Posttranslational Modification of a Neurofilament Protein during Axoplasmic Transport: Implications for Regional Specialization of CNS Axons

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ABSTRACT The possibility that proteins are modified during axoplasmic transport in central nervous system axons was examined by analyzing neurofilament proteins (200,000, 140,000, and 70,000 mol wt) along the mouse primary optic pathway (optic nerve and optic tract). The major neurofilament proteins (NFPs) exhibited considerable microheterogeneity. At least three forms of the "140,000" neurofilament protein differing in molecular weight by SDS PAGE (140,000–145,000 mol wt) were identified. The "140,000" proteins, and their counterparts in purified neurofilament preparations, displayed similar isoelectric points and the same peptide maps. The "140,000" NFPs exhibited regional heterogeneity when consecutive segments of the optic pathway were separately examined on polyacrylamide gels. Two major species (145,000 and 140,000 mol wt) were present along the entire length of the optic pathway. The third protein (143,000 mol wt) was absent proximally but became increasingly prominent in distal segments. After intravitreal injection of [^{3}H]proline, newly synthesized radiolabeled proteins in the "140,000" mol wt region entered proximal mouse retinal ganglion cell (RGC) axons as two major species corresponding to the 145,000 and 140,000 mol wt NFPs observed on stained gels. When transported NFPs reached more distal axonal regions (30 d postinjection or longer), a 143,000 mol wt protein appeared that was similar in isoelectric point and peptide map to the 145,000 and 140,000 mol wt species. The results suggest that (*a*) the composition of CNS neurofilaments, particularly the "140,000" component, is more complex than previously recognized, that (*b*) retinal ganglion cell axons display regional differentiation with respect to these cytoskeletal proteins, and that (*c*) structural heterogeneity of "140,000" NFPs arises, at least in part, from posttranslational modification during axoplasmic transport. When excised but intact optic pathways were incubated in vitro at pH 7.4, a 143,000 NFP was rapidly formed by a calcium-dependent enzymatic process active at endogenous calcium levels. Changes in major proteins other than those in the 145,000–140,000 mol wt region were minimal. In optic pathways from mice injected intravitreally with L-[^{3}H]proline, tritiated 143,000 mol wt NFP formed rapidly in vitro if radioactively labeled NFPs were present in distal RGC axonal regions (31 d postinjection). By contrast, no 143,000 mol wt NFP was generated if radioactively labeled NFPs were only present proximally in RGC axons (6 d postinjection). The enzymatic process that generates 143,000 mol wt NFP in vitro, therefore, appears to have a nonuniform distribution along the RGC axons. The foregoing results and other observations, including the accompanying report (*J. Cell Biol.*, 1982, 94:159–164), imply that CNS axons may be regionally specialized with respect to structure and function.

The 100-Å intermediate filaments of neurons, referred to as neurofilaments, have been characterized biochemically (1, 6, 7, 19, 21, 25, 29, 52) and morphologically (32, 44, 50, 67) in various vertebrate and invertebrate species. In longitudinal

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sections of neurons they appear as nonbranching structures of variable lengths oriented parallel to the long axis of the axon. Neurofilaments are believed to be major components of the neuronal cytoskeleton (14, 23, 46), although their precise structural or functional role has not been clearly established. Purified mammalian neurofilaments contain at least three major protein species in the molecular weight classes 68,000–72,000, 140,000–170,000, and 200,000–220,000 (1, 6, 7, 25, 57). The association of these proteins in a single organelle was initially suggested by their movement within one phase of axoplasmic transport (~0.2 mm/d) (3, 18). The view that these major protein species are physically associated with the neurofilament is supported by the demonstration that isolated neurofilaments were decorated by antibodies directed against each of these proteins (48, 66).

Recent observations that the major neurofilament proteins (NFPs) exhibit microheterogeneity (6, 10, 35, 36) and are modified by phosphorylation (35, 40, 56) indicate that neurofilament structure may be quite complex. Little is known, however, about the nature of this heterogeneity, the spatial organization of the several neurofilament protein forms within neurons and their processes, and whether the structural heterogeneity is related to functional complexity.

In earlier studies from this laboratory, various proteolytic enzymes were shown to be active within CNS axons incubated in vitro (33, 34). We hypothesized that if posttranslational modification of proteins, by any enzymatic mechanism, occurs in vivo within axons this process may become evident as alterations of protein composition along the axonal length. In the present study, we have tested the relevance of this concept to neurofilament protein heterogeneity and distribution within retinal ganglion cell (RGC) neurons of the mouse. Our results demonstrate a greater degree of heterogeneity in the molecular weights of the 140,000–145,000 ("140,000") neurofilament species than was previously recognized. Furthermore, the heterogeneity was greater in distal segments of the RGC axon than in more proximal segments. In radioisotopic studies we found that different proportions of these multiple "140,000" forms along RGC axons are consistent with the posttranslational modification of NFPs during axoplasmic transport. Additional studies of excised but intact optic pathways demonstrated that the enzymatic process generating a 143,000 mol wt NFP in vitro is calcium-dependent and appears to be more active in distal than in proximal regions of RGC axons. In the accompanying paper (5) axonal processing during axoplasmic transport was also shown to lead to proximodistal variations in tubulin subunits along RGC axons. The implications of these findings for regional specialization of structure and function within axons are discussed. A portion of the results was presented in preliminary communications (35, 36).

MATERIALS AND METHODS

Animals

Male or female mice of the C57Bl/6J inbred strain, aged 10–14 wk, were used in all experimental studies. In several cases, however, neurofilaments prepared from C57Bl/6J-C3H or C57Bl/6J-CBA F_1 hybrids by the axonal flotation method (see below) were used to identify neurofilaments on SDS gels; these preparations exhibited a protein composition identical to that of C57Bl/6J neurofilament preparations. The mouse strains, initially obtained from Jackson Laboratories (Bar Harbor, ME), were bred at our facility. Mice were housed at 23°C on a 12-h light-dark cycle and maintained on Wayne Blox (Allied Mills, Chicago, IL) supplied ad libitum.

Tissue Dissections

The mice were sacrificed by cervical dislocation followed by decapitation. After cooling the brain for several minutes in aluminum foil on ice, the primary optic pathway was dissected out with the aid of a stereomicroscope. After removal of meninges, the optic nerves and tracts were freed from basal brain tissue and severed on each side at a point overlying the lateral geniculate body 1.5 mm from the superior colliculus. Referred to as the primary optic pathway, this dissected segment measured 10 mm in length and consisted of the optic nerve severed at the scleral surface of the eye, the optic chiasm, and a length of optic tract extending to but not including terminals in the lateral geniculate nucleus. In certain cases, the optic pathway samples were cut into consecutive 1.1-mm segments on a micrometer-calibrated slide. Tissues were frozen and stored at −70°C for up to 1 mo before analysis. Extensive preliminary experiments, including enzyme inactivation studies using microwave irradiation (described below), revealed no postmortem changes in protein composition during the dissection and storage procedures.

In several experiments, mice were killed by a 1.5-s pulse of irradiation in a Litton microwave apparatus before analysis of proteins in the optic pathway by SDS PAGE. The completeness of enzymatic inactivation was indicated in preliminary experiments by the total inactivation of proteolytic enzyme activity in vitro (33). Due to the extreme friability of the irradiated tissue, the most distal 2 mm of the optic tract was not included in subsequent analyses.

Neurofilament Preparation

Fractions enriched in neurofilaments were prepared from white matter using the general procedure for axon flotation described by De Vries et al. (11), except that 5 mM EGTA was added to all solutions. The myelinated axons were then disrupted by treatment with Triton X-100 (21) and the neurofilaments were purified as previously described (6). Samples were stored at −70°C until used.

One-dimensional PAGE

One-dimensional SDS PAGE was performed by the procedure of Laemmli (20) using 5–15% linear polyacrylamide gradients (6). Long slab gels were used in all experiments (dimensions, $140 \times 260 \times 1.5$ mm^3). Samples for electrophoresis were prepared in such a way as to minimize proteolysis and protein loss. All samples were always stored at −70°C and subsequent manipulations were performed at 0°–4°C. Typically 1–2 mg of wet weight of tissue (per well) was thawed in 150 μl of ice cold 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, and 0.002% bromophenol blue inside a small glass homogenizer. After the addition of 2 μg each of RNase A and DNase I, the sample was homogenized with a Teflon pestle and kept on ice for 5 min. The sample was made 2% in SDS and 5% in β-mercaptoethanol by the addition of 10% SDS and concentrated β-mercaptoethanol. Finally the tissue sample was heated for 5 min in boiling water and subjected to electrophoresis. When radiolabeled samples were used, 3- or 5-μl aliquots were taken for scintillation counting before electrophoresis. After electrophoresis, gels were stained in 0.1% Coomassie Brilliant Blue, 50% methanol, and 10% acetic acid for 30 min and were destained in 5% methanol and 10% acetic acid. Fluorographic procedures were described previously (31).

Preparation of Samples for Two-dimensional PAGE (2D PAGE)

NEUROFILAMENTS: A neurofilament preparation, previously stored as a pellet at −70°C, was dissolved in solution A of O'Farrell (39) and immediately applied to an isoelectric focusing gel (29). LKB ampholytes were used in all experiments (LKB Instruments, Inc., Rockville, MD). Samples were not stored in buffer A before electrophoresis. Trial experiments were used to determine the amount of each pellet used per gel.

OPTIC PATHWAY: Samples of frozen optic pathway (optic nerve and/or optic tract, 2–5 mg wet wt) were transferred to a small conical centrifuge tube containing 0.05 ml of 0.01 M Tris-HCl, pH 7.4, 5 mM $MgCl_2$, RNase A (0.05 mg/ml), and leupeptin (0.04 mg/ml). All procedures were carried out at 4°C except as noted. The tissue was homogenized using a Teflon pestle. The homogenate was subjected to four sonications, 5 s each, using a Biosink sonicator Bronwill Scientific, Rochester, NY at a power output setting of 50 U. DNase I was added to 0.05 mg/ml and the solution was incubated for 5 min at 26°C. Urea was added to a final concentration of 9.5 M in a final volume of 0.1 ml. The sample was then diluted with an equal volume of solution A (39), and an aliquot was removed for scintillation counting. The mixture was applied to an isoelectric focusing gel for electrophoresis.

Two-dimensional Gels

Two-dimensional gels were prepared using the general procedures of O'Farrell (39), modified as previously described (6, 29). Gels were stained and fluorographed as described for one-dimensional gels. As before, long slab gels were used in all experiments.

Peptide Mapping

Staphylococcus aureus V8 protease was used to generate peptide patterns during electrophoresis on 10–15% gradient acrylamide gels. The protein samples used were either homogenized individual gel bands (6, 8) or were intact segments of gel lanes using the general procedure of Bordier and Crettol-Jarvinen (4). When intact gel segments were to be digested the procedure was modified by embedding the gel segment directly in the stacking gel (29).

Axonal Transport Studies

Intravitreal injections of radiolabeled amino acids were made with a glass micropipette apparatus into anesthetized mice as previously described (33). Depending on the experiment, 10–100 μCi of L-[2,3-^{3}H]proline (sp act 30–50 Ci/mmol) or L-[^{35}S]methionine (sp act 1,000 Ci/mmol), purchased from New England Nuclear (Boston, MA), was administered in a volume of 0.25 μl of phosphate-buffered saline, pH 7.4.

To determine the rates of transport of various proteins along the retinal ganglion cell axons, consecutive 1.1-mm segments of the labeled primary optic pathway at postinjection time intervals between 2 h and 120 d were subjected to SDS PAGE. The labeled proteins were first detected by fluorography and selected bands were then cut from these gels and from additional gels not subjected to fluorography. After rehydrating the dried gel samples in 100 μl of water, the proteins were eluted by incubation at 37°C for 24 h in 10 ml of Econofluor containing 8% protosol (both reagents from New England Nuclear). The radioactivity in the gel slices was measured in a Beckman LS 2000 liquid scintillation counter equipped with an external standard mode. The disintegrations per minute in each sample were determined from the external standard value and quench curves based upon the efficiency of counting a [^{3}H]toluene internal standard in selected samples. The radioactivity in aqueous samples was determined in Aquasol (New England Nuclear). The counting efficiency was, in each case, >40%.

The relative radioactivity in consecutive segments of the primary optic pathway was plotted and the transport rate(s) for a given protein were calculated by linear regression analyses of the distance moved by the radioactive wave vs. the postinjection interval. Measurements of the distance traveled were made using as the reference point a position midway between the peak and the leading edge of each wave.

In Vitro Studies

Primary optic pathways were dissected from mice sacrificed at various intervals after intravitreal injection of L-[^{3}H]-proline (12 μCi/eye). The freshly dissected samples were incubated at 37°C in 0.75 ml of prewarmed HEPES buffer (25 mM HEPES, 6 mM KCl, 110 mM NaCl, 5.6 mM glucose, pH 7.4, as previously described [34]). In certain cases, the incubation buffer contained 4 mM $CaCl_2$ or 10 mM EGTA (see Results and the legend to Fig. 8). At various time-points after incubation, the pathways were removed and subjected to one-dimensional SDS PAGE; the radioactively labeled species were identified by fluorography. The position of neurofilament proteins was confirmed by electrophoresing purified neurofilament preparations in adjacent gel lanes.

RESULTS

Neurofilaments of Optic Nerve and Optic Tract

Neurofilaments isolated from mouse CNS by the axon flotation procedure were previously shown to be composed of proteins with approximate molecular weights of 200,000, 140,000, and 70,000 (referred to as *200*, *140*, and *70*, Fig. 1, lanes *B* and *F*). On a SDS-containing gel with a 5–15% acrylamide gradient, the 140,000 mol wt neurofilament protein region was resolved into at least three major protein species that span the size range of 140,000–145,000. After PAGE the proteins appear as a diffuse upper band and two intensely stained lower bands with the apparent molecular weights of 145,000, 143,000, and 140,000. The 145,000 mol wt band may be comprised of more than one incompletely resolved bands; however, in the present study it will be regarded as a single band. On the gel of Fig. 1 (lane *E*) proteins of the mouse primary optic pathway, consisting of optic nerve and optic tract (ON and OT), were compared with those in purified neurofilament preparations and shown to contain 200,000 and 70,000 mol wt species, and multiple proteins at approximately 145,000, 143,000, and 140,000 mol wt. The latter are collectively referred to as "140,000" proteins. On the basis of experiments described in succeeding sections, these proteins were identified as neurofilament proteins. Apparent differences in the proportions of "140,000" proteins in ON and OT (Fig. 1, lanes *C* and *D*) were more clearly defined when smaller segments of the optic pathway were analyzed separately (see below).

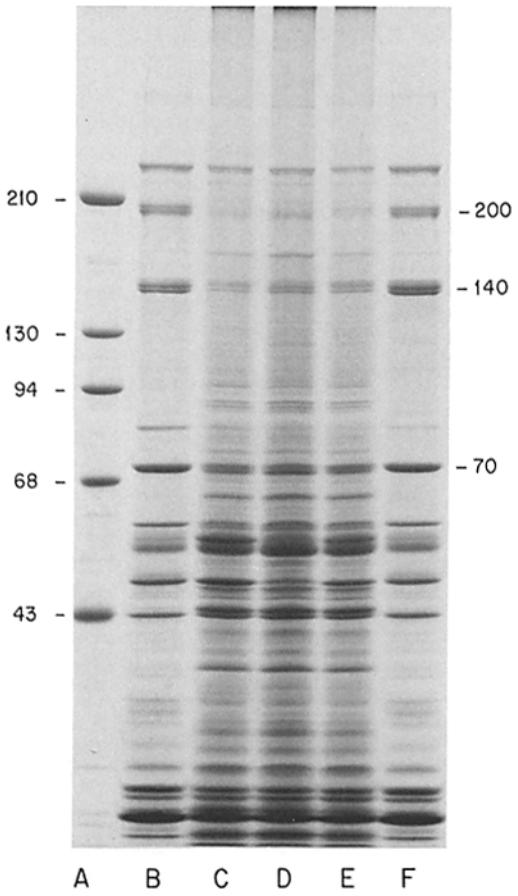

FIGURE 1 SDS gels of neurofilament proteins of the mouse primary optic pathway. The gel shown contains the following protein samples: (*A*) molecular weight standards (× 10^{-3}) (myosin, *210*; β-galactosidase, *130*; phosphorylase *b*, *94*; bovine serum albumin, *68*; ovalbumin, *43*); (*B*) neurofilament protein preparation from mouse brain; (*C*) optic nerve; (*D*) optic tract; (*E*) optic pathway (optic nerve and optic tract); (*F*) same as lane *B*. The gel contained a 5–15% acrylamide gradient and protein bands were visualized by Coomassie Brilliant Blue staining. Neurofilament proteins are indicated as bands at 200, 140, and 70 (mol wt × 10^{-3}).

Multiple "140,000" Neurofilament Proteins in ON and OT—Peptide Mapping and 2D PAGE

The identity of the multiple "140,000" species in the primary optic pathway as neurofilament proteins was established by comparison of peptide maps of "140,000" proteins from ON, OT, and mouse CNS neurofilament preparations. Overloaded SDS gels of ON, OT, and NFPs were prepared. The "140,000" band from each stained gel was excised and applied to a second SDS gel containing a 10–15% acrylamide gradient. The gel was overlaid with *S. aureus* V8 protease and partial digestion was carried out during electrophoresis. As shown in Fig. 2, peptides of the "140,000" proteins from each source were identical.

Further data on the identity of multiple "140,000" proteins was obtained by two-dimensional gel electrophoresis. On two-dimensional gels, neurofilament proteins of the primary optic pathway have isoelectric points and electrophoretic mobilities that correspond precisely to those of NFPs obtained by means of the axon flotation procedure (5) (compare Fig. 3 *A* and *B*). All "140,000" species of neurofilament preparations are present in the two-dimensional gel of optic pathway proteins.

Distribution of NFPs along the Primary Optic Pathway

To further examine apparent differences between optic nerve and optic tract "140,000" NFPs, we analyzed consecutive 1.1-mm segments of the optic pathway extending from the scleral surface to the lateral geniculate body by means of SDS PAGE. In Fig. 4, each lane represents a segment of the optic pathway; the optic chiasm occurs at segment 5 (lane *5*). Most proteins displayed a uniform distribution along the entire length of the optic pathway although numerous exceptions were observed. Among proteins exhibiting the most striking regional differences were proteins comprising the "140,000" neurofilament species and tubulin (50,000–55,000). The tubulin changes are addressed in the accompanying report (5). Within the "140,000" region, only the upper (145,000 mol wt) and lower (140,000 mol wt) protein species were present in the most proximal segments of the optic nerve (lanes *1* and *2*, Fig. 4). The "140,000" protein of intermediate size (143,000) was first detectable in segment 3 and increased in prominence in more distal segments to become the most intensely stained of the three major "140,000" bands in the distal optic tract. Identical results were obtained in multiple repetitions of this analysis. This pattern was not generated by postmortem enzymatic mechanisms since similar proximodistal changes were observed in mice killed by microwave irradiation in order to rapidly inactivate enzymatic activity (55, 58).

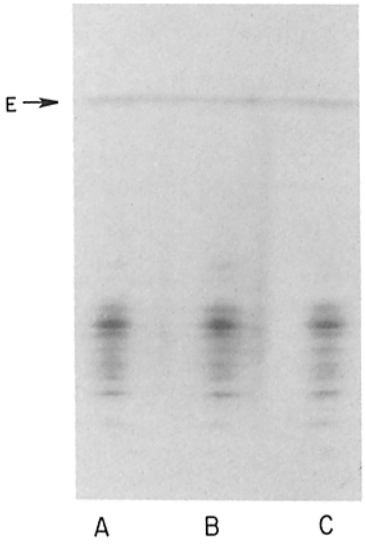

FIGURE 2 Peptide maps of 140,000 mol wt proteins. Preparations of optic tract, optic nerve, and neurofilament proteins were subjected to SDS PAGE using overloaded gels. After staining, the "140,000" proteins were excised and sealed within a second gel containing a 10–15% acrylamide gradient (3, 28). The gel was overlaid with *S. aureus* V8 protease and digestion occurred during electrophoresis. Shown are the peptides of "140,000" from OT (lane *A*), ON (lane *B*), and NF (lane *C*). *E*, position of the proteolytic enzyme.

Axoplasmic Transport Rates of NFPs in RGC Axons

The process by which the 143,000 mol wt NFP is generated in the optic tract was further investigated by examining the fate of newly synthesized neurofilament proteins within the RGC axons. RGC axonal proteins were labeled by intravitreal administration of L-[^{3}H]proline and were subsequently analyzed in consecutive segments of the optic pathway by SDS PAGE followed by fluorography or liquid scintillation counting. Populations of labeled proteins entered the optic nerve axons in multiple waves. At postinjection times >1 d, these waves were observed at progressively greater distances from the eye. When the positions of the wave crests for individual proteins at different postinjection intervals (1.5, 6, and 15 h, and 2, 4, 6, 15, 30, 45, 60, 90, and 120 d) were analyzed, at least five separate transport groups were identified moving at rates (in mm/d) of 0.1–0.4, 2–3, 8–12, 20–28, and >150, corresponding to those previously described in central axons from other species (24, 59, 64). Prominently labeled proteins at 200,000, 140,000, and 70,000 corresponding in position on one-dimensional autoradiographs (not shown) and on two-dimensional autoradiographs (Fig. 5) to purified neurofilament proteins were observed when the radiolabeled species were analyzed 30 d or longer after administration of the radioactive amino acid (see below). In SDS gels radioactive proteins at the 200,000, 140,000, and 70,000 positions, which include the NFPs, were primarily conveyed within the slowest moving group of axonally transported proteins at a rate of 0.2–0.4 mm/d. Since

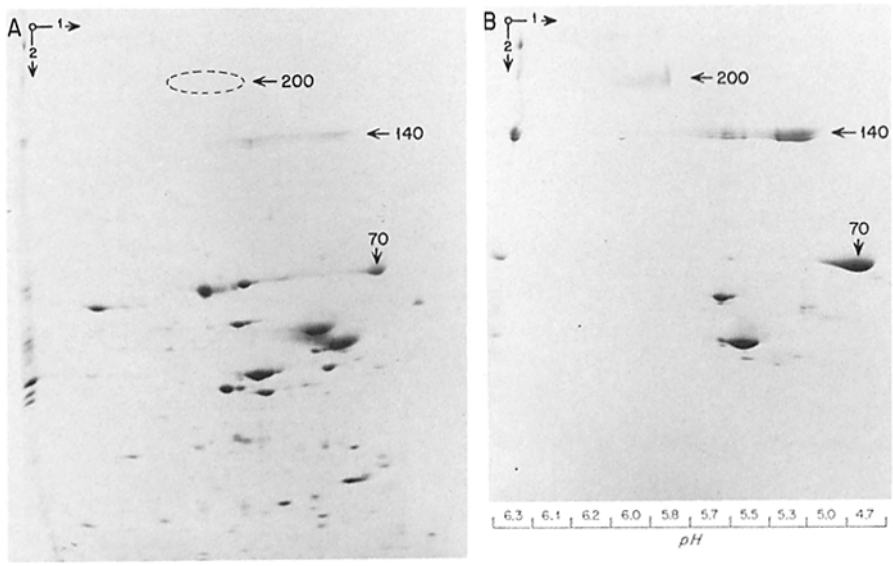

FIGURE 3 Two-dimensional gels of optic pathway proteins and neurofilament proteins. Mouse optic pathway, consisting of optic nerve and tract, was excised and subjected to two-dimensional PAGE. A similar procedure was carried out on mouse neurofilament proteins. (*A*) Optic pathway proteins; (*B*) neurofilament proteins. The positions of the 200,000, "140,000," and 70,000 mol wt NFPs are indicated. On two-dimensional gels the 200,000 mol wt protein in optic pathway preparations is present in only trace amounts (dotted circle).

labeled proteins with molecular weights from 140,000 to 145,000 moved together (see Fig. 6 *A–D*), differences in the distribution of "140,000" neurofilament proteins in consecutive segments of the optic pathway were not accounted for by different transport rates.

Distribution of Radiolabeled NFPs along the Primary Optic Pathway

The distribution and relative proportions of radiolabeled "140,000" NFPs in consecutive segments of the RGC axons were examined next at different intervals after intravitreal injection of [^{3}H]proline to mice (Fig. 6 *A–D*). After 6 d, NFPs were primarily in the proximal half of the optic nerve (Fig. 6 *A*). At this time point, the "140,000" NFP region was comprised of two major protein species corresponding to the 140,000 and 145,000 bands that are evident in the stained pattern of Fig. 6, gel *E*. By 15 d after injection, the leading edge of the NFP wave entered the optic tract (Fig. 6 *B*). At this time the labeled pattern did not yet correspond to the stained pattern (Fig. 6 *E*), since optic tract segments containing the three major stained bands at "140,000" (Fig. 6 *E*) still displayed only the 145,000 and 140,000 mol wt labeled forms upon autoradiography (Fig. 6 *B*). When stained gels were compared with autoradiographs after 39 d of labeling, the leading edge of the transport wave containing NFPs had just reached the preterminal region of the axons, and the pattern of radiolabeled "140,000" NFPs (Fig. 6 *C*) along the RGC axons approximated the pattern in the stained gel (Fig. 6 *E*). As shown in Fig. 6 *C*, the 143,000 mol wt protein became increasingly evident in distal segments of the optic pathway (segments 5–9). As the NFP wave progressed further within the optic tract at 60 d postinjection, the proportions of labeled "140,000" proteins along the RGC axon remained the same (Fig. 6 *D*). These observations indicate that the generation of the 143,000 mol wt protein species within RGC axons begins before the major portion of the labeled NFPs has reached the axon terminals.

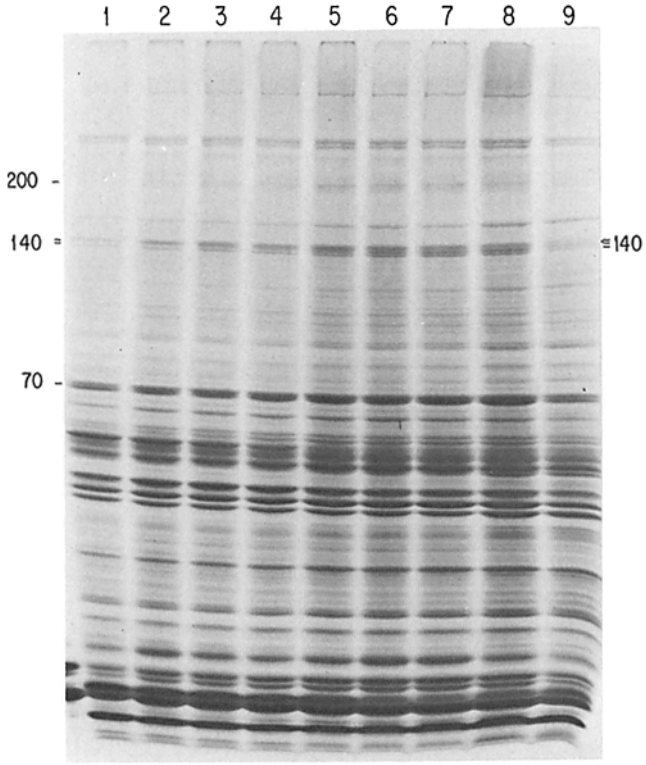

FIGURE 4 Proteins in consecutive segments of the optic pathway. Optic pathway was dissected and cut into 1.1-mm segments and the entire protein content of each segment was analyzed by SDS gel electrophoresis. Segment 1 (lane *1*) was adjacent to the scleral surface of the eye, segment 5 (lane *5*) includes optic chiasm, and segment 9 (lane *9*) was overlying the lateral geniculate body. The neurofilament proteins are indicated. Horizontal lines at 140,000 indicate that these proteins are separated into at least two bands in proximal segments (lanes *1–3*) and as at least three bands in distal segments (lanes *4–9*). See Fig. 6 *E* for an enlarged view. On the gel shown an entire 1.1-mm segment was loaded per well.

Analysis of Radiolabeled "140,000" Proteins

Peptide mapping of the "140,000" region of radiolabeled ON and OT was carried out to ascertain whether the two "140,000" proteins in proximal segments of the RGC, and the three "140,000" proteins of distal segments are structurally related. One-dimensional SDS PAGE was carried out on the proximal half of the optic nerves from mice 15 d after intravitreal injection of L-[^{35}S]methionine; a similar procedure was carried out on optic tracts from mice 45 d after L-[^{35}S]methionine administration. Gels containing the radioactive proteins were stained to visualize the protein bands. Each of the two major stained "140,000" proteins from ON, and of the three "140,000" proteins from OT were cut from the gel and subjected to partial digestion using *S. aureus* V8 protease (6, 8). The resulting fluorograph derived from the two "140,000" ON proteins (Fig. 7 *A* and *B*) demonstrated the similarity between radioactively labeled peptides. Furthermore, although the intensity of the fluorograph of Fig. 7 *C–E* is not as great, the more abundant peptides were visualized and were present in each of the three "140,000" OT proteins. These data are

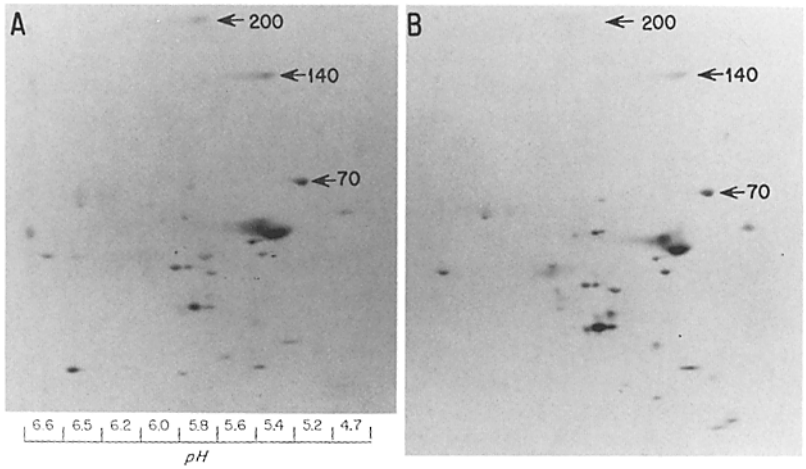

FIGURE 5 Two-dimensional autoradiograph of radiolabeled ON and OT. Optic nerve and optic tract were radiolabeled by injecting mice intravitreally with L-[^{3}H]proline. After 60 d, ON and OT were rapidly excised and prepared for two-dimensional gel separation, as described. After electrophoresis the gel was fixed in acid, dried, and fluorographed. Shown are optic nerve proteins (panel *A*) and optic tract proteins (panel *B*). The neurofilament proteins are indicated by molecular weight values.

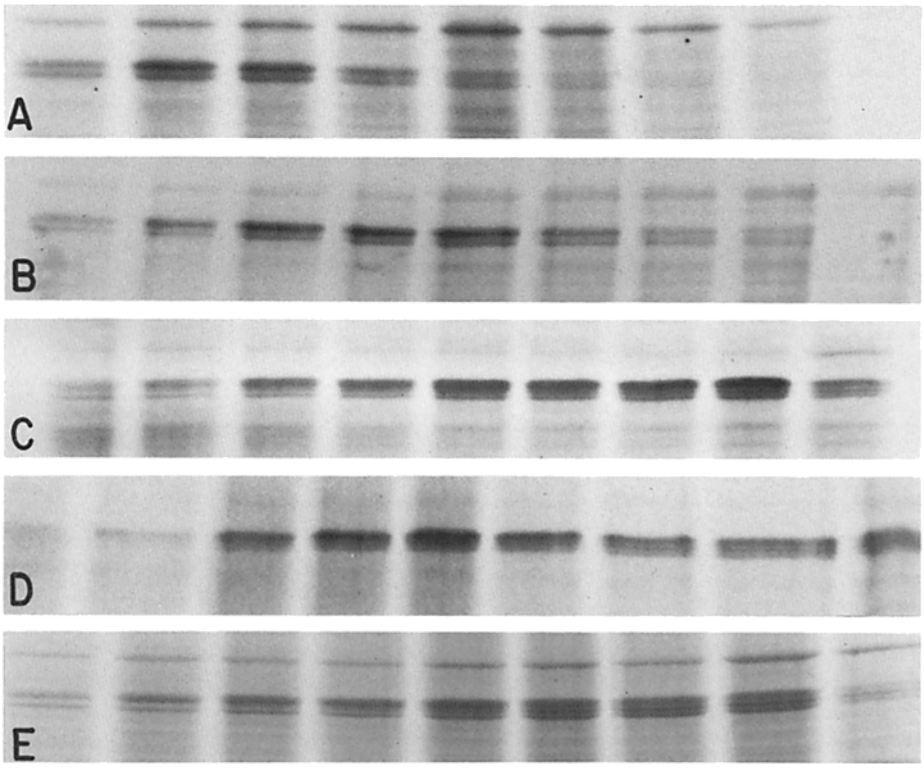

FIGURE 6 Radiolabeled "140,000" proteins in consecutive segments of the mouse optic pathway. Proteins were labeled by intravitreal injection of L-[^{3}H]proline. After various postinjection intervals (see below), the optic pathway was excised and cut into 1.1-mm segments. Each segment was applied to a separate lane of a SDS gel (see legend to Fig. 5). After electrophoresis, gels were fluorographed for appropriate intervals. Only the "140,000" region of each gel is shown. (*A*) 6, (*B*) 15, (*C*) 39, and (*D*) 60 d after injection of radioactive proline. The amounts of radioactivity loaded per gel lane ranged from 100,000 to 700,000 dpm. *E* shows the pattern of the Coomassie-stained gel.

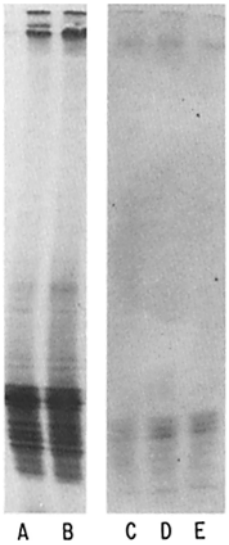

FIGURE 7 Peptide maps of radiolabeled ON and OT "140,000" proteins. Mouse optic pathway proteins were labeled by intravitreal injection of L-[^{35}S]methionine. After 15 d, the proximal half of ON was removed from one group of mice. OT was dissected from a second group of mice after 45 d. ON and OT proteins were separated by SDS PAGE and visualized by staining. The two major stained "140,000" proteins of ON and the three "140,000" proteins of OT were each cut from gels and subjected to partial digestion by *S. aureus* V8 protease. The gels were fluorographed and the results demonstrate the similar digestion products of all "140,000" protein gel bands. Left panel: (ON) (*A*) 145,000, (*B*) 140,000. Right panel: (OT) (*C*) 145,000, (*D*) 143,000, (*E*) 140,000.

consistent with the conclusion that the 143,000 mol wt protein that is generated during axonal transport within RGC axons is structurally related to the 140,000 and 145,000 mol wt proteins.

Modification of 140,000–145,000 mol wt Neurofilament Proteins In Vitro

The distribution of the enzyme activity that generates the 143,000 mol wt NFP along RGC axons was further examined in vitro. Freshly excised but intact primary optic pathways from mice at appropriate times after L-[^{3}H]proline injection were incubated at 37°C in HEPES buffer, pH 7.4 containing calcium (4 mM $CaCl_2$) for various time intervals and then subjected to SDS PAGE and fluorography (Fig. 8*A–C*, lanes *1–6*). Fig. 8*A* shows the 140,000–145,000 mol wt region of optic pathways on a stained gel. In unincubated tissue, the 140,000–145,000 mol wt region is comprised of a major 145,000 mol wt NFP and somewhat less prominent 143,000 and 140,000 mol wt NFP species (Fig. 8*A*, lane *1*). Within 30 s of incubation at 37°C, changes in the proportions of these proteins were noted (Fig. 8*A*, lane *2*). With further incubation (1–5 min), the 143,000 mol wt protein and to some extent the 140,000 protein were rapidly generated concomitantly with disappearance of the 145,000 mol wt NFP (Fig. 8, lanes *3–6*). This process occurred at about the same rate when calcium was excluded from the medium (Fig. 8*A*, lane *7*); however, EGTA (10 mM) was markedly inhibitory (Fig. 8*A*, lane *8*) suggesting that calcium ions are required for enzymatic activity. Preincubation with higher concentrations of EGTA completely inhibited the process (results not shown). Activity was nearly absent at 0°C.

To localize this enzyme activity further, optic pathways were obtained from mice after intravitreal injection of L-[^{3}H]proline. Since radioactively labeled NFPs are located only in proximal optic nerve at 6 d postinjection (Fig. 6*A*), any alterations of these proteins in vitro may be assumed to reflect enzyme activities residing in this region of RGC axons. Similarly, at 31 d postinjection when transported NFPs are predominantly within the optic tract, modifications of radioactively labeled NFPs primarily reflect enzyme activities in distal axonal sites. When optic pathways from mice 6 d postinjection were incu-

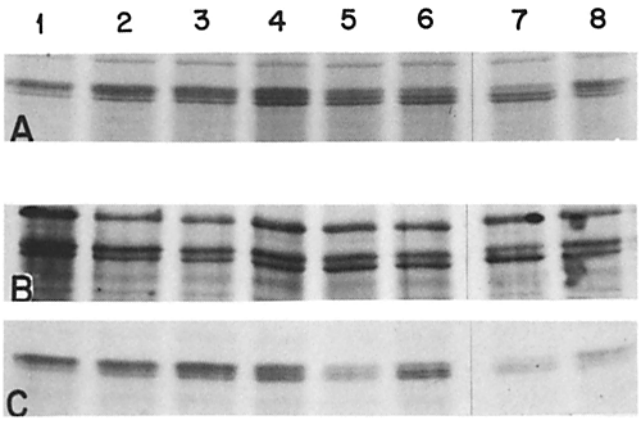

FIGURE 8 In vitro modification of 145,000 and 140,000 mol wt neurofilament proteins in the primary optic pathway. Mice were injected intravitreally with L-[^{3}H]proline. After 6 or 31 d, freshly dissected optic pathways from these mice were incubated at 37°C in prewarmed HEPES buffer pH 7.4 under the conditions described below. After incubation, each optic pathway was analyzed by SDS gel electrophoresis. The gels were stained with Coomassie Brilliant Blue and fluorographed. Only the "140,000" region of each gel is displayed. Lanes *1–6* were derived from optic pathways incubated in vitro in the presence of 4 mM $CaCl_2$ for (*1*) 0, (*2*) 0.5, (*3*) 1, (*4*) 2, (*5*) 3, or (*6*) 5 min. Lane *7* shows a 5-min incubation in the absence of $CaCl_2$. Lane *8* depicts a 5-min incubation in buffer lacking $CaCl_2$ but containing 10 mM EGTA. The Coomassie-stained protein pattern (panel *A*) and corresponding fluorogram (panel *B*) of optic pathway proteins are from mice 6 d after L-[^{3}H]proline injection. A fluorogram of optic pathways from mice 31 d postinjection is displayed in panel *C*.

bated at 37°C in vitro, no conversion of 145,000 NFP to the 143,000 mol wt form was observed (Fig. 8 *B*, lanes *1–8*). By contrast, in optic pathways from mice 31 d postinjection (Fig. 8 *C*), radiolabeled 143,000 mol wt protein was generated in vitro by a calcium dependent process (Fig. 8 *C*, lane *8*) with a time course similar to that observed for stained 140,000–145,000 mol wt proteins (Fig. 8 *A*). These results are consistent with a nonuniform axonal distribution of the enzyme(s) mediating 143,000 NFP formation in vitro.

DISCUSSION

The present study of mouse retinal ganglion cell neurons has revealed greater complexity to the composition of CNS neurofilaments than was previously recognized. The major neurofilament proteins, like several other cytoskeletal proteins of the mammalian nervous system (28, 30), were earlier shown to exhibit microheterogeneity in each size class (6, 10). The most extensive NFP microheterogeneity has been reported for the 200,000 mol wt species (10) although multiple forms each of the 140,000 and 70,000 mol wt NFPs have also been noted (6, 9, 10). In this study, we have observed that the composition of neurofilament proteins within the molecular weight range of 140,000–145,000 is particularly complex. Using SDS PAGE, at least three forms with different molecular weights were identified. These three proteins appear to be structurally related neurofilament proteins since they, and their apparent counterparts in purified neurofilament preparations, had the same molecular weight and isoelectric point on two-dimensional gels and displayed the same peptide maps after limited digestion with *S. aureus* V8 protease. In addition, these 140,000–145,000 mol wt NFP forms exhibited properties during axoplasmic transport that are characteristic of NFPs in several mammalian central axons (3, 18, 24, 59, 65). With the other NFPs and tubulin, the 140,000 mol wt NFPs composed the slowest of five major groups of transported proteins (24, 59, 64). The transport rate of mouse neurofilament proteins (0.2–0.4 mm/d) was the same as that reported in guinea pig optic axons (3) and was slightly slower than the rate in rabbit RGC axons (65).

In addition to structural heterogeneity of "140,000" neurofilament proteins, we have also observed regional heterogeneity of "140,000" neurofilament protein composition within mouse retinal ganglion cell neurons. Since the primary optic pathway is comprised of axons from a single neuronal population, the retinal ganglion cells, we could infer that changes in the proportions of neurofilament species in different regions of the otpic nerve and optic tract reflected alterations along the length of RGC axons. All forms of the "140,000" NFP species identified in whole brain neurofilament preparations appear to be present in this single neuronal population; however, microheterogeneity of the "140,000" protein was shown to be greater in distal segments of the RGC axon than in proximal segments. On SDS gels the pattern of the "140,000" NFPs shortly after synthesis in the RGC neuron differs from the pattern displayed by NFPs from whole brain. As the NFPs are transported, the composition of the "140,000" proteins evolves towards a pattern that is more characteristic of whole brain NFPs.

Since protein synthesis is negligible within axons (22), the appearance of the 143,000 mol wt NFP species in distal portions of RGC axons is consistent with posttranslational modification(s) of existing neurofilament proteins. The 200,000, 140,000, and 70,000 mol wt NF proteins have dissimilar peptide maps (6); thus, the close structural similarities we have demonstrated by peptide map analysis among the three "140,000" proteins suggest that the intermediate size 143,000 mol wt protein may be derived from the 145,000 or, less likely, from the 140,000 mol wt species or a larger precursor.

A posttranslational event such as proteolytic cleavage, glycosylation, or phosphorylation could account for the appearance of the 143,000 mol wt protein. We have previously demonstrated that the major neurofilament proteins, including the three "140,000" proteins, are phosphorylated in vivo in RGC neurons when $^{32}PO_4$ is administered intravitreally (35). Proteolytic enzymes are also present in RGC axons (33, 34), including calcium-activated neutral proteinase(s) with particular affinity toward neurofilaments (16, 41, 51, 53). Recently we described a calcium-activated neutral proteinase (CANP) in RGC axons which rapidly converts the 145,000 mol wt neurofilament protein into 143,000 and 140,000 mol wt forms while having minor effects on other major proteins (37, 38). The enzyme is highly active at the endogenous calcium concentration in freshly excised optic pathways. Its activity is partially inhibited by the serine proteinase inhibitors phenylmethylsulfonyl fluoride and diisopropylfluorophosphate, but is relatively unaffected by leupeptin. These properties distinguish it from the major CANP activity in RGC axons (38), which displays characteristics similar to CANPs from other neural sources (16, 41, 51, 53, 54). The relative selectivity of the former proteinase for 140,000–145,000 mol wt NFPs and its activity at endogenous Ca^{++} levels raise the possibility that a proteolytic mechanism may be responsible for the observed 140,000–145,000 mol wt NFP modifications during axoplasmic transport. Proteolytic cleavage during axonal transport has been demonstrated for several neurosecretory polypeptides in mammalian (15) and molluscan neurons (2).

Whatever the precise mechanism, the enzymatic activity

mediating the formation of the 143,000 mol wt neurofilament protein does not seem to be uniformly distributed along the retinal ganglion cell axon. The 143,000 mol wt neurofilament protein appeared only after transported NFPs had reached distal axonal regions 30 d or longer after injection. Residence time of the precursor NFP within the distal axon may be a factor in 143,000 mol wt NFP generation since the earliest fraction of NFP to reach distal axonal regions at 15 d does not yet contain the 143,000 mol wt NFP. It is unlikely, however, that the appearance of the 143,000 mol wt NFP is dependent exclusively upon residence time; although significant proportions of the 145,000 and 140,000 mol wt species were still present in proximal axonal segments at long postinjection time points, the 143,000 mol wt protein was absent or minimally observable in these proximal sites. These findings suggest that the enzyme(s) responsible for appearance of the 143,000 mol wt neurofilament protein may be most active in distal portions of RGC axons. Alternatively, it is possible that the 143,000 mol wt species is produced uniformly along the axon but then rapidly converted or degraded in proximal segments. Either mechanism implies regional differentiation along RGC axons with respect to the enzyme or enzymes mediating "140,000" protein modifications. This conclusion is further supported by results from incubating excised optic pathways in vitro. These studies demonstrated that radioactive 143,000 mol wt NFP formed only if labeled NFPs were present in distal axonal segments. In recent experiments on mice 30–40 d postinjection, proximal segments of optic pathways that were removed and incubated separately from distal segments appeared to be considerably less active than distal segments in converting 145,000 mol wt NFP to 143,000 and 140,000 mol wt forms (Nixon, R. A., B. A. Brown, and C. A. Marotta, manuscript in preparation). These results together imply that the enzyme activity mediating 143,000 mol wt NFP formation in vitro is not uniformly distributed along RGC axons. If the in vitro and in vivo mechanisms of NFP modification are related, the greater activity in distal axonal sites provides a possible basis for the selective appearance of the 143,000 mol wt NFP in distal RGC axons during axoplasmic transport.

Regional heterogeneity along RGC axons is not restricted to "140,000" neurofilament proteins. The accompanying report (5) also demonstrates striking variations in tubulin subunit composition along RGC axons which, in part, reflect processing during axonal transport. Furthermore, the nonuniform proximodistal distribution of other major axonal proteins (Nixon, R. A., B. A. Brown, C. A. Marotta, manuscript in preparation) suggests that protein processing may be an important axonal function. Earlier investigations have emphasized biochemical and ultrastructural differences between the axon and its initial segment (42, 43, 62, 63) or terminal region (17, 27, 42, 45). Recently, however, morphological (12, 47, 60) and physiological (13, 60, 61) specializations of the axonal membrane have been demonstrated along the length of the axon itself. In addition, results from this laboratory (33, 34) and others (26) have revealed that proximodistal gradients exist for certain enzyme activities measured along axons in vitro. The regional differences of cytoskeletal proteins and related enzymatic components along RGC axons established in the present studies may imply even greater complexity of axonal structure and function than has previously been recognized. These observations further suggest that, rather than serving as a static conduit between perikaryon and synapse, the axon may be a highly specialized compartment for modifying the structure or activity of specific axonal and synaptic proteins.

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