optic tract, expressed relative to the total radioactivity in the optic nerve and optic tract, is plotted against distance from the eye. The data shown are the average of the numbers shown in parentheses (vertical bars indicate the standard error of the mean).

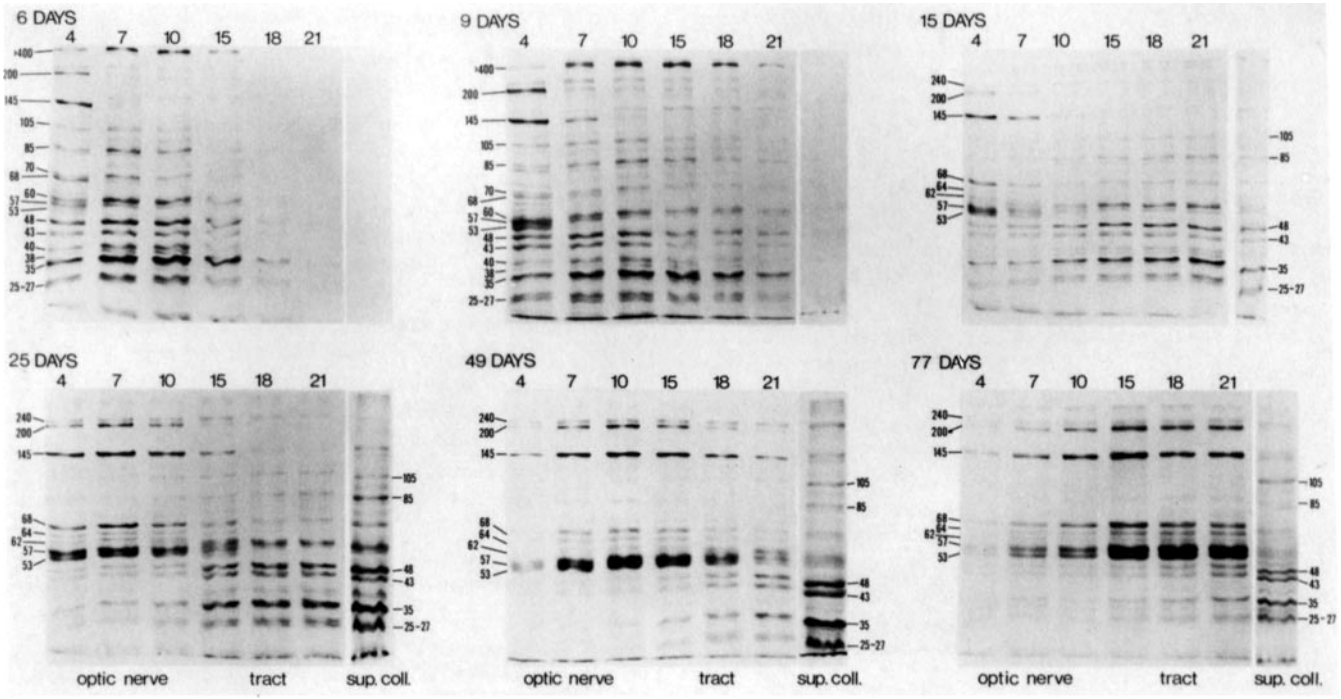

FIGURE 2 Fluorographs illustrating the distribution of labeled polypeptides in the optic axons at several postinjection times between 6 and 77 d. The retinal ganglion cell axons project from left to right, with the optic nerve, contralateral optic tract, and superior colliculus indicated at the bottom. To obtain a picture of the distribution of labeled SCa and SCb polypeptides within the optic axons at the indicated times, band intensities should be "read" from left to right. The numbers to either side of each fluorograph indicate molecular weight (× 10^3). 50 μl of each optic nerve and optic tract sample were applied to the gels; 25 μl of the superior colliculus samples were applied to the gels. The numbers above each fluorograph indicate distance (in millimeters) from the eye.

solubilize the radioactivity. 5 ml of a Toluene-Triton X-100 scintillation cocktail was added to each sample, and the amount of radioactivity was determined. Data were corrected for quenching by the method of internal standardization (counting efficiencies ranged from 18 to 23%).

For determination of molecular weight the following standards were used: vitellogenin (240,000 mol wt, kindly supplied by L. Gehrke, Department of Anatomy, Case Western Reserve University), rabbit skeletal muscle myosin and guinea pig skeletal muscle actin (200,000 and 43,000 mol wt, respectively), phosphorylase *a* and bovine serum albumin (94,000 and 68,000 mol wt, respectively, from Sigma Chemical Co., St. Louis, Mo.).

RESULTS

Two slowly moving groups of axonally transported proteins can be identified in guinea pig retinal ganglion cells (Fig. 1). The more rapidly moving group of proteins was clearly evident in the optic axons at 6 and 9 d postinjection. At these times, the distribution of total radioactivity within the optic nerve and contralateral optic tract exhibited a wavelike shape. At 6 d postinjection, the crest of the wave was located 4–7 mm from the eye, whereas at 9 d postinjection the crest was situated 12–15 mm from the eye. The proximodistal shift in the position of this wave corresponds to a transport rate of 2–3 mm/d. By 15 d postinjection, and to a lesser degree at 25 d postinjection, the trailing (proximal) portion of this wave was apparent in the optic axons (specifically within the portion of the optic axons situated in the optic tract). The labeled material comprising the remaining portion of this wave entered the terminal regions of the optic axons located principally in the lateral geniculate nucleus and superior colliculus.

The slowest moving group of axonally transported proteins first appeared as a wave of radioactivity within the optic axons at 25 d postinjection (the leading [distal] portion of this wave can be seen within the optic nerve at 15 d postinjection). At progressively longer postinjection times, the position of this wave within the optic axons was found at greater distances from the eye. We subjected the position of the wave crest at postinjection times of 25, 35, 49, and 63 d to linear regression analysis. The slope (± SE) of the regression line, which represents the average transport rate of the labeled material comprising this wave, is 0.25 ± 0.02 mm/d. The data in Fig. 1 show the transport rates of two populations of axonally transported proteins. To determine the transport kinetics of the individual proteins of SCa and SCb, we analyzed the distribution of the individual labeled polypeptides comprising SCa and SCb within the optic axons as a function of the postinjection time. The results of these analyses are shown in Figs. 2 and 3.

At 6 d postinjection, the labeled polypeptides comprising SCb were distributed throughout the optic nerve and proximal optic tract (Fig. 2). The major labeled polypeptides transported in SCb have molecular weights of >400,000, 105,000, 85,000, 70,000, 60,000, 48,000, 43,000 (actin), 40,000, 38,000, 35,000, 27,000, and 25,000 daltons (the labeled polypeptides with molecular weights of 200,000, 145,000, 68,000, 57,000,[1] and 53,000 daltons seen in the segment of nerve located 1–4 mm from the eye are from the leading edge of SCa [see below]). These major labeled SCb polypeptides correspond to major stained bands within the optic nerve and tract (data not shown; see also reference 69).

Two features of the distribution of labeled SCb polypeptides at 6 d postinjection deserve note. First, the distribution of each of the labeled SCb polypeptides exhibits a wavelike shape that

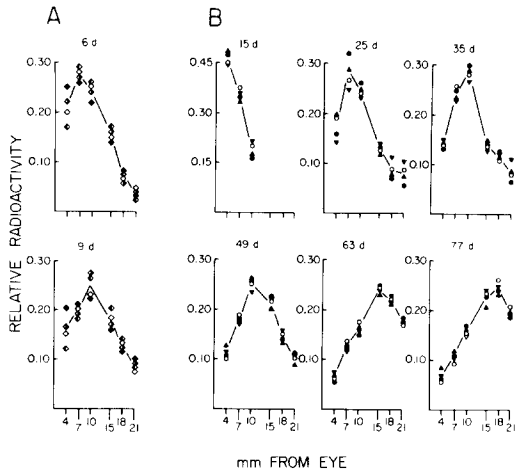

FIGURE 3 Quantitative analyses of the distribution of radioactively labeled SCb (*A*) and SCa (*B*) polypeptides in the optic axons at several postinjection times between 6 and 77 d. The SCb polypeptides analyzed were as follows: 48,000 daltons (◑), 43,000 daltons (actin [4], ◆), 35,000 daltons (◐), and 25,000–27,000 daltons (◇). The SCa polypeptides analyzed were as follows: tubulin (○), 200,000 daltons (▲), 145,000 daltons (●), and 68,000 daltons (▼). The radioactivity associated with each polypeptide was determined as described in Materials and Methods. For each polypeptide, its radioactivity in consecutive 3-mm segments of the optic nerve and contralateral optic tract is expressed relative to its total radioactivity in the optic nerve and tract. In *A*, each point is representative of two determinations, whereas in *B* each point is the average of from four to six determinations (as indicated in Fig. 1). At 15 d postinjection, the distribution of the SCa polypeptides in the optic nerve is shown; because of similarities between the electrophoretic mobilities of the SCa polypeptides and certain SCb polypeptides (see Fig. 2), the distribution of the SCa polypeptides in the optic tract could not be determined accurately.

is very similar to the waveform exhibited by the distribution of total radioactivity within the optic axons at 6 d postinjection (see also reference 4). This is apparent both from visual inspection of the fluorographs in Fig. 2 and from the quantitative analysis in Fig. 3*a*. Three regions of each wave can be distinguished (see especially Fig. 3): a crest, which corresponds to the highest point of the wave; a leading portion, which extends distally (relative to the eye) from the wave-crest; and a trailing portion, which extends proximally from the wave-crest. Second, the major and many of the minor labeled polypeptides of SCb appear to have coincident distributions within the optic axons (Fig. 2). Fig. 3*a* provides quantitative proof that the waves exhibited by the labeled SCb polypeptides with molecular weights of 48,000, 43,000 (actin), 35,000, and 27,000–25,000 daltons are in phase with one another. The coincidence between the distributions of the labeled SCb polypeptides at 6 d postinjection continues at 9 d postinjection, after these polypeptides have moved ~8 mm within the optic axons (Figs. 2 and 3*a*).

By 15 d postinjection, the entry of the labeled SCb polypeptides into the superior colliculus (a major termination site for retinal ganglion cell axons) is apparent (Fig. 2). These polypeptides were also seen in the lateral geniculate nucleus, the other major termination site of the optic axons, at 15 d postinjection (data not shown). Labeled SCb polypeptides were detectable in the superior colliculus (and lateral geniculate nucleus) at all postinjection times studied (Fig. 2). Previous studies have shown that the actin transported in SCb has a half-life in the superior colliculus of ~4 wk (4).

[1] SCb also has a polypeptide of ~57,000 daltons. This polypeptide behaves differently from the 57,000-dalton polypeptide of SCa in IEE-SDS gels (64).

The major labeled polypeptides of SCa have molecular weights of 200,000, 145,000, 68,000, 57,000, and 53,000 daltons (Fig. 2). The latter two polypeptides correspond to the subunits of tubulin, where as the other three polypeptides correspond to the slow component triplet, the subunits of mammalian neurofilaments (29, 41). The five major SCa polypeptides correspond to major stained bands of the optic nerve and tract (data not shown; see also reference 70). In addition to tubulin and the slow component triplet polypeptides, we have identified four other polypeptides which are minor labeled constituents of SCa of guinea pig retinal ganglion cells; two of these have molecular weights of ~240,000 daltons, where as the other two have molecular weights of 62,000 and 64,000 daltons.

At 6 d postinjection, labeled SCa polypeptides from the leading edge of the SCa wave can be seen in the segment of optic axon situated 1–4 mm from the eye (Fig. 2). These labeled polypeptides moved away from the eye at a rate of 0.25 mm/d and, by 25 d postinjection, the radioactivity associated with each polypeptide was distributed in a bell-shaped wave within the optic axons. A similar bell-shaped wave was also noted for the distribution of labeled SCa proteins within the axons of rat anterior horn cells (29, 37) and other peripheral neurons (36, 40). The shape of the wave exhibited by each polypeptide was maintained throughout the course of its movement within the optic axons (Figs. 2 and 3 *b*); there was no apparent proximal skewing of these wave forms as a function of time. At all postinjection times examined, the polypeptides transported in SCa had completely coincident distributions within the optic nerve and contralateral optic tract. The fluorographs in Fig. 2 and the quantitative analyses shown in Fig. 3 *b* demonstrate that the wave forms exhibited by the SCa polypeptides were in phase with each other at all postinjection times studied.

We determined the relative amount of radioactivity associated with the polypeptides of SCa as they moved within the optic axons. The ratio of the individual triplet polypeptides with respect to one another remained constant between 25 and 77 d postinjection. The ratio of the 68,000:145,000:200,000 was 0.26:0.39:0.34. Because the values obtained for the amount of radioactivity associated with the 200,000-dalton polypeptide were contaminated with the 240,000-dalton SCa polypeptides, the contributions of the 68,000- and 145,000-dalton polypeptides to the total radioactivity in the slow component triplet are somewhat higher than indicated. The ratio of the amount of radioactivity associated with tubulin relative to that associated with the slow component triplet (the sum of the radioactivity associated with the 68,000, 145,000 and 200,000-dalton polypeptides) was 0.88 ± 0.03 (n = 5) at 25 d postinjection, 0.90 ± 0.06 (n = 5) at 35 d postinjection, 1.15 ± 0.16 (n = 4) at 49 d postinjection, 1.24 ± 0.13 (n = 4) at 63 d postinjection, and 1.12 ± 0.11 (n = 4) at 77 d postinjection.

Although labeled polypeptides of SCa and SCb were present in the optic axons at each postinjection time studied (Fig. 2), the wave forms exhibited by the labeled SCb polypeptides were always out of phase with those exhibited by the labeled SCa polypeptides, indicating that each group of polypeptides moved separately from one another. Additional information regarding the movement of SCa relative to SCb can be obtained by studying the labeled material within a focal region of the optic axons as a function of the postinjection time (that is, holding the distance constant and using the time as a variable; see reference 45).

Fig. 4 *a* shows the total radioactively labeled material in the segment of optic axons examined as a function of the postinjection time. The labeled material associated with SCb entered the region shortly before 6 d postinjection, and reached a maximum at 9 d postinjection. By 25 d postinjection, most of the mobile fraction of SCb had passed through this region. At 35 d postinjection, the entry of radioactivity associated with SCa into the region studied is apparent. Because of the slow transport rate of SCa, its movement through this region occurred over a long period of time and was not quite completed by 100 d postinjection (Fig. 4 *b*).

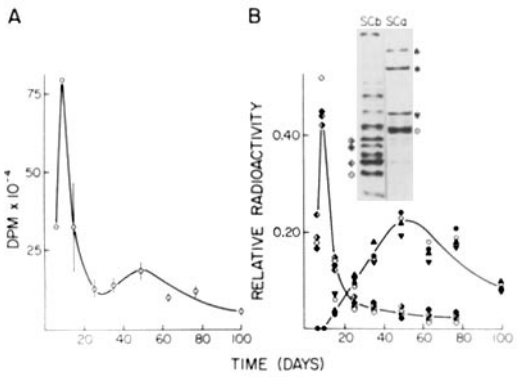

FIGURE 4 The amount of (a) total radioactivity and (b) radioactivity associated with individual SCa and SCb polypeptides in a 3-mm segment of the optic axons at several postinjection times between 6 and 100 d. The region of optic axon analyzed was situated 12–15 mm from the eye. In 4 *a*, the total radioactivity in this region is plotted against time after injection (each point is the average of from two to six determinations, as indicated in Fig. 1; vertical bars indicate the standard error of the mean). In 4 *b*, the relative amount of radioactivity associated with each polypeptide in the 3-mm segment studied is plotted against postinjection time. The *inset* shows typical profiles of the labeled polypeptides that comprise SCa and SCb; symbols are adjacent to the polypeptides they represent (also, see the legend to Fig. 3). The data were normalized as follows. The average amount of radioactivity associated with each polypeptide was determined at each postinjection time (n ranged from 2 to 6 as indicated in Fig. 1, except at 100 d, when the n = 1). For each polypeptide, the average value at each time is expressed relative to the average values summed for all of the postinjection times examined. At 6 and 9 d, the filled circles are representative of all of the SCa polypeptides.

The transport of the individual labeled polypeptides comprising SCb and SCa through the region of optic axons examined is illustrated in Fig. 4 *b*). The labeled SCb polypeptides entered and passed through relatively quickly, such that by 25 d postinjection relatively little radioactivity remained associated with these polypeptides. Note that subsequent to 25 d postinjection, the amount of radioactivity associated with the polypeptides transported in SCb declined steadily. There was no indication of a second wave of SCb polypeptides accompanying the SCa polypeptides in the optic axons, indicating that SCb polypeptides are not transported in SCa. The presence of low levels or radioactivity associated with SCb polypeptides at postinjection times >25 d may indicate that a small fraction of these polypeptides were deposited in the axon during their transit from cell body to axon terminal. It is also possible that some of these polypeptides remain in the cell body and are transported into the axon over a long period of time.

We were unable to detect radioactivity in SCa polypeptides at postinjection times of 6 and 9 d (Fig. 4 *b*), indicating that these polypeptides are not transported in SCb. At 25 d postinjection, labeled SCa polypeptides were clearly evident in the

region of the optic axons examined (trace amounts were detectable at 15 d), and by 49 d the amount of radioactivity associated with them reached a maximum (Fig. 4*b*). Subsequently, the amount of radioactivity associated with the SCa polypeptides decreased.

The amount of radioactivity transported in SCb was compared with that transported in SCa. The total radioactivity in the optic axons at 9 d postinjection was compared with that at 49 d postinjection. These times were chosen because the crest region of each wave was situated in the optic axons at approximately the same position (Fig. 1). The amount of radioactivity comprising SCb was 2.55×10^6 dpm (average of two samples), whereas for SCa this value was $7 \pm 1.7 \times 10^5$ dpm (mean ± SE, $n = 4$), indicating that SCb contained approximately four times more radioactivity than did SCa. A similar comparison, using 6 and 25 d postinjection for SCb and SCa, respectively (the total radioactivity in the optic nerve and tract at 6 d was compared with that in the optic nerve and proximal 3 mm of optic tract at 25 d), indicated that SCb contained ~7 times more radioactivity than did SCa. Similar results have been noted for components of axonal transport comparable to SCa and SCb in rabbit retinal ganglion cells (70). Comparisons of the amount of radioactivity associated with individual polypeptides of SCa and SCb demonstrated that the extent of labeling of major SCb polypeptides also exceeded that of the major SCa polypeptides (tubulin and neurofilament triplet).

DISCUSSION

SCa and SCb Represent Structural Complexes in Axons

Our observations fully support the hypothesis that SCa and SCb represent the axonal transport of protein complexes. Figs. 2 and 3 demonstrate that the SCa polypeptides are transported as a cohesive unit. The fluorographs in Fig. 2 also suggest that the polypeptides of SCb are transported coherently, and our quantitative analyses have proven this for four of these polypeptides (Fig. 3*a*). More recent studies have increased this number to 18 (20, 22). The simplest explanation of these results is that SCa and SCb represent the axonal transport of tightly associated protein complexes. This conclusion is reinforced by the facts that SCa and SCb have completely different transport rates and that the polypeptides comprising SCa differ completely from those of SCb. The lack of mixing between SCa and SCb further indicates that they represent highly ordered protein complexes rather than nonspecific aggregates. This point is also supported by the demonstration that polypeptides of SCa and SCb are the subunits of identified axonal structures. The major polypeptides of SCa are tubulin and neurofilament triplet (29, 41), the principal constituents of microtubules and neurofilaments. Slow component *b* contains actin (4, 71) and clathrin (21), structural proteins that comprise microfilaments (28, 58) and clathroids (33, 57), respectively. In the ensuing paragraphs, we relate SCa and SCb to cytologically identified structures.

SCa: Axonal Transport of Tubulin and Neurofilament Protein

The labeled polypeptides with molecular weights of 53,000 and 57,000 correspond to tubulin because they comigrate with tubulin in one-dimensional SDS gels (29) and in two-dimensional IEF-SDS gels,[2] and they copurify with tubulin through cycles of thermally induced assembly and disassembly.[2] In retinal ganglion cells, tubulin is transported only in SCa; we found no evidence for tubulin in SCb. Tubulin is also transported completely separately from SCb in guinea pig hypoglossal and phrenic neurons (3). The separation between tubulin and SCb is less clear in other systems (29, 32, 52). This apparent discrepancy is related to the fact that transport is studied in populations of axons. The average transport rate of SCa and SCb varies from one axon population to another (3). Thus, it is likely that the variation in the degree of separation of the transport of SCa proteins (tubulin and neurofilament triplet) and SCb proteins in different axon populations reflects variation in the heterogeneity of these different axon populations.

The SCa polypeptides with molecular weights of 68,000, 145,000, and 200,000 are collectively referred to as the slow-component triplet (29). Hoffman and Lasek (29, 41) first suggested that these polypeptides were the subunits of mammalian neurofilaments. Recent observations on mammalian neurofilaments leave little doubt as to the validity of their hypothesis (46, 60).

We have identified four minor labeled polypeptides that are also transported in SCa (Fig. 2). Similar polypeptides have also been identified in SCa of guinea pig hypoglossal neurons (3), rabbit retinal ganglion cells (70), and rat dorsal root ganglion cells (52). The labeled SCa polypeptides with molecular weights of 62,000 and 64,000 have been identified as the "tau" class of microtubule-associated proteins on the basis of phosphocellulose chromatography and two-dimensional gel electrophoresis.[2]

Although tubulin and neurofilament protein comprise SCa of every mammalian neuronal system examined, certain properties of SCa vary from system to system. For example, the amount of radioactivity associated with tubulin relative to that associated with neurofilament protein depends upon the system examined (3, 29, 52). The transport rate of SCa also varies according to the system studied (3, 34, 38), although it is always the slowest rate class of axonal transport. The relationship, if any, between the tubulin-to-neurofilament protein ratio and the transport rate of SCa is unclear.

SCA: The Movement of the Microtubule-Neurofilament Network in Axons

Hoffman and Lasek (29, 41) have shown that tubulin and neurofilament protein move together in peripheral motor axons. Our studies demonstrate that these proteins also move coherently in axons intrinsic to the central nervous system (these proteins also move coherently in mouse retinal ganglion cell axons [Black, unpublished data]). On the basis of their transport behavior, it has been suggested that tubulin and neurofilament protein are transported as microtubules and neurofilaments (29, 41). Independent support for this hypothesis has been provided by recent studies. Morris and Lasek (53) demonstrated that >98% of neurofilament protein in squid giant axons exists in a stable polymeric form. Similar observations have also been made for the subunit protein of 10 nm filaments in other cell types (30, 59). These observations suggest that intermediate filament proteins are almost entirely polymerized in cytoplasm. That this is essentially true of mammalian neurofilament protein is further suggested by the observation

[2] Tytell, M. Personal communication. Department of Anatomy, Case Western Reserve University.

that neurofilaments are readily isolated from nervous tissue (46, 60). Because neurofilament protein appears to exist almost entirely polymerized in axoplasm, it must be transported as neurofilaments. Similarly, it is likely that tubulin is transported as microtubules because >80% of axoplasmic tubulin exists in a polymeric form (53). This conclusion is reinforced by the identification of "tau" in SCa.

Because the axonal transport of tubulin is indistinguishable from that of neurofilament protein (Figs. 2 and 3), microtubules and neurofilaments are transported coherently. To explain how structures with such different morphology exhibit such similar transport behavior, we suggest that they comprise a structural unit, a microtubule-neurofilament (MT-NF) network, that is conveyed in axons. Several additional observations support this hypothesis. First, filamentous bridges are often observed interconnecting microtubules and neurofilaments (14, 43, 50, 74, 75). Second, microtubules and neurofilaments form bundles in the neuron soma which extend without interruption into the axon (67, 68, 74). Third, the normal distribution of neurofilaments in axons is apparently dependent upon microtubules (16). Fourth, recent studies have demonstrated interactions between microtubules and neurofilaments in vitro (2, 61).

The minor labeled polypeptides of SCa are also transported coherently with microtubules and neurofilaments (Fig. 2). Thus, it is likely that these polypeptides are part of the MT-NF network, and that one or more of them mediates the interaction between microtubules and neurofilaments.

SCb: a Major Structure in Axons

The major labeled polypeptides of SCb correspond to major stained polypeptides in the optic nerve and tract, raising the possibility that they are subunits of major axonal proteins. Reinforcing this possibility are the observations that the amount of radioactivity associated with SCb is several times greater than that associated with SCa (see also reference 70), and that the amount of radioactivity associated with individual SCb polypeptides exceeds that associated with either tubulin or neurofilament protein. Assuming that the polypeptides of SCa and SCb are synthesized at similar rates and from precursor pools with similar specific activities, then the SCb polypeptides are in greater abundance in axoplasm than those comprising microtubules and neurofilaments, and hence are the subunits of major axonal proteins. These considerations suggest that the structure transported in SCb is abundant in axons. Because the SCb polypeptides are not present in other rate classes of axonal transport (Figs. 2 and 4; and references 63, 64, 69), the structure which these polypeptides comprise is conveyed only in SCb.

SCb Includes the Microfilament Network

Several structures commonly observed in axons are not conveyed in SCb. Microtubules and neurofilaments are not transported in SCb because tubulin and neurofilament protein are not constituents of SCb. Mitochondria and other membrane structures are not transported in SCb because these structures move at rates that greatly exceed that of SCb (1, 12, 13, 17, 47, 48, 76, 77) and because the solubility properties of these structures differ from those of the SCb proteins (12, 31, 47).

Another structure observed in axons is the microfilament (MF) network. This consists of a three-dimensional lattice of microfilaments (9, 15, 35, 43, 44, 49–50, 75), some of which contain actin (9, 44, 50, 51). The demonstration that actin is transported principally and probably only in SCb (4, 71) identifies actin microfilaments in SCb. Because actin accounts for only 1–2% of the total radioactivity associated with SCb (4), other structures must be transported in SCb. Another component of the MF network has been designated the microtrabecular network (8, 15, 72, 73). Several observations raise the possibility that this structure is also transported in SCb. First, actin microfilaments and microtrabeculae appear to form a structural complex in cytoplasm (66, 72). Second, because microtrabeculae are abundant in axoplasm (15, 43, 75), it is likely that their transport corresponds to a major rate class of axonal transport. The major rate classes of axonal transport are SCa, SCb, and the fast component. The major polypeptides of SCa and the fast component comprise the MT-NF network and membrane structures, respectively. Thus, SCb is the most likely vehicle for the microtrabecular network. Finally, microtrabeculae are labile structures (8, 72), suggesting that their constituent proteins are soluble in "physiological" buffers. The proteins of SCb are soluble in such buffers (31, 47). This is in contrast to the proteins of the fast and intermediate components (12, 31, 47) and neurofilament protein of SCa (46, 47, 53).

This picture of SCb which emphasizes the MF network may be too limited. Recent studies have identified several enzymes of intermediary metabolism in SCb (6). In fact, the extent of labeling of some of these enzymes is equivalent to or exceeds that of actin and clathrin.[3] Thus, it is inappropriate to view SCb simply as a cytoskeletal entity. This is further suggested by the observation that enzymes of intermediary metabolism are capable of forming structural complexes (10, 19, 54, 55). To convey an image of SCb broader than that provided by our emphasis of the MF network, we will refer to the structural equivalent of SCb as the "axoplasmic matrix."

The hypothesis that SCb is equivalent to the axoplasmic matrix is related to the more general hypothesis that each rate class of axonal transport represents the movement of cytologically identified structures (39). This general hypothesis is consistent with all of the presently available information. Furthermore, Hoffman and Lasek (29, 41) used this hypothesis to deduce the identity of the polypeptide subunits of neurofilaments at a time when the literature on neurofilaments was dominated by hypotheses that have since been proven incorrect. Thus, there is a precedent for using axonal transport to identify the protein composition of axonal structures. This precedent lends additional credence to our hypothesis that the polypeptides of SCb comprise a structure. The specific hypothesis equating SCb with the axoplasmic matrix is based on the identification of actin, clathrin, and enzymes of intermediary metabolism in SCb. If our hypothesis proves correct, then we will have identified the proteins comprising the MF network as well as all of the other proteins that are complexed with microfilaments as they move within axons.

Two Cytoskeletal Networks in Axons

Our studies provide an image of the axonal cytoskeleton while it is in motion. In this image, the cytoskeleton consists of two networks, the MT-NF network and the MF network. These networks can be distinguished from one another by their transport rate, protein composition, and structure. The concept of two networks is reinforced by studies that demonstrate that the MT-NF network and the MF network have distinctive

[3] Brady, S. Personal communication. Department of Anatomy and Neurobiology Center, Case Western Reserve University.

distributions in axons; the MF network is especially prominent in the region immediately beneath the axolemma (9, 26, 35, 49–51, 75), whereas the MT-NF network is most prominent in the central region of the axon (49, 50, 75). Similar distinctions between the distribution of the MF network and that of the microtubule-intermediate filament (MT-IF) network have also been described for many nonneuronal cells (7, 28, 62, 65). The rate of movement of the MF network also appears to exceed that of the MT-IF network in nonneuronal cells. For example, in spreading cells, the MF network appears in peripheral regions in advance of the MT-IF network (27, 65). Thus, the notion of two cytoskeletal networks appears to be general.

Although the two cytoskeletal networks are distinguishable from one another, numerous studies have demonstrated associations between them (7, 35, 43, 49, 50, 75). Thus, the two networks interact in cells. Because they move at different rates, the interactions between the networks are transient.

The biochemical and structural uniqueness of each cytoskeletal network implies that they have different roles in producing and maintaining cell structure and behavior. Growing axons readily illustrate this point. Growth cones can be distinguished from axons because of differences between their cytoskeletal constituents. Growth cones contain an abundance of microfilaments but relatively few microtubules and neurofilaments, whereas axons have an abundance of all of these structures (35, 75). Growth cones also differ from axons in many other ways, among which are the motile behaviors they exhibit and the relative stability of their form. It is likely that these and other differences between the properties of axons and those of growth cones are related to differences in the composition of their cytoskeletons. Considerations such as these, when combined with studies of the properties of cytoskeletal networks, will result in a better understanding of how each network is functionally specialized.

We are grateful to Dr. Scott Brady for many discussions and to Ms. Shirley Ricketts for her excellent technical assistance. We would also like to thank Mr. Ronald Rutchick for preparation of the line drawings, and Mr. Wolfgang Busse for the photographic work.

This work was supported by National Institutes of Health grants NS14900 and NS13658 to Dr. Lasek, and National Institutes of Health traineeship HD00020 to Dr. Black.

Received for publication 29 January 1980, and in revised form 7 April 1980.

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