

Proteins Transported in Slow Components a and b of Axonal Transport Are Distributed Differently in the Transverse Plane of the Axon

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ABSTRACT The distribution of the proteins migrating with the slow components a (SCa) and b (SCb) of axonal transport were studied in cross-sections of axons with electron microscope autoradiography. Radiolabeled amino acids were injected into the hypoglossal nucleus of rabbits and after 15 d, the animals were killed. Hypoglossal nerves were processed either for SDS-polyacrylamide gel electrophoresis fluorography to identify and locate the two components of slow transport, or for quantitative electron microscope autoradiography. Proteins transported in SCa were found to be uniformly distributed within the cross-section of the axon. Labeled SCb proteins were also found throughout the axonal cross-section, but the subaxolemmal region of the axon contained 2.5 times more SCb radioactivity than any comparable area in the remainder of the axon.

The axon has emerged as one of the primary models for studying intracellular motility in differentiated metazoan cells (9, 22). The basic mechanisms of motility in axons appear to be similar to those in other cell types; however, cell motility has become highly specialized during the evolution of axons, which makes some aspects of motility more accessible to investigation (9, 22). In the axon, all materials required for growth and maintenance are actively transported from the neuron cell body (15, 23). These axonal constituents move by fast and slow axonal transport.

Fast axonal transport is responsible exclusively for the movement of membranous organelles (15). By contrast, slow transport corresponds specifically to the movement of the axonal cytoplasmic matrix, including microfilaments, microtubules, and neurofilaments (7). Slow axonal transport can be divided into two subcomponents, slow components a and b (SCa and SCb).¹ SCa consists principally of the neurofilament and microtubule proteins that apparently move as constituent components of the microtubules and neurofilaments (3, 18). By contrast, SCb is heterogeneous and consists of more than 100 different cytomatrix proteins, including actin, clathrin,

fodrin (brain spectrin), myosin-like protein, calmodulin, and a variety of metabolic enzymes (2, 6, 8, 13, 25, 38, 39). Cytochemical mappings of SCb proteins such as actin, fodrin, and myosin indicate that these proteins are concentrated at the periphery of the axon subjacent to the axolemma (17, 19, 20, 25, 27, 35). These observations suggest that SCb proteins may move preferentially in the cortical region of the axon.

Axons are basically cylindrical, and the structure of the axon is defined principally by the axonal cytoskeleton which is organized in a relatively simple pattern. This pattern is fairly constant along the length of the axon. When analyzed in cross-section, axons are essentially radially symmetrical and can be divided into two general regions: an outer cortical region which is subjacent to the plasma membrane, and an inner core which comprises the majority of the axoplasm (17, 28, 34). The cortex of the axon is specialized and contains a fine filamentous network that is associated with the plasma membrane. Some slowly transported proteins, such as actin and fodrin, are concentrated at the cortex of the axon. This specialization of the axonal cortical region suggests that it may have a special role in intraaxonal motility (17, 26).

Electron microscopic autoradiography can be used to map the regional distribution of radiolabeled axonally transported components as they move through the axon (16, 24, 36). In these analyses, we measured the distribution of radioactivity along the radial dimension by sectioning the axons trans-

¹ Abbreviations used in this paper: SCa and SCb, two components of slow axonal transport. SCa consists primarily of neurofilament and microtubule proteins. SCb is heterogeneous and consists of more than 100 different cytomatrix proteins.

versely, and dividing the axon into a series of concentric annuli drawn parallel to the axolemma. Previous autoradiographic analyses of axonally transported proteins have focused on fast axonal transport. These studies demonstrate that labeled rapidly transported proteins are uniformly distributed in the radial dimension of the axon (16, 31). In this study, we extend these analyses to slow axonal transport to determine whether the cytomatrix components are transported in a specific region of the axon.

MATERIALS AND METHODS

We used the hypoglossal axons for our analyses because they are relatively homogeneous in diameter, and are large enough to resolve differences in the distribution of radioactivity between different regions of the axon. Further, the SCa and SCb waves are well separated in these axons. Albino rabbits weighing 2.8–3.2 kg were used. Under halothane anesthesia, 500 mCi of [³⁵S]methionine (953.15 Ci/mmol) or 1,000 mCi of a 1:1 mixture of L-2,3,4,5-[³H]proline and L-4,5-[³H]lysine (74.3 Ci/mmol) (New England Nuclear, Boston, MA), were administered with four microinjections into each hypoglossal nucleus according to a modification of a previously described method (36). 15 d after injection, rabbits were killed. Hypoglossal nerves from animals injected with [³⁵S]methionine were used to identify and locate the SCa and SCb waves by using the distribution of radioactivity and fluorography of polyacrylamide gels. It is important to note that 15 d after injection, essentially all of the radioactivity in the hypoglossal nerve is TCA precipitable. Therefore, there are no problems with soluble radioactivity that could generate an interference in the autoradiographic analysis.

Polyacrylamide Gel Electrophoresis: Hypoglossal nerves were cut into 3-mm segments that were separately homogenized in 250 μ l of the following buffer: 1.0% SDS, 2.0% β -mercaptoethanol, and 8.0 M urea, buffered with 0.1 M Tris to pH 6.8. An aliquot was run on 4–17.5% gradient gels according to the procedure of Laemmli (21). Fluorography was done according to the procedure of Bonner and Laskey (4). Quantitation of labeled proteins was carried out by cutting out individual bands and counting in a liquid scintillation counter. The data were corrected for background and quenching so that the relative contribution of each band to the total amount of radioactivity in each nerve segment could be obtained.

Autoradiography: Animals were killed by intracardiac perfusion of 1% paraformaldehyde (370 mosmol) for 30 s followed by 1 liter of 3.5% glutaraldehyde (350 mosmol) at 37°C. Both fixatives were in 25 mM Na-cacodylate (pH 7.35). This fixative was chosen to minimize distortion of the axon (11). After dissection, the two hypoglossal nerves were placed in 3.0% glutaraldehyde at 4°C overnight and then cut into 3-mm pieces. Even-numbered segments were solubilized in 2.0 M NaOH and counted in a liquid scintillation counter to locate the SCa and SCb waves. Odd-numbered segments were placed in 4.0% osmium tetroxide for 3 h, dehydrated in graded ethanol, and embedded in Epon 812 (Ernest F. Fullam, Inc., Schenectady, NY). Segments containing the moving front of SCa and SCb were prepared for light and electron microscope autoradiography (32, 36).

Sections were analyzed from four hypoglossal nerves obtained from two animals. The grids were systematically scanned, and myelinated axons with four or more nonclustered grains over them that measured from 4.0 to 5.5 μ m in diameter were photographed. Negatives were enlarged to a final magnification of 22,000. 112 Axonal profiles including 1,354 grains were analyzed for SCb, and 70 axonal profiles including 836 grains, for SCa. Histograms of grain density distribution were obtained according to the method of Salpeter et al. (33) for each nerve. Since the four histograms for SCa and the four histograms for SCb were not statistically different among themselves, they were combined to obtain one histogram for each component. To analyze the distribution of radioactivity, we compared these experimental histograms with computer-generated theoretical curves for radioactive sources of size and shape similar to those of the axons examined. The curves were generated from those of Salpeter et al. (33), starting with curves of uniform distribution, and increasing the radioactivity present in the compartment containing the axolemma or the compartment immediately adjacent to the axolemma on the axoplasmic side.

RESULTS

Kinetics of Slow Axonal Transport in the Rabbit Hypoglossal Nerve

To analyze slowly transported proteins that are in transit in the axon, we chose the fronts of the SCa and SCb waves.

The wave fronts contain the most rapidly moving components. By contrast, the trailing parts of the wave probably contain proteins whose progress has been retarded by interactions either with slower moving structures or with stationary components in the axon. This is particularly important in the case of SCb, because many of the SCb proteins trail behind the moving wave (14).

A postlabeling period of 15 d was chosen to obtain the fronts of both the SCa and SCb waves in the same preparation (Fig. 1). At 15 d, the crest of SCb was 32 mm from the medulla, which is consistent with a transport rate of \sim 2.1 mm/d. By comparison, SCa only reached the proximal end of the hypoglossal nerve. Analyses of the transport of SCa at later postlabeling intervals suggested a rate of \sim 1.0 mm/d for SCa (Bizzi, A., L. Autilio-Gambetti, and P. Gambetti, personal communication). When these preparations were analyzed by SDS PAGE, we found that only the slowly transported proteins were present in the hypoglossal nerve (Fig. 2). The proximal portion of the nerve contained the neurofilament triplet and tubulin. The distal portion of the nerve, on the other hand, showed the typical SCb pattern. Two SCb proteins, actin and the 35-kD protein, trailed behind the SCb wave and were still present in the region of the nerve containing the SCa wave.

To accurately define the positions of the SCa and SCb waves, we analyzed the individual distributions of representative slowly transported proteins (Fig. 3). The neurofilament triplet and beta tubulin were selected as representative proteins of SCa (3). Alpha tubulin was not analyzed because it was not completely separated from a similar-sized SCb protein on one-dimensional SDS PAGE (14). The neurofilament triplet proteins were distributed in a wave that extended out to a position 22 mm from the medulla (Fig. 3). The peak of the distribution of the beta tubulin coincided with that of the neurofilament triplet. However, a fraction of the tubulin extended into the SCb wave.

Actin and the 35-kD protein, which are representative proteins of SCb (14), were distributed in a wave located 22–40 mm from the medulla. Although actin and the 35-kD protein moved primarily with the SCb wave, they left a small trailing component that overlapped with the SCa wave. The overlap between SCa and SCb was modest, and labeled tubulin accounted for only 2% of the total radioactivity present in the nerve segments that were used for autoradiography of

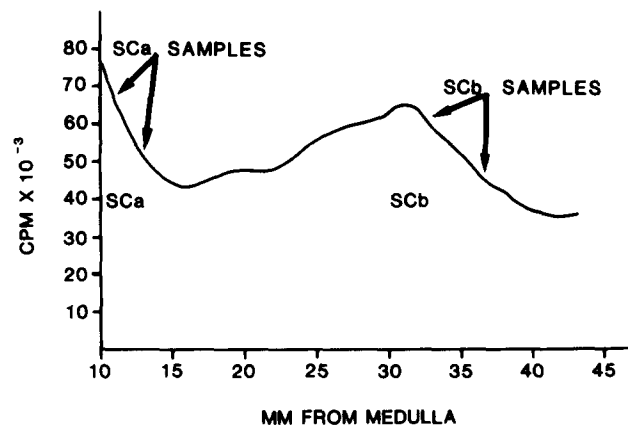


FIGURE 1 Distribution of slowly transported radioactivity in the rabbit hypoglossal nerve 15 d after labeling. The locations of the nerve segments containing SCa and SCb that were sampled for autoradiography are noted by arrows.

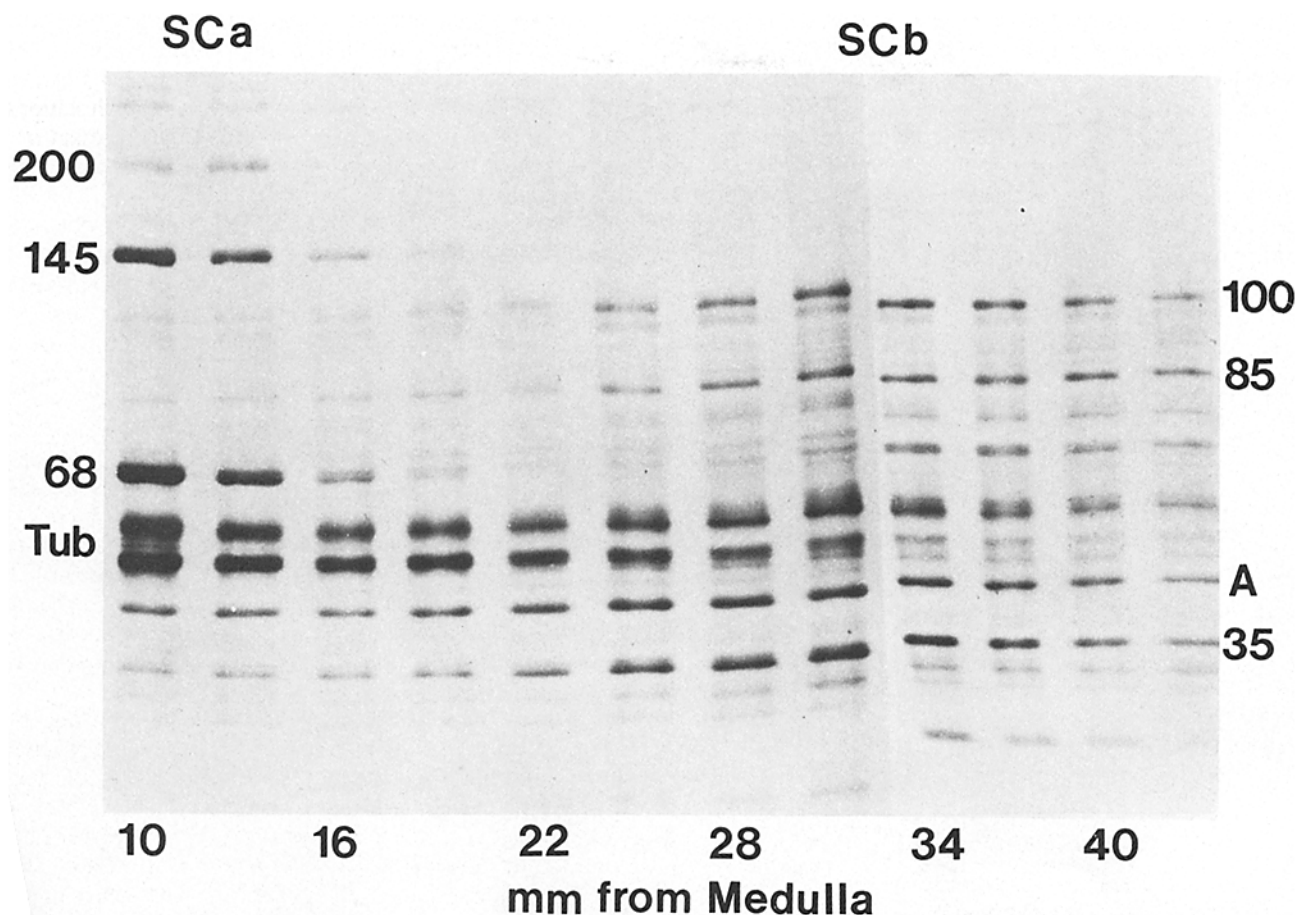


FIGURE 2 Gels obtained with the same hypoglossal nerve as in Fig. 1. The proximal segments show the typical SCa pattern (neurofilament triplet and α - and β -tubulin), whereas the distal segments show the SCb profile. The identities of the two slow components, as well as the location of peaks of radioactivity, are established by gels.

SCb, i.e., those located 33–35 mm from the medulla. Likewise, actin and the 35-kD protein, which are the major trailing components from SCb, accounted for 10% of the label in the segment of the nerve, used for autoradiography of SCa, which was located 13 mm from the medulla.

Autoradiography

Light microscope autoradiographs showed that virtually all of the axons of the hypoglossal nerve obtained from the segments corresponding to the fronts of SCa and SCb contained silver grains (Fig. 4). Visual examination of the electron microscope autoradiographs, such as the one shown in Fig. 5, did not reveal a difference in SCa and SCb grain distribution. By contrast, quantitative analysis revealed a significant difference between the distributions of the silver grains corresponding to the radiolabeled proteins migrating with the fronts of SCa and of SCb (Figs. 6 and 7). These curves were compared with models of theoretical radioactive distributions that were generated by computer using Salpeter's analytic method. In our computer modeling, we could vary the relative percentage of radioactivity in various annuli. Fig. 6 shows the theoretical distribution of grains that would be generated by a uniform distribution of radioactivity or if 100% of the radioactivity were located in the outermost annulus. The curve for a uniform distribution of radioactivity fits the distribution of SCa very closely, i.e., the theoretical curve fell within the standard error bars for most of the points. The only region of

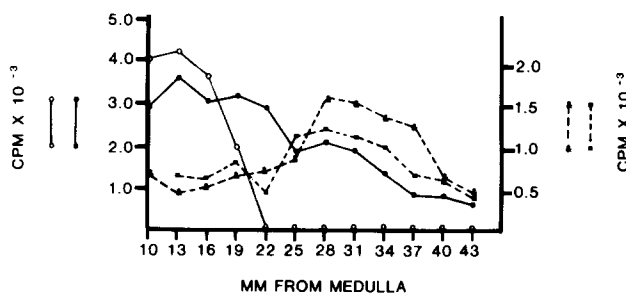


FIGURE 3 Distribution of radioactivity associated with individual polypeptides. Four SCa polypeptides, β -tubulin and the neurofilament subunits (the latter considered as one polypeptide), and two SCb polypeptides, actin and the 35-kD protein, were analyzed. Tubulin and the neurofilament polypeptides show a peak at, or very near to, the SCa peak, whereas the two SCb polypeptides, although trailing into SCa, have only one peak, which is in the SCb region. \circ , neurofilament proteins; \bullet , β -tubulin; \blacksquare , actin; \blacktriangle , 35-kD proteins.

the curve that departed from the theoretical was between 0.96 and 1.92 μ m from the axolemma, and this was not statistically significant. These results indicate that the radiolabeled proteins migrating with the front of SCa were uniformly distributed along the radial axis of the axon.

The grain density distribution of SCb differed substantially from that of SCa, and the difference was statistically significant for each of the annuli within the axon (asterisks in Fig.

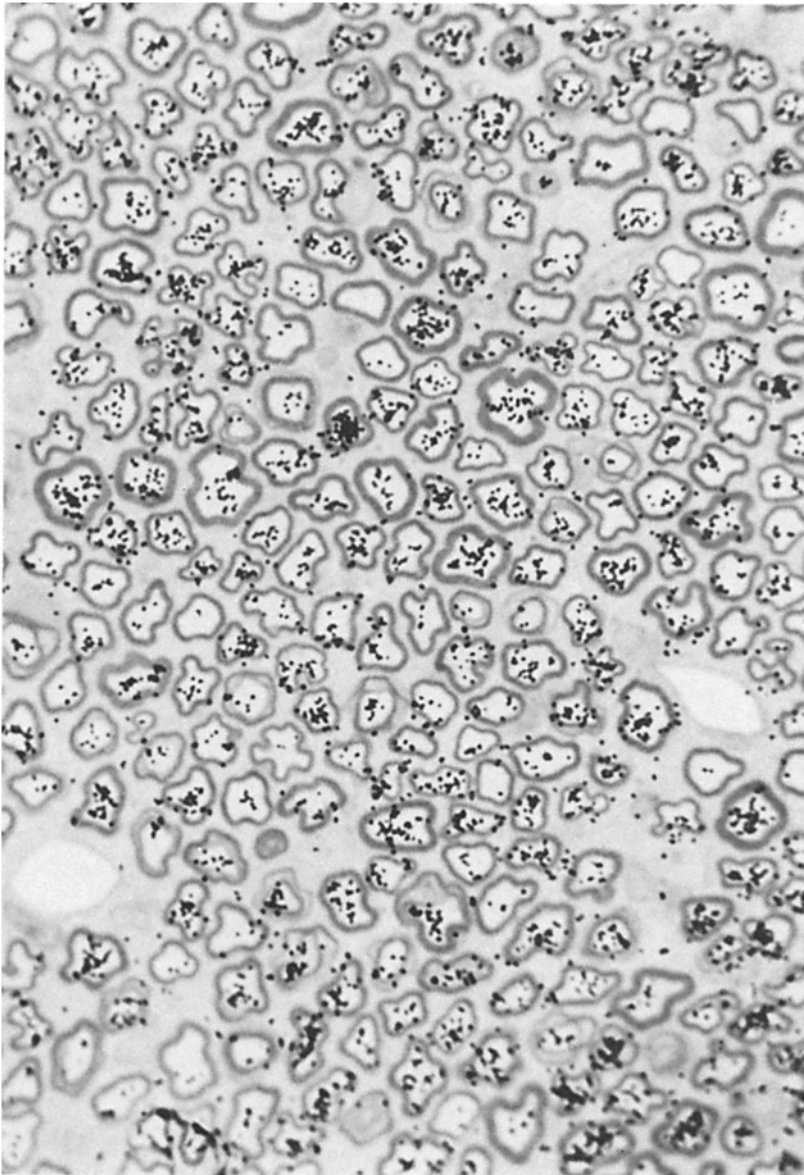


FIGURE 4 Light microscope autoradiograph of SCb. As in Fig. 5, the labeling is excellent, background is low, and there appears to be radioactivity throughout the axonal cross-section, although more definitive statements cannot be made at the light microscope level. The stain is toluidine blue.

6). Comparison of the SCb curve with a variety of computer-generated theoretical distributions demonstrated that the SCb radioactivity was neither uniformly distributed in the axon nor all concentrated near the axolemma (Fig. 7). Instead, the intraaxonal distribution of SCb appeared to be a combination of these two curves. The theoretical curve that best fit the histogram of SCb grain density distribution was that in which 8–12% of the radioactivity was concentrated on the axolemma, while the remaining 88–92% of the radioactivity was uniformly distributed throughout the rest of the axonal cross-section (Fig. 7). Using the computer model, we analyzed the effects of moving the concentrated radioactivity to different locations within the axon. When the concentrated radioactivity was moved inside the axolemma by either 0.5 half distance units or 1.0 half distance units, the theoretical distribution did not fit the observed histogram of SCb grain density distribution normalized at the same units. Therefore, we conclude that ~10% of the labeled SCb is either associated with or within 0.08 μm of the inside of the axolemma, while the remainder is uniformly distributed throughout the axonal cross-section. If the radioactivity is expressed in terms of

concentration, then the subaxolemmal region contains 2.5 times more labeled SCb than any other comparable area of the axon.

DISCUSSION

Intraaxonal Distribution of Axonally Transported Sca Proteins

Our autoradiographic analyses demonstrate that the labeled microtubule and neurofilament proteins that move with the front of the Sca wave are uniformly distributed in the radial dimension of the axon. This distribution corresponds with that of microtubules and neurofilaments in rat motor axons (31) and rabbit hypoglossal axons (P. Gambetti, unpublished results). The correspondence of the spatial distributions of these labeled transported proteins and their cytological counterparts is consistent with the biochemical evidence that indicates that tubulin and neurofilament proteins are actively transported in the axon as component parts of their cytological counterparts, the microtubules and the neurofilaments (10, 23, 29, 30, 37).

Intraaxonal Distribution of SCb Proteins

SCB PROTEINS MAY CONSTITUTE THE SUBAXOLEMMAL MATRIX: Our autoradiographic results demonstrate that the proteins transported with SCb are 2.5 times more concentrated in the peripheral region of the axon than in the core of the axon. This observation that SCb proteins are not uniformly distributed within the axon supports the hypothesis

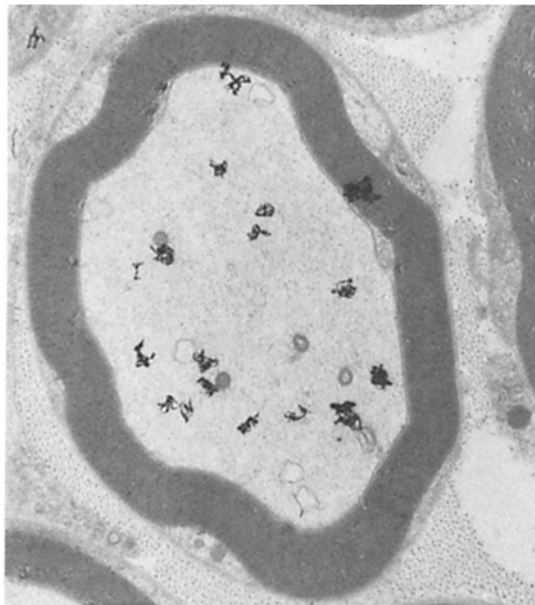


FIGURE 5 Electron microscope autoradiograph of a labeled hypoglossal axon. The microtubules and neurofilaments are visible within the axon, and the myelin is well preserved. There are numerous silver grains, but no grain clusters. The axon is sufficiently rounded so that it can be reasonably divided into concentric annuli.

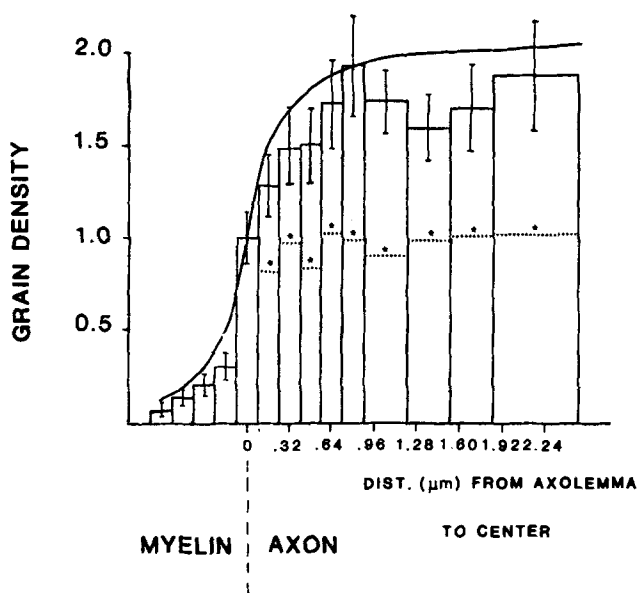


FIGURE 6 Histogram obtained for SCa. The theoretical curve of uniform distribution of the radioactivity fits the histogram, indicating that the SCa proteins were uniformly distributed throughout the axon. The SCb histogram is also plotted within the SCa histogram (dotted line). There is a significant difference in the two histograms, and therefore, in the intraaxonal distributions of SCa and SCb.

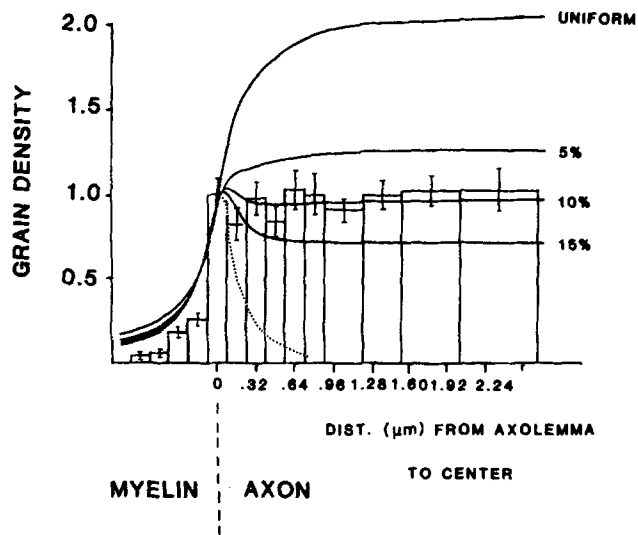


FIGURE 7 Histogram of SCb grain density distribution. The histogram does not fit either the theoretical curve for uniform distribution (uppermost curve) or the theoretical curve that would be obtained if all of the labeled SCb proteins were on or near the axolemma (dotted line). Also shown are three computer generated curves representing 5, 10, and 15% of the radioactivity located at the axolemma (in each case, the remaining radioactivity is distributed uniformly throughout the axon). The best fit occurs at 10% of the label at the axolemma.

that at least some of the SCb proteins are associated with nondiffusible structures of the axoplasmic matrix (2, 14, 23). Furthermore, our findings indicate that some of these SCb transport structures are concentrated next to the axolemma.

Electron microscopic observations of axons have identified axoplasmic matrix structures near the axolemma, and some of these may correspond to the transported SCb proteins. For example, actin microfilaments are concentrated in the subaxolemmal zone within 100 nm of the axolemma (12, 17, 27). Brain spectrin binds to microfilaments, and it is also concentrated near the axolemma (25). Previous studies have considered these structures to be stationary and stably connected to the axolemma (17, 34). However, some of these cytomatrix components near the axolemma appear to move, because actin and brain spectrin are transported coordinately with the front of SCb (2, 25, 38).

SCB PROTEINS CONSTITUTE CYTOMATRIX STRUCTURES THAT MOVE IN THE CORE OF THE AXON: Although the axonally transported SCb proteins were concentrated preferentially in the cortex of the axon, the remaining 88–92% of the SCb proteins were distributed throughout the core of the axon. Thus, most of the axonal cytomatrix components that are conveyed with the front of SCb (i.e., proteins that are actively moving in the axon) are located in the core of the axon. The axonal core can be divided into two structural domains: one contains bundles of microtubules, and the other contains bundles of neurofilaments (28, 34). Kinetic studies of axonal transport suggest that the SCb proteins are preferentially associated with the microtubule domains rather than the neurofilament domains (23). This relationship between the microtubules and the SCb proteins is supported by the observation that tubulin moves in association with SCb, whereas the neurofilament proteins move significantly behind the SCb wave (Fig. 3).

The Intraaxonal Distribution of SCb and the Translocation of the Axonal Cytomatrix

SCb may play a pivotal role in the mechanisms of motility in the axon. In fact, it has been proposed that SCb contains the force-generating components for the translocation of the cytomatrix and the cytoskeleton in slow axonal transport (23, 40). This proposal is based on the following evidence. SCb supplies the proteins that constitute the fabric of the actively motile growth cone, and comparisons between the rate of SCb and axonal elongation suggest that SCb is a rate-limiting factor in axonal elongation (40). Moreover, actin and a myosin-like protein are transported preferentially with SCb, suggesting that SCb contains an actomyosin-based motile system (2, 38, 39). SCb also contains other proteins that could be involved in acto-myosin function and energy production for force generation. For example, SCb is the vehicle for calmodulin (8) and for metabolic enzymes (5, 6).

Our finding of a dense concentration of the faster moving SCb proteins near the axolemma places SCb in a strategic location to produce force for the movement of the cytoskeleton (26). The axolemma represents a site where the cell has stable attachments to the surrounding connective tissue elements. Thus, the contractile components in SCb could exert force against the stationary components of the axolemma, possibly through cross-linking proteins such as brain spectrin (1, 25).

The generation of force for slow axonal transport may not be limited to the cortex of the axon. In large myelinated axons, such as hypoglossal axons, the cortex represents only a small fraction of the total axonal mass, and most of SCb is transported in the core of the axon. Moreover, the core of the axon exhibits a great deal of motility (9), and studies with the pharmacological agent β, β' -iminodipropionitrile indicate that microtubules and the cytomatrix components of SCb can move through the axon even when they are separated from the plasma membrane by a stationary population of neurofilaments (31). This observation indicates that the movement of slowly transported elements does not require a direct association with the plasma membrane. Instead, some of the forces for the movement of the cytomatrix and cytoskeleton may also be generated in the core of the axon.

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