THE IDENTIFICATION OF TWO INTRA-AXONALLY TRANSPORTED POLYPEPTIDES RESEMBLING MYOSIN IN SOME RESPECTS IN THE RABBIT VISUAL SYSTEM

MARK WILLARD

From the Departments of Anatomy and Neurobiology, and Biochemistry, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT

Two polypeptides (M1 and M2) which co-sediment with F-actin in an ATP-reversible way have been detected in extracts of tissue from the rabbit visual system. Both polypeptides resemble skeletal muscle myosin in their ATP-sensitive co-sedimentation with actin, while they resemble the heavy chain of myosin and the lighter polypeptide of erythrocyte spectrin in their electrophoretic mobilities. (The estimated molecular weights are: M1 $\simeq$195,000; myosin $\simeq$200,000; M2 and spectrin $\simeq$220,000). M1 and M2 were labeled in the cell bodies of the retinal ganglion cells with a radioactive amino acid and subsequently recovered in tissues (optic nerve, optic tract, lateral geniculate nucleus, and superior colliculus) containing segments of the retinal ganglion cell axons. The temporal sequence of labeling M1 and M2 in these tissues indicated that both polypeptides were synthesized in the cell bodies of retinal ganglion cells and subsequently transported down their axons at different maximum velocities. The estimated velocities were: M1, 4–8 mm per day; and M2, 2–4 mm per day.

KEY WORDS axonally transported proteins · myosin · rabbit visual system · polyacrylamide gel electrophoresis · autoradiography

Many neuronal proteins are conveyed from their site of synthesis in the cell body to the axon and synaptic terminals by the process of intraaxonal transport. Since the somatofugal movement of these proteins suggests that they are involved in axonal and synaptic events, it is important to establish their identities and functions. I report here evidence indicating that two of the transported proteins have one of the properties of muscle myosin, i.e., ATP-sensitive binding to actin. Since the generation of force in muscle requires the binding of myosin filaments to actin filaments and their subsequent dissociation by ATP (10, 25, 29), ATP-sensitive actin binding (ASAB) is a prerequisite for a myosin-like function of a protein. It has been suggested that proteins with this property may play a role in such neuronal functions as neurotransmitter release (1), process elongation during development (3), and protein transport in the axons (23, 26).

A suitable experimental system for studying the transport of ASAB proteins is provided by the centrally projecting fibers of the rabbit retina (the axons of the retinal ganglion cells), which pass through the optic nerve and contralateral optic tract to form synaptic terminations in the lateral geniculate nucleus and the superior colliculus (Fig. 1). Because of their proximity to the vitreous, the cell bodies of these neurons are able to take up radioactive amino acids injected into the eye and

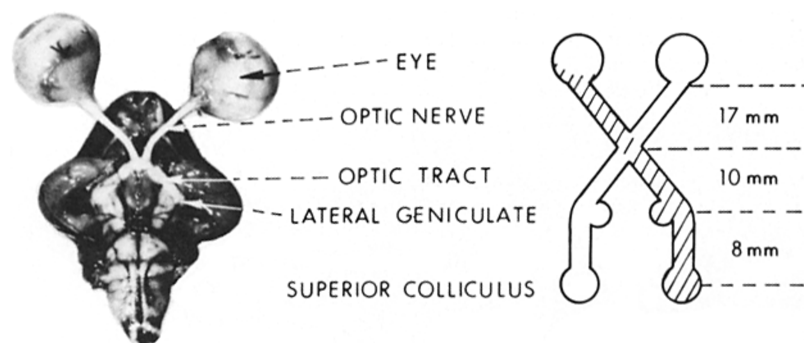

FIGURE 1 Left: A ventral view of a rabbit's brain and eyes. The superior colliculus, on the dorsal side, is not visible. Right: The approximate dimensions of the visual system structures containing axons of the rabbit retinal ganglion cell. The diagonal shading indicates the pathway taken by the great majority of retinal ganglion cell axons originating in one eye (7).

to incorporate them into proteins. Some of the labeled proteins (comprising more than 40 polypeptides [32]) are transported down the axon in at least five groups, each group defined by a characteristic polypeptide composition and transport velocity (12, 32, 33). The estimated transport velocities are: group I, >240 mm per day; group II, 34–68 mm per day; group III, 4–8 mm per day; group IV, 2–4 mm per day; Group V, 0.7–1.1 mm per day. To determine whether any of the transport groups include ASAB proteins, I measured the radioactivity associated with ASAB proteins in tissues containing sequential segments of the retinal ganglion cell axons at increasing time intervals after labeling the retina with ^{35}S-methionine.

MATERIALS AND METHODS

Labeling of Transported Proteins

^{35}S-methionine (0.5 or 1 mCi, from New England Nuclear, Boston, Mass. at a sp act of ~400 Ci/mmol) was injected into the left eyes of adult albino (New Zealand White) rabbits as described previously (32). Two animals were sacrificed by an intravenous injection of sodium pentabarbitol at each of several intervals (3, 6, and 12 h; 1, 2, 4, 5, 5.8, 8, 10.8, 16, and 24 days) after the injections, and the left optic nerves, right optic tracts, right lateral geniculate nuclei, and right superior colliculi were removed. The corresponding structures from the two animals were combined and processed together. To estimate the amount of local incorporation of blood-borne ^{35}S-methionine into ASAB proteins, the right optic nerves, left optic tracts, left lateral geniculate nuclei, and left superior colliculi (which contain primarily axons originating in the uninjected eye) were also analyzed.

Extraction of ASAB Proteins

The following procedures were carried out at 0°–4°C. The dissected tissues were homogenized in 7 ml of LS buffer (0.01 M KCl, 1 mM dithiothreitol, 2 mM KH_2PO_4, pH 6.8) and centrifuged for 1 h at 100,000 *g*. The solubility properties of muscle myosin suggest that myosin-like ASAB proteins should be insoluble under these conditions. The pellet was, therefore, re-homogenized in 3 ml of HS buffer (0.6 M NaCl, 1 mM dithiothreitol, 1 mM $MgCl_2$, 1% Triton X-100 [Packard Instrument Co., Inc., Downer's Grove, Ill.], 20 mM $Na_4P_2O_7$, pH 8.2) in order to solubilize the ASAB proteins. The purpose of the pyrophosphate was to dissociate ASAB proteins from actin. The homogenate was agitated gently for 45 min and then centrifuged at 100,000 *g* for 1 h.

Assay for ASAB Proteins

The supernates (containing the solubilized ASAB proteins) were dialyzed (to remove the pyrophosphate) for at least 16 h against three 1-liter portions of assay buffer (5 mM $MgCl_2$, 0.3 M NaCl, 0.2 mM dithiothreitol, 0.1% Triton X-100, 25 mM imidazole, pH 7). Sedimentable material was removed by centrifugation of the dialysates for 1 h at 100,000 *g* and the supernates were immediately divided into two equal portions (1.5 ml each). F-actin was added to both portions, while ATP (0.17 mM, pH 7) was added to only one of the portions. About 100 μg of F-actin was used in the experiments reported here in order to assure the formation of pellets; however, as little as 5 μg of actin was sufficient to cause maximum sedimentation of M2 from one superior colliculus. The actin was prepared from rabbit back and leg muscle by either the procedure of Martinosi (19) or the method of Spudich and Watt (27). Sedimentation of the F-actin by means of centrifugation at 100,000 *g* for 35 min gave rise to a translucent pellet in both portions. The supernates were removed and the pellets were prepared for electrophoresis by suspending them in 0.1 ml (0.2 ml in the case of the samples from the superior colliculi) of a solution containing EDTA (5 mM), dithiothreitol (2

mM), sodium dodecyl sulfate [SDS] (1.5%), glycerol (30%), bromophenol blue tracking dye, and Tris (10 mM), pH 8. The samples were heated at 90°C for 5 min and aliquots (0.05 ml) were electrophoresed.

Electrophoresis

The samples were electrophoresed on discontinuous slab gels (13 cm high and 1.2 mm thick) containing a linear gradient of polyacrylamide (5–12%) and urea (5.2–7.3 M) (32). With the exception of the added urea, the buffer systems were those described by Laemmli (15). After electrophoresis for about 12 h at 100 V, the gels were stained with Coomassie Brilliant Blue dye and destained according to the procedure of Fairbanks et al. (5).

The relative degree of recovery of ASAB polypeptides from different assays was estimated by scanning the stained electrophoretic bands with a Joyce-Loebl microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England). A constant amount of muscle myosin was electrophoresed on one track of each gel, and its intensity of staining was used to normalize the peak areas of the ASAB proteins in order to correct for any variations in staining intensity between gels.

Estimate of the Radioactivity Associated with the ASAB Polypeptides

The polyacrylamide gels were impregnated with 2, 5-diphenyloxazole (PPO) (2), dried, and exposed to X-ray film (Kodak RP/R54) at −78°C for periods ranging from 1 to 30 days. The PPO served to convert the energy of the β-particles to light energy, and increased the efficiency of the autoradiography by about an order of magnitude (2). The autoradiographs were developed under standard conditions in a Kodak X-omat processor, and the intensity of labeling of the ASAB polypeptides was determined by inspection. In addition, a numerical value indicative of the relative intensity of labeling of ASAB polypeptides was determined by scanning their position on the autoradiograph with a microdensitometer. The resulting peak area was corrected for ATP-insensitive radioactivity at the same electrophoretic position and then normalized for exposure time, radioactive decay, the quantity of isotope injected into the vitreous, and the protein recovered (as judged from densitometry of the stained gel bands). The resulting measure of relative specific activity is a monotonic function of the radioactivity associated with the ASAB proteins; however, since at low intensities of light the response of X-ray film is a sigmoidal rather than linear function of light intensity (16), the relative specific activity calculated in this way may not be directly proportional to the actual specific radioactivity.

In some instances, the radioactivity associated with ASAB proteins was determined directly by liquid scintillation counting; the ASAB protein was excised from the gel, and the gel was dissolved in 30% H_2O_2 and counted in the presence of 3A70 scintillation fluid (Research Products International Corp., Elk Grove, Ill.).

RESULTS

The Detection of the ASAB Proteins in the Rabbit Visual System

When F-actin was sedimented through extracts of visual system tissue, electrophoretic analysis of the sediments revealed, in addition to actin, several polypeptides which co-sedimented with actin (Fig. 2*A*). When ATP (0.17 mM) was included in the actin-sedimentation mixture, the concentration of a major high molecular weight polypeptide (*M2,* Fig. 2*A*) was significantly reduced in the sediment. In addition, a minor polypeptide (*M1,* Fig. 2*A*) (which was apparent only when the gels were heavily loaded with protein) also appeared to be ATP-sensitive in its actin co-sedimentation. The presence of M1 and M2 in the sediment required both the addition of actin (Fig. 2*A*, column *2*) and the addition of nervous system extract (Fig. 2*A*, column *3*); therefore, M1 and M2 originate in the nervous system and co-sediment with actin in a manner that is inhibited by ATP.

The electrophoretic mobility of M1 on SDS-urea gradient polyacrylamide gels is slightly greater than that of the heavy chain of skeletal muscle myosin (mol wt = 200,000 [17]) (Fig. 2*B*), while the mobility of M2 is close to that of the more mobile polypeptide chain of erythrocyte spectrin (mol wt = 215,000–220,000 [28, 30]) (Fig. 2*B*). These mobilities suggest approximate mol wt of 195,000 and 220,000, respectively, for M1 and M2.

M1 and M2 make up a minor fraction of the Coomassie Blue-staining proteins of the nervous system; neither polypeptide was detected after electrophoresis of unfractionated extracts of the nervous system (Fig. 2*C*). I estimate that the amount of M1 and M2 recovered from the superior colliculus is ~0.02 and 0.1%, respectively, of the total protein in this tissue. (This estimate is based on the intensity of Coomassie Blue staining of M1 and M2 relative to muscle myosin; however, the staining efficiencies of M1 and M2 are not necessarily the same as for muscle myosin). A protein with an electrophoretic mobility indistinguishable from that of M2 can be detected in whole brain, spinal cord, and sciatic nerve, by the same actin-co-sedimentation procedure described here.

The Time-Course of Labeling of M1 and M2 in Tissues Containing Sequential Segments of the Retinal Ganglion Cell Axons

If M1 and M2 are neuronal, intra-axonally transported proteins, it should be possible to label them radioactively in the neuronal cell bodies and subsequently to recover their label from segments of the axons at time intervals dictated by their transport velocities. Therefore, at increasing intervals after the intravitreal injection of ^{35}S-methionine, the radioactivity associated with ASAB proteins was analyzed in the optic nerve, optic tract, lateral geniculate nucleus, and superior colliculus (Figs. 3-7). The label co-sedimenting with actin in the absence of ATP, but not when ATP was present, was assumed to be associated with intra-axonally transported ASAB polypeptides.

M1 became radioactively labeled in a temporal sequence consistent with its transport down the axons at a velocity of 4-8 mm per day. The label associated with M1 was recovered in the distal half of the optic nerve (a minimum distance of 8 mm behind the eye), 2 days after labeling the retina, but at this time it had not yet reached the proximal edge of the optic tract (about 18 mm from the eye) (Figs. 3 and 7). By 8 days after the injection, label associated with M1 had reached the lateral geniculate nucleus (Figs. 4, 6, and 7). 16 days after the injection, the label associated with M1 was diminished in the optic nerve and optic tract, but was still present in the lateral geniculate nucleus and superior colliculus (Fig. 5).

On the other hand, M2 became labeled in axonal segments in a sequence suggesting that it is transported down the retinal ganglion cell axons at a velocity of 2-4 mm per day. Between 2 and 4 days after labeling the retina, M2-associated label first appeared in elevated amounts in the distal half of the optic nerve (Figs. 3 and 7). M2-associated label first appeared in the lateral geniculate nucleus between 8 and 11 days after the isotope injection (Figs. 4 and 7). By 24 days after the injection, the label associated with M2 had diminished in the optic nerve and optic tract, but was still present in the lateral geniculate nucleus and superior colliculus (Figs. 5 and 7).

The sequence of labeling M1 and M2 in the visual system most likely reflects the transport of these polypeptides down the axons of the retinal ganglion cells. The alternative, i.e., that M1 and M2 are locally synthesized from blood-borne radioactive amino acids by glial cells in the optic nerve, optic tract, lateral geniculate, and superior colliculus, is unlikely; visual system structures which are equally exposed to the blood but contain primarily retinal ganglion cell axons from the

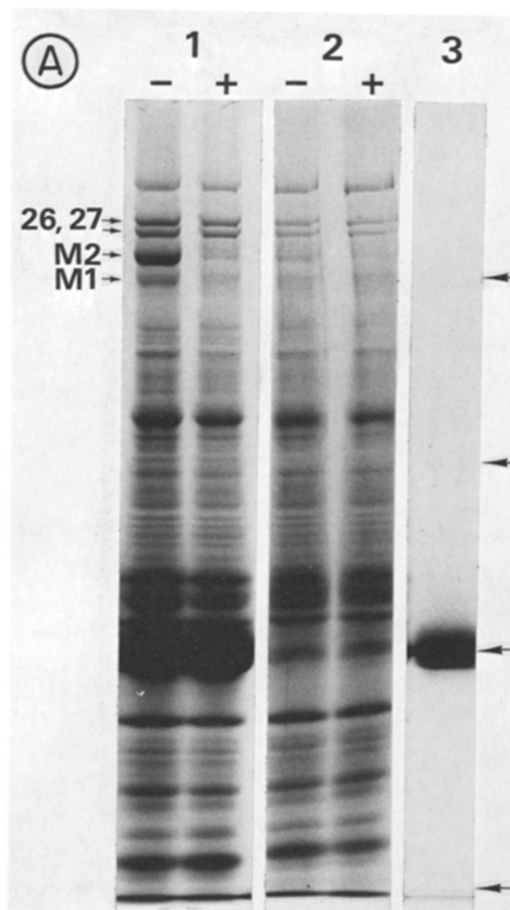

FIGURE 2*A* The actin-co-sedimenting polypeptides in an extract of the superior colliculus. Columns *1*: Actin (about 10 μg) was sedimented through an extract of the superior colliculus (Material and Methods) in the absence (−) or presence (+) of 1.7 × 10^{-4} M ATP. The polypeptides in the pellet were electrophoresed on SDS-polyacrylamide gels and the gels were stained. Columns *2* are identical to columns *1*, except that no actin was added to the extracts before centrifugation. Column *3* shows the composition of the pellet when actin was sedimented in the absence of the brain extract. The large arrows show the approximate electrophoretic mobilities of proteins of known molecular weight, i.e., from top to bottom, skeletal muscle myosin (200,000), phosphorylase *a* (94,000), actin (46,000), and lysozyme (14,000). Polypeptides 26 and 27 are discussed in the text.

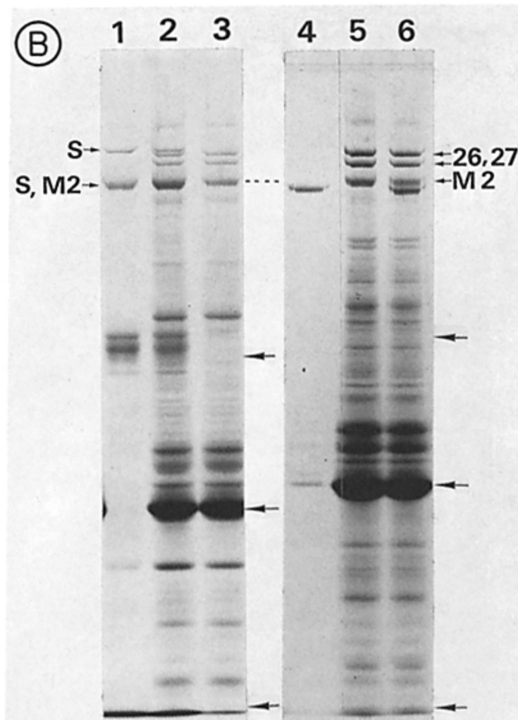

FIGURE 2*B* A comparison of the electrophoretic mobilities of M2, spectrin, and skeletal muscle myosin. Column *1*: An SDS extract of rabbit erythrocyte ghosts electrophoresed and stained. The bands labeled *S* are the two components of spectrin. Column *2*: A mixture of spectrin and M2 (the same samples and amounts as in columns *1* and *3*) electrophoresed on the same channel of the gel. The electrophoretic mobilities of M2 and the lower band of spectrin are indistinguishable in this electrophoretic system. Column *3*: M2 prepared by sedimenting actin through an extract of the superior colliculus. Column *4*: Skeletal muscle myosin prepared by actin co-sedimentation. To reduce the chance of proteolysis, a sample of skeletal muscle was homogenized directly in HS buffer (Materials and Methods) containing phenylmethylsulfonylfluoride (10^{-3} M) and *o*-phenanthroline (10^{-3} M). Instead of the usual dialysis step (Materials and Methods), the proteins were precipitated from the HS buffer with $(NH_4)_2 SO_4$ at 65% of saturation (0°C), and then resuspended in assay buffer (Materials and Methods). The myosin prepared by this method had the same electrophoretic mobility as myosin prepared by conventional methods. Column *5*: M2 prepared from the cerebral cortex by actin co-sedimentation, but with the same procedural modifications as described above (column *4*) for the preparation of myosin. Column *6*: A mixture of myosin and M2 (same samples and amounts as in columns *4* and *5*) showing that the two polypeptides are electrophoretically distinguishable. However, preliminary results indicate that the relative mobilities of myosin and M2 may be dependent upon the conditions of electrophoresis. The large arrows show the approximate electrophoretic mobilities of phosphorylase *a* (94,000), actin (46,000), and lysozyme (14,000).

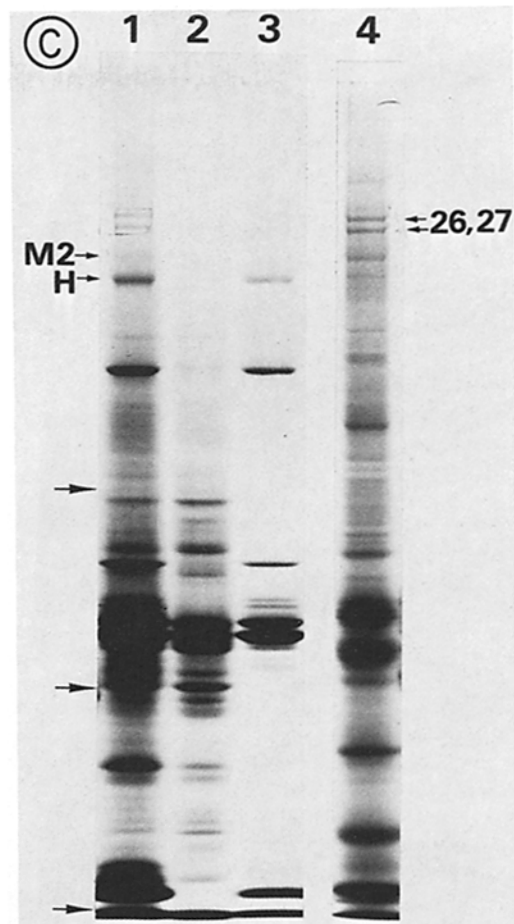

FIGURE 2*C* The polypeptides in intermediate fractions of an M1 and M2 preparation from the optic nerve and optic tract. Column *1*: The stained polypeptides from the initial homogenate in LS buffer (Materials and Methods). Column *2*: The polypeptides which are soluble in the LS buffer. Column *3*: The polypeptides which are insoluble in the HS buffer (Materials and Methods). Column *4*: The HS-buffer extract just before the addition of actin. The large arrows show the approximate electrophoretic mobilities of the same molecular weight markers shown in Fig. 2*B*. *H*, *26*, and *27* are polypeptides which are discussed in the text.

unlabeled retina, e.g., the contralateral optic tract, do not accumulate labeled M1 and M2 (Fig. 4).

While the autoradiographs of Figs. 3–6 most clearly demonstrate the relatively slow intra-axonal transport of M1 and M2, they also suggest

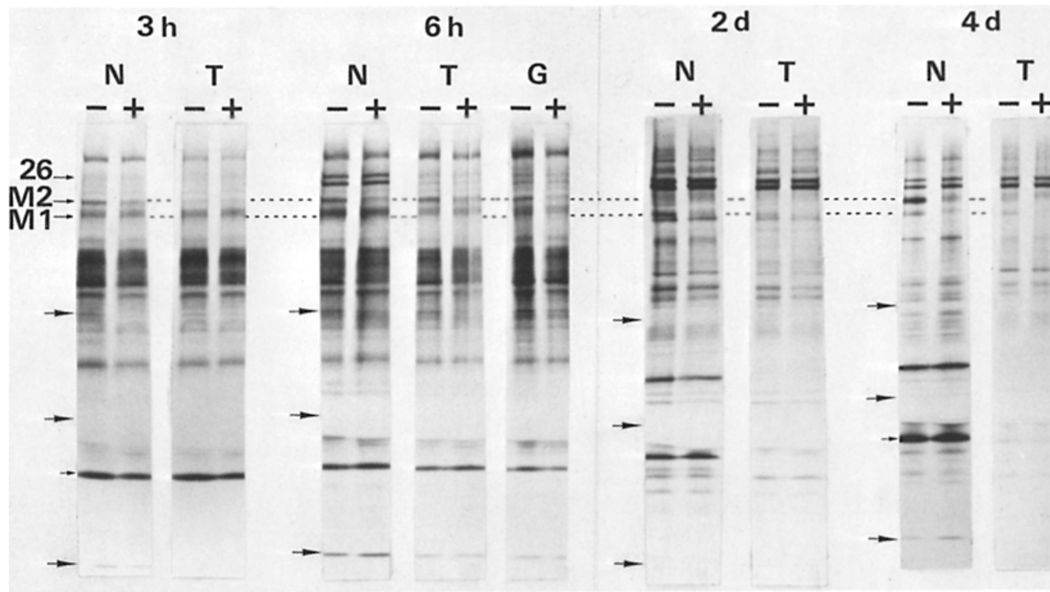

FIGURE 3 Autoradiographs of electrophoresed actin-co-sedimenting polypeptides extracted from the visual system at 3 h, 6 h, 2 days, and 4 days after an eye injection of ^{35}S-methionine. The symbols – and + indicate the absence or presence, respectively, of 0.17 mM ATP in the actin-sedimentation mixture. The designations at the left indicate the electrophoretic positions of polypeptides referred to in the text. The upper row of numbers indicates the survival time in hours (h) or days (d) after the eye injection of ^{35}S-methionine. The portion of the visual system which was analyzed is indicated by *N* (distal half of the left optic nerve), *T* (right optic tract), or *G* (right lateral geniculate nucleus). The autoradiographic exposure times were 17 days' exposure for the 3-h, 6-h, and 2-day survival times, and 8 and 25 days' exposure for the optic nerve and optic tract, respectively, from the animal which survived for 4 days. The large arrows indicate the approximate electrophoretic mobilities of the same molecular weight markers as described in Fig. 2*B*. The small arrows indicate labeled polypeptides discussed in the text. The label at the M1 position before the 2-day time-point is not sensitive to ATP; presumably, it is associated with an unrelated transported protein having the same electrophoretic mobility as M1.

(although less convincingly) that several other intra-axonally transported polypeptides may interact with actin. First, a small amount of label was associated with a low molecular weight polypeptide (20,000–30,000; a range which would include the molecular weights of some myosin light chains) which also appeared to co-sediment with actin in an ATP-sensitive fashion. (The small arrows in Figs. 4 and 6 show the position of this labeled polypeptide on the gels). While the small amount of radioactive label associated with this polypeptide makes its transport velocity uncertain, the time at which it is labeled is consistent with its movement down the axon at a velocity similar to M2 (2–4 mm per day). Second, although the major fraction of M2-associated label traveled slowly down the axons, a much smaller amount of ASAB-polypeptide-associated label of similar electrophoretic mobility appeared in the axons in a temporal sequence suggesting a rapid transport velocity of >100 mm per day. (I estimate [based upon densitometric measurements] that the rapidly moving M2-associated label may be as little as 1% of the slowly moving M2-associated label.) The amount of rapidly moving M2-associated label is near the lower limits of detection; however, it was reproducibly observed in the distal half of the optic nerve at 3 h after an eye injection, and by 6 h it had reached the lateral geniculate nucleus (Fig. 3). Third, in addition to the ASAB polypeptides, several other polypeptides are concentrated in the actin pellet but in a manner that is not inhibited by ATP. These include two polypeptides (bands *26* and *27* in Figs. 2–5) which were previously shown to be transported in group II (34–68 mm per day [32]). One of these (band *26*) electrophoreses slightly faster than the less mobile component of erythrocyte spectrin (mol wt, 240,000–

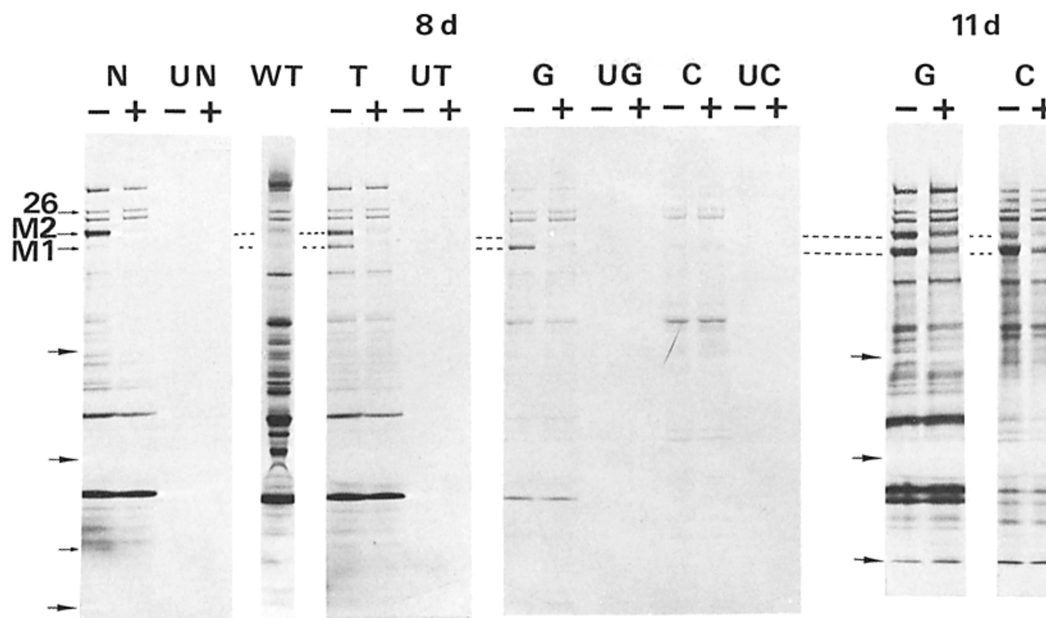

FIGURE 4 Autoradiographs of electrophoresed actin-co-sedimenting polypeptides extracted from the visual system 8 and 11 days after an eye injection of ^{35}S-methionine. The designations are the same as for Fig. 3, except that *C* indicates the right superior coliculus, while *UN, UT, UG,* and *UC* indicate the control structures (right optic nerve, left optic tract, left lateral geniculate nucleus, and left superior colliculus) containing predominantly retinal ganglion cell axons originating in the uninjected right eye. The column labeled *WT* shows labeled proteins present in an SDS extract of one-twentieth of the particulate fraction of the optic tract, before extracting myosin-like polypeptides from it with HS buffer. The autoradiographic exposure times were 56 h and 4 days for the optic nerve and the WT sample, respectively, and 8 days for all other samples.

250,000 [28, 30]) (Fig. 2*B*). Two other labeled polypeptides with molecular weights close to 30,000 (which is also the molecular weight of brain-tropomyosin [6]) appear in the actin pellet. One of these (at the 3- and 6-h time-points in Fig. 3 [small arrow]) is transported in group I at a velocity of >200 mm per day (32), while the other (at the 4-day time in Fig. 3 [small arrow]) (Fig. 4) is transported in group IV at 2–4 mm per day.

DISCUSSION

The intra-axonal transport of M1 and M2 in the axons of the rabbit retinal ganglion cells implies that this single type of cell requires two different polypeptides which are able to bind to actin in an ATP-reversible way. The nature of this requirement can presently be only surmised by considering the possible relationship of M1 and M2 to other actin-co-sedimenting proteins, to each other, and to other intra-axonally transported polypeptides.

In certain respects, M1 and M2 resemble other actin-co-sedimenting polypeptides. They are strikingly similar to muscle myosin. First, M1, M2, and the heavy chain of muscle myosin each have a mol wt close to 200,000. Second, all three polypeptides co-sediment with actin in an ATP-reversible way. In the case of muscle myosin, ATP-reversible actin co-sedimentation results from a direct binding of myosin to actin; it seems likely that the actin co-sedimentation of M1 and M2 also reflects a direct interaction of these polypeptides with actin. Alternatively, actin co-sedimentation would occur if M1 or M2 bound in an ATP-sensitive way to some other actin-binding protein, or if one of the two polypeptides bound to the other, which in turn bound to actin in an ATP-sensitive way. Preliminary experiments show that M2 retains its ATP-reversible actin-binding properties in a partially purified brain extract, suggesting that at least M2 interacts directly with actin (M2 is the major protein in this extract, making up more than one-half of the Coomassie Blue-staining protein). A third consideration in evaluating the possibility

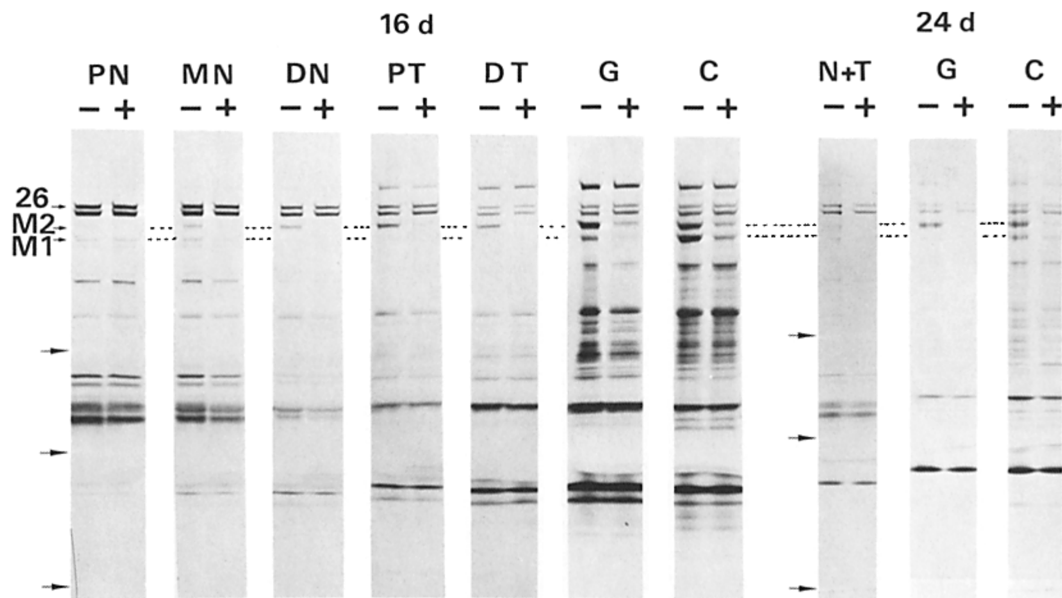

FIGURE 5 Autoradiographs of electrophoresed actin-co-sedimenting polypeptides extracted from the visual system at 16 and 24 days after an injection of ^{35}S-methionine into the left eye. In the case of the animal which survived for 16 days, the entire optic nerve was divided into three equal segments (about 5 mm in length) designated *PN, MN,* and *DN,* in order of increasing distance from the eye. The optic tract was divided into two equal segments (about 5 mm in length) designated *PT* and *DT* in order of increasing distance from the eye. In the case of the animal which survived for 24 days after the eye injection, the optic nerve and optic tract were combined ($N + T$) before analysis. Autoradiographic exposure times were 8 days for all samples from animals which survived for 16 days, 16 days for the combined optic nerve and optic tract of the 24-day survival, and 13 days for the remaining 24-day survival samples. The large arrows indicate the approximate electrophoretic mobilities of the same molecular weight markers as shown in Fig. 2*B*.

that M1 and M2 are related to myosin is the extensive documentation that a protein physically and enzymatically similar to muscle myosin is located in the nervous system (1, 4). It is quite possible that M1 or M2, or both, are related to this "brain myosin," although the intra-axonal transport of brain myosin has not been conclusively demonstrated (Khan and Ochs reported that a $Mg^{++}Ca^{++}$-activated ATPase similar in some additional respects to actomyosin is transported slowly down the sciatic nerve of the cat; however, this ATPase differed substantially from myosin in molecular weight [14]). If, in fact, M1 and M2 are related to myosin, they would most likely be involved in carrying out mechanical processes in conjunction with actin. Several mechanical roles for myosin in neurons have been proposed (1, 3, 23, 26); M1 and M2 might each be specialized to carry out different mechanical functions.

In addition to being similar to myosin, M2 electrophoretically resembles certain actin-binding proteins isolated from rabbit alveolar macrophages (8) and sea urchin eggs (11) and also resembles the lighter polypeptide of erythrocyte spectrin (Fig. 2*B*). These proteins have a molecular weight (220,000) that is greater than that of the heavy chain of muscle myosin but is apparently very close to that of M2. (It is interesting to consider the possibility that proteins that are similar to spectrin might interact with proteins which penetrate the axonal membranes [as spectrin interacts with proteins that penetrate the erythrocyte membrane (18, 20–22, 24)] and participate in their intramembrane movements, including their transport down the axon.) On the other hand, unlike M2, these other polypeptides have not been shown to be ATP-sensitive in their interactions with actin.

While it seems most likely that M1 and M2 are the products of different genes, the presence of the two polypeptides in a single cell type suggests,

alternatively, the possibility of a genetic relationship between them, e.g., that one of the two polypeptides is post-translationally converted to the other. Although the postsynthetic conversion of M1 to M2 in the axon could account for the sequential appearance of first M1 and then M2 in consecutive axonal segments, such a conversion seems unlikely. First, the conversion could not be proteolytic in nature (since the apparent molecular weight of M2 is greater than that of M1); second, the interval between the appearance of labeled M1 and that of labeled M2 after an intra-ocular injection of ^{35}S-methionine is much shorter in the retina (<15 min, suggesting a simultaneous synthesis or very rapid post-translational conversion [data not shown]) than in the optic nerve and optic tract. While it is possible, on the other hand, that M2 is postsynthetically converted to a more rapidly transported form, i.e., M1, the simplest interpretation is that the two polypeptides are synthesized independently and transported at different velocities down the axons.

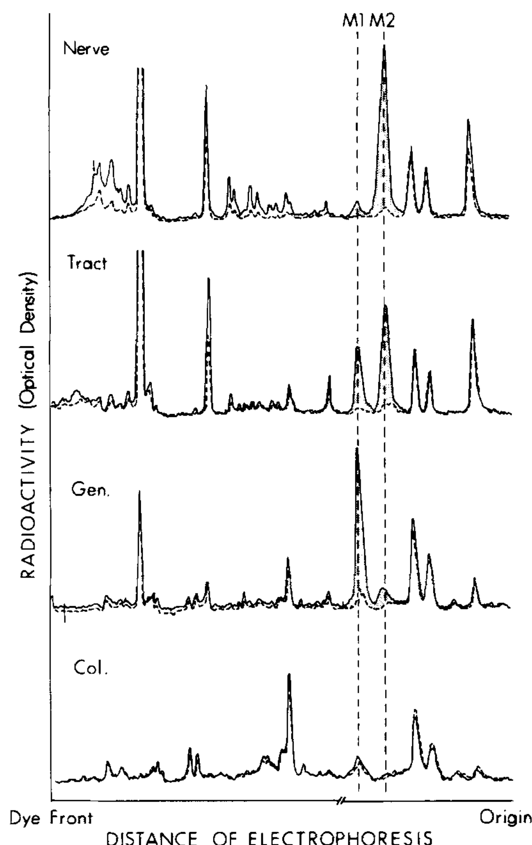

FIGURE 6 Densitometric scans of autoradiographs of electrophoresed actin-co-sedimenting polypeptides extracted from the visual system 8 days after an eye injection of ^{35}S-methionine. The solid lines show scans derived from samples where ATP was absent during the actin sedimentation. The superimposed broken lines show scans derived from identical samples, except that ATP (0.17 mM) was included in the medium during the actin sedimentation. The shaded areas emphasize the ATP-sensitive actin-co-sedimenting radioactivity with the electrophoretic mobilities of M1 and M2. The visual system structures which were analyzed were, from the top, the distal half of the optic nerve, the optic tract, the lateral geniculate nucleus, and the superior colliculus. The small arrow indicates the electrophoretic position of a low molecular weight polypeptide which may also show ATP-sensitive actin co-sedimentation (see text). The autoradiographic exposure times were the same as those in Fig. 4.

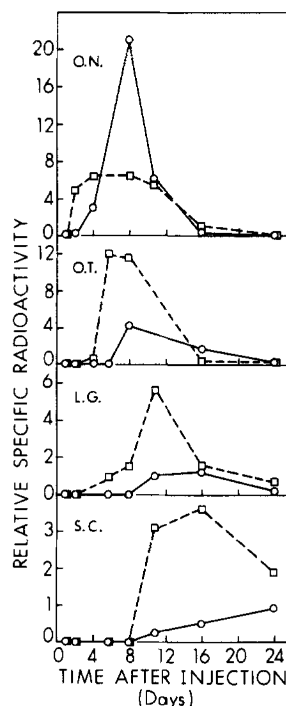

FIGURE 7 A summary of the arrival of M1 (broken line) and M2 (solid line) associated radioactivity in structures of the visual system as a function of time after labeling the retina with ^{35}S-methionine. The "relative specific radioactivity" indicates the order of intensity of labeling at different times, but is not necessarily directly proportional to the actual specific radioactivity (Materials and Methods). The visual system structures are, from the top: the half of the optic nerve most distal to the eye (O.N.), the optic tract (O.T.), the lateral geniculate nucleus (L.G.), and the superior colliculus (S.C.).

M1 and M2 are among more than 40 polypeptides that are transported down the axons of the

rabbit retinal ganglion cells. The transported polypeptides appear to be organized into at least five groups; each group has a characteristic composition and transport velocity (32). While the identities of individual transported polypeptides are known in only a few instances, the available evidence suggests that the polypeptides of each group are related to each other either functionally, or by virtue of sharing the same subcellular compartment. For example, the polypeptides of the most rapidly transported group (group I, velocity >200 mm/day) are associated with material which has hydrodynamic properties similar to those of the plasma membrane (T. Lorenz, unpublished observations from this laboratory). On the other hand, group II polypeptides (with the exception of polypeptides 26 and 27 [reference 32 and Fig. 2]) appear to be associated with mitochondria (T. Lorenz, unpublished experiments). None of the polypeptides of group III have previously been identified. Group IV probably includes tubulin (13) and actin (Willard, manuscript in preparation), while group V contains polypeptides similar to those which Hoffman and Lasek have suggested are associated with neurofilaments (9). The relationship of M1 and M2 to this organization of intra-axonally transported polypeptides is indicated by their transport velocities (4-8 and 2-4 mm per day, respectively); M1 is most likely a group III polypeptide, while M2 is probably transported in group IV. M1 is, therefore, the first group III polypeptide to which an activity, i.e., ATP-sensitive binding to actin) can be assigned. Group IV probably includes three proteins (M2, actin, and tubulin) with properties suggesting their involvement in intracellular movements; two of these proteins (M2 and actin) interact with each other in a manner that could be analogous to the interaction of actin and myosin. It will be of interest to learn whether additional group IV polypeptides have properties which also suggest a relationship to actin, myosin, or tubulin, and an involvement in intracellular movements.

M1 and M2 appear to differ from any of the transported polypeptides previously described in the rabbit retinal ganglion cells (32, 33). While several of these previously described transported polypeptides are electrophoretically similar to M1 and M2 (and myosin), they differ from M1 and M2 in their transport velocities. For example, polypeptide number 34 (32) has an electrophoretic mobility similar to that of M2; however, it is transported more rapidly than M2, in group III. On the other hand, polypeptide H (Fig. 2*C*) (31) has an electrophoretic mobility similar to that of M1, but is transported more slowly than M1, in group V (33). (H is probably analogous to the 212,000 "slow component triplet" polypeptide of the sciatic nerve described by Hoffman and Lasek [9]). It appears that M1 and M2 were not previously detected due to their low level of labeling relative to many other transported polypeptides. This conclusion emphasizes the sensitivity of the methods used here to detect the ASAB polypeptides (the present methods are able to resolve ASAB-polypeptide-associated label estimated to constitute as little as 0.001% of the total protein-associated label) and raises the possibility that the number of transported polypeptides in the rabbit retinal ganglion cells may be considerably in excess of the 43 polypeptides resolved previously (32).

I am indebted to K. Hulebak and M. Wiseman for invaluable technical assistance; to T. Lorenz, M. Estridge, and Drs. C. Kent, P. R. Vagelos, and D. A. Fischman for helpful discussion; to Drs. A. Martinosi, M. C. Beinfeld, L. Kurz, and B. Ashby for generous gifts of actin, myosin, and advice; to M. W. Rhoades for photography, and to Dr. W. M. Cowan for helpful suggestions on the manuscript.

This work was supported by grant NS-1245-01 from the National Institutes of Health and by a Jerry Lewis Neuromuscular Research Center Grant.

Received for publication 22 March 1976, and in revised form 11 April 1977.

REFERENCES

1. Berl, S., S. Puszkin, and W. J. Nicklas. 1973. Actomyosin-like protein in brain. *Science (Wash. D. C.)*. **179**:441-446.
2. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83-88.
3. Bray, D. 1973. Model for membrane movements in the neural growth cone. *Nature (Lond.)*. **224**:93-96.
4. Burridge, K., and D. Bray. 1975. Purification and structural analysis of myosins from brain and other non-muscle tissues. *J. Mol. Biol.* **99**:1-14.
5. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*. **10**:2606-2617.
6. Fine, R. E., A. L. Blitz, S. E. Hitchcock, and B. Kaminer. 1973. Tropomyosin in brain and growing

neurons. *Nat. New Biol.* **245:**182-186.

7. Giolli, R. A., and M. D. Guthrie. 1969. The primary optic projections in the rabbit: an experimental degeneration study. *J. Comp. Neurol.* **136:**99-126.
8. Hartwig, J. H., and T. P. Stossel. 1975. Isolation and properties of actin, myosin, and a new actin-binding protein in rabbit alveolar macrophages. *J. Biol. Chem.* **250:**5696-5705.
9. Hoffman, P. N., and R. J. Lasek. 1975. The slow component of axonal transport. *J. Cell Biol.* **66:**351-366.
10. Huxley, H. E. 1973. Muscular contraction and cell motility. *Nature (Lond.).* **243:**445-449.
11. Kane, R. E. 1975. Preparation and purification of polymerized actin from sea urchin egg extracts. *J. Cell Biol.* **66:**305-315.
12. Karlsson, J. E., and J. Sjöstrand. 1971. Synthesis, migration and turnover of protein in retinal ganglion cells. *J. Neurochem.* **18:**749-767.
13. Karlsson, J. O., and J. Sjöstrand. 1971. Transport of microtubular protein in axons of retinal ganglion cells. *J. Neurochem.* **18:**975-982.
14. Khan, M. A., and S. Ochs. 1974. Magnesium or calcium activated ATPase in mammalian nerve. *Brain Res.* **81:**413-426.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680-685.
16. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56:**335-341.
17. Lowey, S. 1971. Myosin-molecule and filament. *In* Subunits in Biological Systems. Part A. S. N. Temasheff and G. D. Fasman, editors. Marcel Dekker, New York. 201-259.
18. Marchesi, V. T., and E. Steers. 1968. Selective solubilization of a protein component of the red cell membrane. *Science (Wash. D. C.).* **159:**203-209.
19. Martinosi, A. 1962. Studies on actin VII. Ultracentrifugal analysis of partially polymerized actin solutions. *J. Biol. Chem.* **237:**2795-2803.
20. Nicolson, G. L., V. T. Marchesi, and S. J. Singer. 1971. The localization of spectrin on the inner surface of human red blood cell membranes by ferritin-conjugated antibodies. *J. Cell Biol.* **51:**265-272.
21. Nicolson, G. L., and R. G. Painter. 1973. Anionic sites of human erythrocyte membranes. II. Antispectrin-induced transmembrane aggregation of the binding sites for positively charged colloidal particles. *J. Cell Biol.* **59:**395-406.
22. Nicolson, G. L., and S. J. Singer. 1971. Ferritin-conjugated plant agglutinins as specific saccharide stains for electron microscopy: applications to saccharides bound to cell membranes. *Proc. Natl. Acad. Sci. U. S. A.* **68:**942-945.
23. Ochs, S. 1972. Fast transport of materials in mammalian nerve fibers. *Science (Wash. D. C.).* **176:**252-260.
24. Painter, R. G., M. Sheetz, and S. J. Singer. 1975. Detection and ultrastructural localization of human smooth muscle myosin-like molecules in human non-muscle cells by specific antibodies. *Proc. Natl. Acad. Sci. U. S. A.* **72:**1369-1363.
25. Pollard, T. D., and R. R. Weihing. 1974. Actin and myosin and cell movement. *CRC Crit. Rev. Biochem.* **2:**1-65.
26. Schmitt, F. O., and F. E. Samson, Jr. 1968. The molecular biology of neuronal fibrous proteins. *Neurosci. Res. Program. Bull.* **6**(no. 2)**:**119-144.
27. Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. *J. Biol. Chem.* **246:**4866-4871.
28. Steck, T. L. 1972. Crosslinking the major proteins of the isolated erythrocyte membrane. *J. Mol. Biol.* **66:**295-305.
29. Taylor, E. W. 1973. Mechanism of actomyosin in ATPase and the problem of muscle contraction. *Curr. Top. Bioenerg.* **5:**201-231.
30. Tilney, L. G., and P. Detmers. 1975. Actin in erythrocyte ghosts and its association with spectrin. *J. Cell Biol.* **66:**508-520.
31. Willard, M. B. 1976. Genetically determined protein polymorphism in the rabbit nervous system. *Proc. Natl. Acad. Sci. U. S. A.* **73:**3641-3645.
32. Willard, M., W. M. Cowan, and P. R. Vagelos. 1974. The polypeptide composition of intra-axonally transported proteins: evidence for four transport velocities. *Proc. Natl. Acad. Sci. U. S. A.* **71:**2183-2187.
33. Willard, M. B., and K. L. Hulebak. 1977. The intra-axonal transport of polypeptide H: evidence for a fifth (very slow) group of transported polypeptides in the retinal ganglion cells of the rabbit. *Brain Res.* In press.