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The Polypeptide Composition of Intra-axonally Transported Proteins: Evidence for Four Transport Velocities

(visual system/rabbit/gradient gel electrophoresis/autoradiography)

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ABSTRACT **Using a method of gradient gel electrophoresis coupled with autoradiography, we have analyzed the polypeptide composition of the proteins being transported down the axons of the projecting neurons of the rabbit retina. This analysis reveals: (1) the molecular weight distribution of 43 transported polypeptides; (2) the existence of at least four components of intra-axonal protein transport, each characterized by an unique polypeptide composition as well as by an unique velocity of transport; (3) the disappearance of individual labeled intra-axonally transported proteins from the axons and synaptic terminals with half-times ranging from several hours to more than 8 days.**

The possibility that intra-axonal transport functions as a temporal mediator of alterations in axonal or synaptic events is discussed in relation to these findings.

The axons and synaptic terminals of neurons are critically dependent upon proteins supplied to them by their cell bodies (1). To satisfy this requirement, proteins synthesized in the neuronal somata are transported intra-axonally to their axonal or synaptic sites of utilization (1–3). The phenomenon of intra-axonal transport of protein is generally considered to include a "slow" component (transported at a rate on the order of 1–5 mm/day) and a "fast" component (transported at a rate on the order of hundreds of millimeters per day) (1–3). Proteins transported "rapidly" differ from those transported "slowly" with regard to their subcellular distribution (4–6), their oligosaccharide content (4, 7–9), and their electrophoretic mobility (9–12). Thus, the "rapid" and "slow" components of intra-axonal transport are defined not only by their velocity of transport, but also by their composition.

Several lines of evidence suggest that proteins are transported intra-axonally at additional velocities intermediate between the velocities of the "rapid" and "slow" components (13–18). However, the existence of a compositionally distinct "intermediate" component of intra-axonal transport has not been demonstrated; on the contrary, it has been reported that proteins that appear to be transported at an intermediate velocity are electrophoretically indistinguishable from those of the "rapid" component (11). If in fact the "rapid" and "slow" components are the only compositionally distinct classes of intra-axonally transported proteins, the significance of intermediate velocities of transport would be questionable. Since a knowledge of the number of components involved is prerequisite for a comprehensive understanding of intra-axonal transport, it is critical to determine whether intra-axonal transport comprises exclusively a "slow" and a "fast" component, or whether a multiplicity of intermediate components exist.

The centrally projecting fibers of the rabbit retina (the axons of the retinal ganglion cells) are well suited for studying this problem. Because of their proximity to the vitreous of the eye, the cell bodies of these fibers are able to take up intraocularly injected radioactive amino acids and to incorporate them into proteins. Some of the labeled proteins are transported down the axons and can be recovered from these fibers in the optic nerve (which is about 16 mm long) and the contralateral optic tract (about 12 mm long), and from the synaptic terminations of these fibers in the lateral geniculate nucleus and the superior colliculus.

We report here a method for analyzing the polypeptide composition of labeled, transported proteins in the axons of the rabbit retinal ganglion cells. We have used this method to answer the question of how many changes occur in the polypeptide composition after intraocular injection of a radioactive amino acid. A single change (corresponding to a transition from the "rapid" component to the "slow" component) would indicate only two compositionally distinct components of transport. Multiple changes would indicate that there must be compositionally distinct intermediate components of transport. In addition to answering this question, our observations give some indication of the rate of disappearance of certain of the labeled transported polypeptides from different segments of the retinal ganglion cell axons.

MATERIALS AND METHODS

[^{35}S]Methionine (New England Nuclear Corp., 140 Ci/mmol, 1 mCi/ml) was lyophilyzed to dryness in the presence of neutralized cysteine (20 μg/mCi of [^{35}S]methionine), which functioned as carrier. Shortly before injection, the isotope was resuspended in a solution of 10 mM dithiothreitol in a volume of 80 μl/mCi of ^{35}S.

The left eyes of adult albino rabbits were anesthetized by the topical application of a 0.5% ophthaine solution (Squibb), and 0.04 ml (0.5 mCi) of [^{35}S]methionine was injected into the vitreous. After an appropriate survival period, animals were killed by intravenous injections of sodium pentabarbitol. The distal half of the optic nerve, the contralateral optic tract, lateral geniculate body, and superior colliculus were immediately removed and stored frozen on dry ice. Each tissue sample was homogenized in a motor-driven 7-ml glass-on-glass tissue grinder (Kontes) containing 5 ml of H buffer [10 mM Tris, pH 8–5 mM EDTA–1 mM *o*-phenanthroline–1

Abbreviation: SDS, sodium dodecyl sulfate.

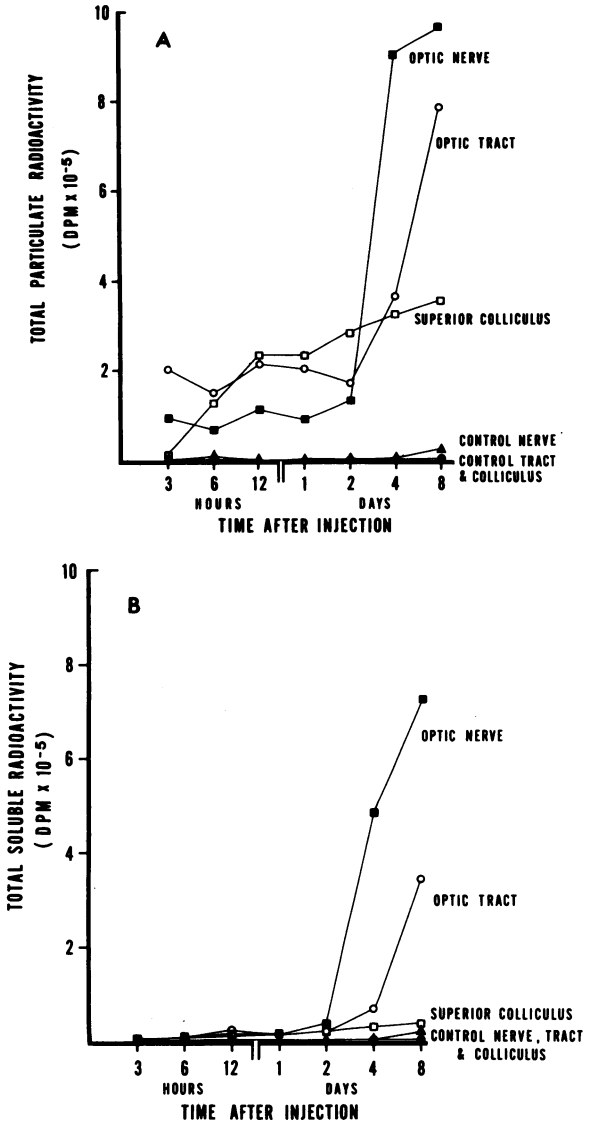

FIG. 1. Time dependence of incorporation of radioactivity into particulate (*A*) and nondialyzable, soluble (*B*) material after the intraocular injection of [^{35}S]methionine. Radioactivity was determined by liquid scintillation counting of aliquots of samples prepared for electrophoresis; values have been multiplied by 10^{-5}.

mM phenylmethyl sulfonyl fluoride (Sigma)]. The homogenate was centrifuged at 100,000 × *g* for 1 hr, and the pellet was resuspended in 0.5 ml of H buffer (without phenylmethyl sulfonyl fluoride) containing 1% sodium dodecyl sulfate (SDS) and 2 mM dithiothreitol. The resuspended pellet was heated to 90° for 5 min, and after it had cooled to room temperature, urea (0.375 g) was added to give a final concentration of 8 M. The samples were centrifuged for 1 hr at 100,000 × *g*, and electrophoresis was performed on the supernatant with bromphenol blue as the tracking dye.

The supernatant from the first centrifugation (containing the soluble proteins) was lyophilyzed to dryness and resuspended in 0.4 ml of H buffer containing 1% SDS. The samples were heated to 90° for 5 min and dialyzed twice (for 5 hr each time) against 1 liter of 5 mM Tris, pH 8–0.5 mM EDTA–0.1 mM *o*-phenanthroline–0.1% SDS–1 mM dithiothreitol. Urea (0.3 g), SDS (0.02 ml of a 10% solution), and tracking dye were added to each sample before electrophoresis.

Slab-gel electrophoresis was carried out on the equipment supplied by Aquebogue Machine and Repair Shop (Aquebogue, N.Y.), using standard glass plates, 0.05-inch (0.13-cm) spacers and a 13-tooth well former. The running gels (7.7 cm high) consisted of a gradient of 5–12% acrylamide (acrylamide/bisacrylamide = 33) and 5.2–7.3 M urea. The gel contained 0.1% SDS in Laemmli's buffer (19). The gradient was formed with a Beckman density gradient former. A stacking gel (4% acrylamide, 0.12% bisacrylamide, 8 M urea) in Laemmli's buffer (19) was polymerized on top. Samples (0.05-ml) for electrophoresis contained either about 70 μg of protein (optic nerve and tract) or 200 μg of protein (superior colliculus and lateral geniculate). Gels were stained and destained as described by Fairbanks, Steck, and Wallach (20) and dried immobilized under a reduced pressure. The dried gels were autoradiographed on Kodak RP/R54 x-ray film. Exposure was for 15 days.

Double-label experiments were analyzed on similar 10-cm gradient gels in cylindrical glass tubes (14 mm inside diameter), with 3 ml of stacking gel polymerized on top. The samples contained about 1 mg of protein. After electrophoresis, the gels were frozen, sliced into 1.5-mm segments, and dried. The dried gel slices were incubated for 12 hr at 80° with hydrogen peroxide (0.5 ml of a 30% solution). Water (2.5 ml) and 10 ml of 3A70 scintillation fluid (Research Products International) were added, and radioactivity was determined in a Packard scintillation counter.

RESULTS

Kinetics of Labeling the Axons of Retinal Ganglion Cells. The left eyes of seven rabbits were injected intravitreally with [^{35}S]methionine (0.5 mCi). The animals were killed 3, 6, and 12 hr, and 1, 2, 4, and 8 days after injection. The distal half of the left optic nerve, the right optic tract, right lateral geniculate body, and right superior colliculus were analyzed for total radioactivity in the particulate (Fig. 1*A*) and soluble (Fig. 1*B*) fractions. Since almost all the axons from the retinal ganglion cells of albino rabbits cross at the optic chiasm (21), the above structures contain almost exclusively axons originating in the retina of the injected eye. The corresponding structures from the other side (containing primarily axons originating in the uninjected eye) served as a control for local protein synthesis from blood-borne [^{35}S]methionine (Fig. 1*A* and *B*). Fig. 1 shows: (*i*) that the level of radioactivity in the control structures was insignificant compared to the level in structures containing axons derived from the injected eye; and (*ii*) that the label is recovered predominantly in the particulate fraction for 2 days after injection. The soluble fraction of the optic nerve and optic tract becomes heavily labeled with the arrival of the "slow" component of transport in these structures at 4 and 8 days after injection, respectively.

The Kinetics of Labeling Individual Polypeptides. Fig. 2 shows the electrophoretic patterns of radioactive polypeptides extracted from the optic nerve, and the contralateral optic tract, lateral geniculate nucleus, and superior colliculus at various time intervals after the intraocular injection of [^{35}S]-methionine. The existence of at least four compositionally distinct components of protein transport, differing in transport velocity, is indicated by the arrival of different polypeptides in the optic tract at four time intervals after the injections. The most rapidly transported proteins (those present in

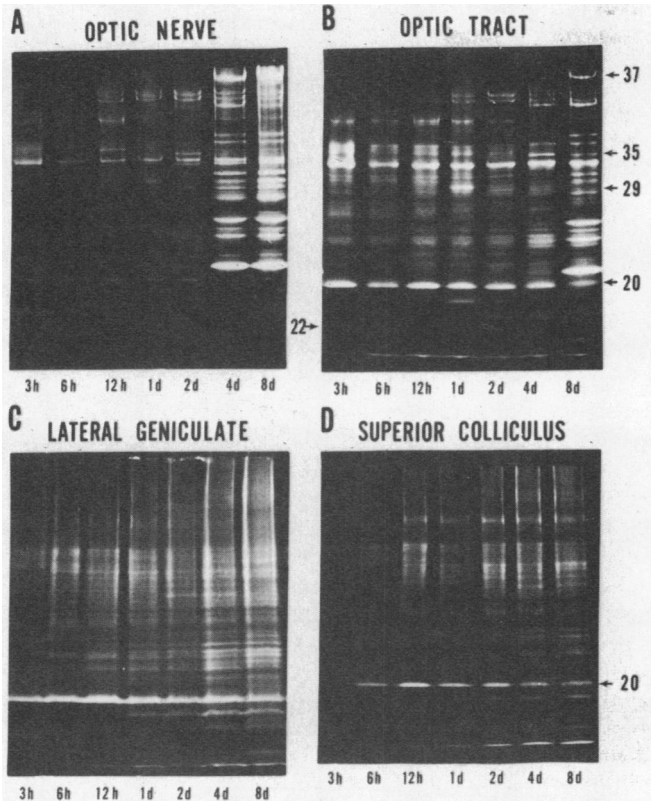

FIG. 2. Autoradiographs of proteins, subjected to electrophoresis, from the different segments of the axons of rabbit retinal ganglion cells. The animals received intraocular injections of [^{35}S]methionine at the indicated times before they were killed, and particulate samples were prepared as described in *Methods*. The autoradiographs have been reproduced on photographic paper so that the *light areas* correspond to polypeptides labeled with ^{35}S. The exposure times used for the photographic reproductions of the gels were adjusted according to the density of the autoradiograph.

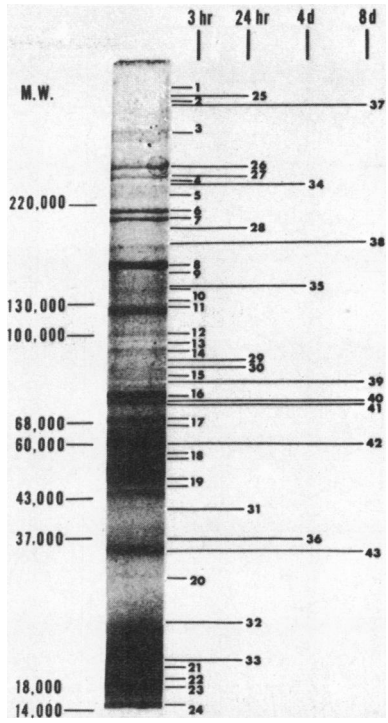

FIG. 3. A summary of the electrophoretic mobilities of polypeptides undergoing intra-axonal transport in the rabbit optic tract. The polypeptides in the tract were subjected to electrophoresis and stained. The numbers on the left of the gel show the position of protein markers of the indicated molecular weights (myosin, β-galactosidase, paramyosin, bovine-serum albumin, catalase, ovalbumin, alcohol dehydrogenase, β-lactoglobulin, and lysozyme, from top to bottom). Molecular weights are those given by Weber and Osborn (26). The lines on the right that terminate at the 3-hr designation indicate the positions in the gel of polypeptides radioactively labeled in the optic tract 3 hr after intraocular injection of [^{35}S]methionine. Lines terminating at the 24-hr, 4-day, and 8-day marks show additional labeled polypeptides present in the optic tract at these times after injection, but absent at the earlier times. This summary was derived from an experiment that differed from that shown in Figs. 1 and 2 only in that the optic tracts were processed without freezing immediately after the rabbits were killed.

the optic tract 3 hr after injection) include at least 24 polypeptides (Figs. 2*B* and 3). Since some of these have reached the lateral geniculate nucleus and superior colliculus (a distance of about 30 mm from the cell bodies, ref. 22) by this time (Figs. 2*C* and *D*), they must have been transported at a rate of at least 240 mm/day. Twenty-four hours after injection, at least nine additional polypeptides have reached the optic tract (Figs. 2*B* and 3). Since some of these can first be detected in the tract 12 hr after injection, they must have traversed the estimated 17 mm between the ganglion cell bodies and the proximal edge of the optic tract at a rate of 34–68 mm/day. Eight days after the injection, at least 10 species of polypeptide that were not present at either 3 or 24 hr can be observed in the optic tract. These represent proteins transported with at least two different velocities, as may be most clearly seen from a comparison of bands 35 and 37 in Fig. 2*B*. Whereas, polypeptide 35 appears to reach a peak in the optic tract at 4 days (suggesting a transport rate of 4–8 mm/day), polypeptide 37 does not appear in the optic tract until 8 days after injection (indicating a transport rate between 2 and 4 mm/day).

Electrophoresis of equivalent amounts of protein from the control structures did not produce detectable radioactive bands. Thus, none of the above classes of transported proteins can be ascribed to the local incorporation of blood-born [^{35}S]methionine.

An analysis of the labeled polypeptides in the soluble fraction of the optic nerve reveals the arrival of heavily labeled polypeptides in the "slow" component of transport 4 days after the injection. Prior to this time, most of the faint polypeptide bands arising from the soluble fraction appear to correspond in electrophoretic mobility to polypeptides that were more heavily labeled in the particulate fraction.

Time-Dependent Changes in the Composition of Transported Proteins in Individual Rabbits. At various time intervals after an initial intraocular injection of [^{35}S]methionine, a second injection of [^{3}H]methionine was made into the eyes of rabbits. Each animal was killed 3 hr after the second injection, and a sample of the particulate fraction from the optic tract was subjected to electrophoresis on cylindrical polyacrylamide gels (see *Methods*). The amount of ^{3}H (3-hr proteins) and of ^{35}S (variable-time proteins) in consecutive slices of the gel were compared. It is clear from Fig. 4 that the ratio of ^{3}H to

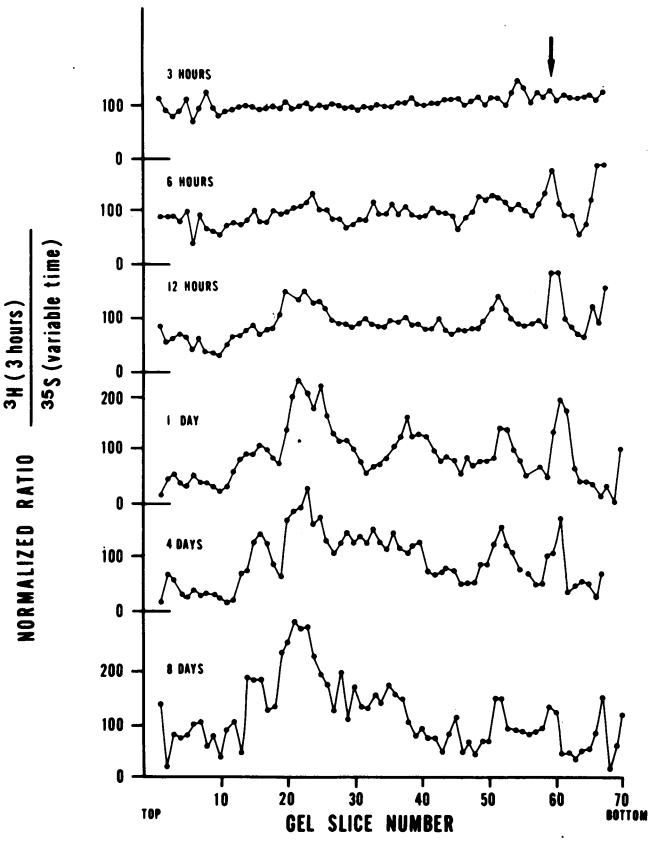

FIG. 4. Changes in the labeled polypeptide composition in the optic tract of individual rabbits as a function of the time between two intraocular injections of labeled amino acids. Each rabbit received an initial injection of [^{35}S]methionine at the indicated time before it was killed. A second injection of [^{3}H]methionine (1 mCi, 0.024 mg) was administered to the same eye 3 hr before the rabbit was killed. The particulate fraction of the optic tract was prepared and subjected to electrophoresis as described in *Methods*. The ratio ^{3}H cpm/^{35}S cpm in the consecutive slices of each gel was calculated. To facilitate comparison of gels, each ratio was multiplied by a number (constant for each gel) to make the average ratio for each gel equal to 100. The *arrow* indicates the approximate expected position of band 22 of Fig. 2*B*.

^{35}S across the gel is more nearly constant in the 3-hr animal (in which both isotopes were injected simultaneously) than in any animal where some time had elapsed between the injection of the two isotopes. This observation confirms that long before the arrival of the "slow" component of intra-axonal transport, the composition of transported proteins in the optic tract undergoes changes in an individual rabbit.

Disappearance of Labeled Polypeptides from the Axons of Retinal Ganglion Cells. After a single injection of [^{35}S]methionine, different labeled polypeptides persist in the axons of retinal ganglion cells for different lengths of time. A quantitative determination of radioactivity in band 20 (Figs. 2 and 3) suggests that it has a halftime ($t_{1/2}$) of disappearance of longer than 8 days in the optic tract and superior colliculus. On the other hand, band 29 (Figs. 2 and 3) first appears in the optic nerve and tract 24 hr after the isotope injection and is greatly diminished by 48 hr, which suggests it has a $t_{1/2}$ of disappearance of less than 24 hr. Band 22 (Figs. 2 and 3) is faintly visible in the nerve and tract at 3 hr but has largely disappeared by 6 hr, which implies a $t_{1/2}$ of 3 hr or less. The rapid disappearance of this polypeptide is also indicated by the data in Fig. 4 (6-hr gel); there is an increase in the ratio of ^{3}H (3-hr protein) to ^{35}S (6-hr protein) in the region of the gel expected to contain this polypeptide (marked by the *arrow* in Fig. 4).

DISCUSSION

Particulate protein fractions can be analyzed with a high degree of resolution by electrophoresis on gradients of polyacrylamide in the presence of SDS and urea. The detection of [^{35}S]methionine-labeled proteins by autoradiography of such gels efficiently preserves this resolution.

We have used this technique to determine the composition of polypeptides in segments of the axons of rabbit retinal ganglion cells at various times after polypeptide synthesis in the retina. The sequential arrival in each axonal segment of four unique classes of proteins demonstrates the existence of at least four velocities of protein transport in rabbit retinal ganglion cells. Karlsson and Sjostrand (16) have arrived at a similar conclusion based on an analysis of the kinetics of accumulation of ^{3}H label in the rabbit visual system after intraocular injections of [^{3}H]leucine. Our observations confirm their interpretation, and extend it by demonstrating that the proteins transported at different velocities differ in composition.

This latter conclusion differs from that of Marko, Susz, and Cuenod (11). From observations of the transport of proteins to a synaptosomal fraction obtained from the pigeon optic tectum, these authors suggest that two different components of transported proteins (defined by the kinetics of accumulation of label) contain qualitatively identical proteins. Although this discrepancy could reflect a species difference, it seems more likely that the resolution provided by the autoradiographic method has allowed us to observe compositional changes that could not be resolved by their analytical techniques.

The functional significance of multiple velocities of intra-axonal transport is unknown, but two possible alternatives may be considered. On the one hand, each velocity might simply reflect the most expedient mode of transfer of a protein from its site of synthesis in the cell soma to its destination in the axon or its synaptic endings. For example, multiple velocities of intra-axonal transport could arise as a result of the application of a single transport mechanism to different protein-containing organelles. Thus, if two different organelles differing in size were subjected to identical driving forces by identical mechanisms, the larger organelle would be expected (because of its greater frictional coefficient) to move more slowly through a viscous medium such as axoplasm.

On the other hand, the velocity of transport of a protein could serve as an important temporal mediator of alterations in axonal or synaptic function. If the amount of a protein committed to transport is responsive to changes in certain physiological or developmental parameters, the transport velocity of that protein would dictate the minimal time between a change in such a parameter and the resulting change in the concentration of the protein at its destination. However, the velocity of transport could only be an important temporal factor in reducing the concentration of a protein at its destination if its half-life at its destination were not greatly in excess of its transport time. Otherwise the cessation of transport of a protein would result in its disappearance from its

destination at a rate determined by its half-life at its destination, rather than by its transport velocity. Among the most rapidly transported proteins in the rabbit retinal ganglion cells, we have observed one (band 22, Figs. 2*B* and 3) that disappears from the axon with a $t_{1/2}$ of less than 3 hr. Since there appear to be no long-lived pools of this protein in the system, the time lag between a modulation of its transport from the cell soma and the resulting alteration of its concentration at its destination would largely reflect its transport time. Thus, polypeptide 22 disappears with a $t_{1/2}$ that might be expected if the function of its rapid rate of transport were in fact the rapid communication of changes in protein concentration to the ganglion cell axons or their synaptic terminals.

Conversely, if the slow rate of disappearance of some rapidly transported polypeptides (for example band 20 in Fig. 4) reflects their rate of turnover at their destination, their rapid rate of transport could not be a critical temporal factor in reducing their concentration at their destination. However, long disappearance times could alternatively reflect (*i*) the presence of a long-lived pool of these proteins in the bodies of the retinal ganglion cells, or (*ii*) the transport at more than one rate of either the same or electrophoretically indistinguishable polypeptides.

Intra-axonal transport could also influence events in the axons and at their synaptic terminals through variations in the *rate* (and perhaps as a result of this, in the *amount*) of transport of particular proteins. The rate of protein transport in axons (as judged by the movement of total radioactivity) has been reported to vary with age (22–24) and after axonal injury (25). In view of the existence of multiple rates of protein transport, the possibility should be considered that an apparent alteration in the rate of transport might alternatively reflect the transport of different classes of protein (each characterized by a different and invariant transport velocity) under different physiological or developmental conditions.

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