AXONAL GROWTH: MODES, MOLECULES AND MECHANISMS

by

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ABSTRACT

Proteins of the nerve terminal membrane were examined during the development of the hamster retinofugal pathway in order to further understand the molecular mechanisms that underlie axonal growth. Intraocular injections of [^{35}S]-methionine were made at various ages and the labeled, rapidly transported proteins in developing axons were analyzed by 2-D gel electrophoresis and fluorography. The growth-associated, neuron-specific phosphoprotein, GAP-43, and the neural cell adhesion molecule, N-CAM, were positively identified and found to be associated with early stages of axon growth. The expression of several other proteins was precisely correlated with the elaboration of terminal arbors, including a 67kD species putatively identified as pp60^{src}. Several proteins were shown to be primarily associated with establishment and maintenance of mature retinofugal connections including species of 27kD, 64kD and a 94kD protein similar to integrin. The time course of a 64kD, 67kD and a 230kD species similar to the neuron-glia cell adhesion molecule was significantly altered after retinal fibers were induced to form abnormal projections, suggesting that these proteins may participate in the interactive events between developing axons and target cells.

Immunolocalization of GAP-43 showed that this protein underwent a marked shift in its cellular localization. It was distributed throughout elongating axons, concentrated in terminals during the formation of synaptic relationships and then virtually disappeared from optic fibers. The formation of abnormal connections showed that the anomalously projecting retinal axons express a competitive advantage for terminal space and induce an anatomical reorganization of the heterotypic target.

These studies show that specific nerve terminal proteins are associated with such morphogenetic events as axon elongation, terminal arborization and synaptogenesis, and that some of these proteins may directly participate in interactive developmental events. The identification and characterization of these proteins suggest specific molecular mechanisms whereby consitituents of the nerve terminals contribute to axonal growth.

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CHAPTER 1

INTRODUCTION

The complex organization of the vertebrate central nervous system provides the structure upon which the wide array of brain functions and behaviors are based. This complexity is marked by a precise pattern of connections among subsets of neurons that are often separated by considerable distances. The challenge to the developmental neurobiologist is to understand the mechanisms that underlie the formation and modification of this neural network in terms of the cellular and molecular events through which individual elements present at early embryonic stages evolve into the mature pattern of organization. These mechanisms are responsible for such developmental events as cellular migration, differentiation, the extension of neuronal processes, interactions among cells and the formation of functional connections. The studies presented here have focused on the latter events in an attempt to understand the biological basis of axonal growth and synaptogenesis.

One pathway that has been used extensively to study axonal growth and the formation of connections is the projection from the retina to its central targets, the lateral

geniculate nucleus and the superior colliculus. This pathway is the essential link for visual functioning between the receptor organ and the brain. Unlike peripheral ganglia, the receptor organ, or retina, is itself part of the central nervous system, hence, the results and conclusions derived from studies of this pathway may be more readily generalized to the rest of the brain. In addition, the retinofugal pathway offers certain practical advantages for studying many basic aspects of neural development, including the precise, well-defined organization of its connections, the relative isolation and ease of access to the retina, and the convenience of the globe as a receptacle for various precursors, markers and other reagents. These characteristics, combined with the ability of retinal ganglion cells in lower vertebrates to regrow after injury, made the retinofugal pathway well suited for regeneration studies (cf Attardi and Sperry, 1963; Benowitz and Lewis, 1983; Grafstein, 1986; Schmidt et al., 1978; Skene and Willard, 1981; Yoon, 1971).

This visual pathway has also proven to be a valuable model for examining developmental events in a number of mammalian species using a variety of approaches, including neuroanatomy, physiology and behavior (see for example, reviews by Lund, 1978, Sherman and Spear, 1982, and Movshon and Van Sluyters, 1981). One species which has been used extensively to study visual system development is the Syrian hamster, which offers the particular advantage of an exceptionally short gestation period (15.5-16 days) which makes early stages of development accessible in neonatal animals.

The first retinal axons leave the hamster's eye at embryonic day 11.5 (E11.5; day of mating = E0) and elongate rapidly, growing in compact fascicles at a rate of 60-100µm/ hour through the optic nerve, over the surface of the lateral geniculate, and reach the caudal end of the SC by E13.5. At these early stages, retinofugal axons have a simple morphology with numerous vericosities and short filopodial extensions. Around the time of birth (E16), as later arriving axons reach target structures, collateral branches begin to

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form and penetrate target zones. By postnatal day 5 (P5), an adult-like precision in retinotectal topography is nearly established. During the next two weeks axon end-arbors elaborate extensively and give rise to increasing numbers of synapses with target cells in the brain. Eye opening occurs on P14, at which time a pupillary light reflex can be elicited, indicating that at least some retinal axons have formed functional connections. Myelination of optic tract axons begins around the same time and is accompanied be a dramatic increase in the diameter of the axons (Sachs and Schneider, 1984; Jhaveri et al., 1983; Jhaveri and Schneider, 1985; Schneider et al., 1985). The developmental time course of these events is depicted in Figure 1 below.
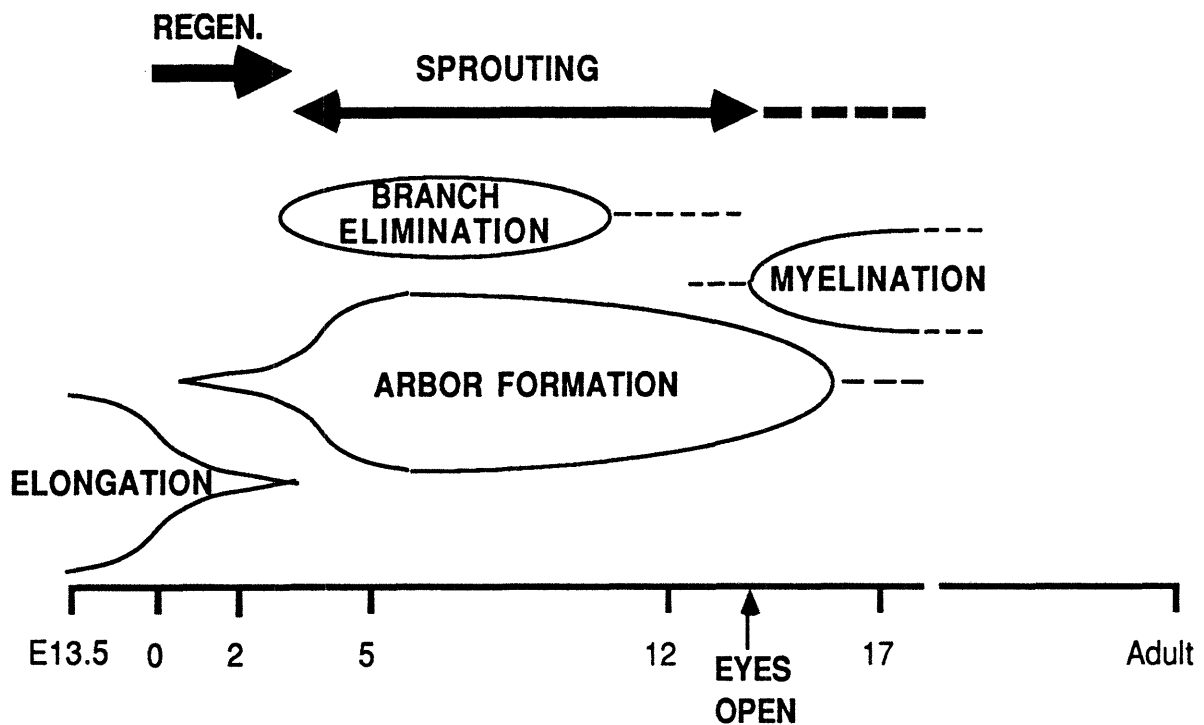

Figure 1. Developmental events in the hamster retinofugal pathway.

The hamster retinofugal pathway has also been well characterized in terms of the plasticity of optic axons in response to injury (Schneider, 1973; Frost et al., 1979; So et

al., 1981; Schneider et al., 1985). If optic fibers are transected prior to terminal arbor formation, i.e., up through P3, damaged axons can regenerate across the cut; however, axons cut after P3 fail to regrow across the region of transection. After P3, damaged retinal axons often sprout collaterals proximal to the lesion site, and occupy available terminal space, a response which declines over the first few weeks. Thus, the critical period for regeneration ends soon after birth, while the potential for collateral sprouting of retinal arbors persists for at least 2 more weeks.

The nerve terminal plasma membrane is the site of contact between developing neurons and the extracellular environment which includes substrates, markers and other cells. Thus molecular constituents of the membrane are likely to participate in developmental events such as axon elongation, target recognition, terminal arborization and synaptogenesis through the regulation of growth cone motility, extension of microspikes, cell-cell adhesion, axonal branching, sprouting of collaterals and responses to ion fluxes or other extracellular cues. Therefore, in order to understand the contribution of membrane proteins to neural development, it is necessary to identify and functionally characterize these molecular species. Many of these proteins are synthesized in the cell body and conveyed to the growth cone or terminal membrane in the rapid phase of axonal transport (Grafstein and Forman, 1980; Hammerschlag and Stone, 1982; Lorenz and Willard, 1978; Willard et al, 1974), where they may be exposed to the extracellular environment, become integral components of membrane specializations or form links between the plasma membrane and the cytoskeleton.

The studies presented here have examined proteins of the nerve terminal membrane of hamster retinofugal axons in an attempt to begin to understand the molecular mechanisms that underlie axon growth and the formation of connections. Five of following chapters take the form of published or submitted manuscripts and can be divided into

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studies of normal development (Chapters 2-4) and studies which examine the consequences of experimental manipulations (Chapters 5 and 6). Chapters 2 and 3 include molecular studies on the pattern of nerve terminal proteins in developing axons which show that specific proteins are temporally correlated with particular morphogenetic events. Chapter 4 examines developmental changes in the levels and cellular localization of one well-characterized membrane protein, GAP-43. Chapter 5 describes the results from experiments in which retinal axons were induced to form abnormal connections, and shows that the time course of expression of several proteins is altered, suggesting that they may participate in target interactions. It is clear from the results in Chapter 6 that retinal axons do not assume a heterotypic phenotype in terms of GAP-43 when induced to form abnormal connections, although these abnormally projecting axons do alter the organization of the target area, perhaps through competitive interactions. Chapter 7 is a collection of several studies which will be published separately, and describes experiments aimed at characterizing and identifying various proteins in rapid axonal transport in order to understand the molecular mechanisms of neural development. This thesis concludes with a general discussion of the findings and their significance, in which I have proposed a model, which though speculative and incomplete, illustrates how the new information about molecular constituents may be linked to what is already known about axonal growth and the establishment of connections.

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CHAPTER 2

ENHANCED VISUALIZATION OF AXONALLY TRANSPORTED PROTEINS IN THE IMMATURE CNS BY SUPPRESSION OF SYSTEMIC LABELING.

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Key words: Axonal transport; Blood-brain barrier; Visual system; Developmental neurobiology; CNS proteins.

ABSTRACT

In the neonate hamster, visualization of axonally transported proteins in the retinofugal pathway is obscured by high levels of systemic (background) labeling. Radiolabeled precursors injected into the eye diffuse rapidly into the general circulation and then across the immature blood-brain barrier to be incorporated into proteins that are synthesized throughout the brain. Systemic labeling can be suppressed, however, by i.p. injections of large amounts of either non-radioactive methionine 30 min after intraocular labeling with [^{35}S]-methionine, or non-radioactive leucine given at the time of intraocular labeling. Whereas the former competes with the radioactive precursor during incorporation into brain proteins (after most of the retinal labeling has already been achieved), the latter competes at the earlier stage of access to the brain. Both methods reduced background labeling by more than 60%, thereby allowing for unambiguous identification of axonally transported proteins. The pattern of rapidly transported proteins was found to be strikingly different between neonates and mature animals, including marked changes in an identified "growth-associated protein" (50 kDa, pI 4.8).

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The development of retinofugal projections in the hamster is marked by a clear temporal sequence of changes in the morphology of retinal axons as they approach and subsequently invade central targets, and in the response of these axons to experimental manipulations (Finlay et al., 1979; Frost et al., 1979; Schneider and Jhaveri, 1984; Schneider et al., 1985; So et al., 1981). The detailed information available about the time course of this progression makes the hamster optic pathway an ideal system for relating developmental phenomena at the anatomical and molecular levels. Of particular interest to us in this regard were changes in the complement of proteins conveyed down the axon in the rapid phase of transport, since components of this are incorporated into the nerve terminal membrane and presumably play a role in events such as axon elongation, target recognition, and synaptogenesis (Grafstein and Forman, 1980; Hammerschlag and Stone, 1982; Lorenz and Willard, 1978; Skene and Willard, 1981a,b).

In preliminary studies, it became apparent that in neonatal animals, the molecular constituents of axonal transport were difficult to identify using conventional means. Due to the immaturity of the blood-brain barrier (see also Frost et al., 1979), radioactive precursors injected into the eye diffuse readily into the circulation and then throughout the brain, making it difficult to distinguish proteins synthesized in the retina and transported to central structures from those synthesized locally in the target area. The present study shows, however, that large amounts of non-radioactive amino acids administered in conjunction with the intraocular labeling can effectively suppress systemic labeling. Such a technique allows the proteins transported to developing nerve terminals be identified unambiguously.

MATERIALS AND METHODS

Protein labeling in the superior colliculus (SC) following intraocular injections of [^{35}S]-methionine.

Adult Syrian hamsters, 2-day-old pups (P2; day of birth = P0) and 6-day-old pups (P6) were bred at MIT or were received from Charles River Laboratories (Wilmington, MA). Prior to eye injections, pups were anesthetized by hypothermia, whereas adults were administered Chloropent (0.35 ml/100g body wt.; Fort Dodge Laboratories, Fort Dodge, IA). Postoperative recovery occurred under a heat lamp.

Hamsters were injected in the right eye, behind the lens, with 100 μCi of [^{35}S]-methionine (DuPont/New England Nuclear, Boston, MA; spec. act. 1000 Ci/mmol). For P2 and P6 neonates, label was diluted in 0.5 μl or 1.0 μl phosphate-buffered saline (PBS); adult animals received injections of 2.0 μl. Animals were allowed to survive 4 h in order to permit proteins synthesized in the retina and conveyed by rapid axonal transport adequate time to accumulate in the nerve terminals of the contralateral SC. Animals were sacrificed with an overdose of Chloropent, the right and left SC (RSC and LSC) were rapidly dissected out, and these were stored at - 70°C until further processing.

Tissues were homogenized in a 10-fold excess (v/w) of sucrose buffer [0.32 M sucrose (Schwarz-Mann; Cambridge, MA); 50 mM Tris-HCl, pH 7.4 (Sigma); 10 nM RNase and 10 nM DNase (Sigma)] with a Teflon-glass homogenizer (0.25 mm gap, 5,000 rpm, 12 strokes, 4 °C). The particulate fraction, which contains the majority of rapidly-transported proteins (Lorenz and Willard, 1978), was lypholized to remove all water, then solubilized in isoelectric focusing (IEF) gel lysis buffer (200 μg protein in 200 μl of 9.5 M urea (Schwarz-Mann), 5% ß-mercaptoethanol (Bio Rad); 6% ampholines in a ratio of 2:2:1 of pH 3.5-5.0, pH 5.0-8.0, pH 3.5-10.0 (LKB, Gaithersburg, MD), 2% NP-40 (Nonidet, Particle Data Laboratories, Elmhurst, IL) at 30 °C. Proteins were separated by IEF using the method of O'Farrell (1975) modified by using 6% ampholines in the gel

matrix in the same ratio as above. At the completion of the IEF run (300 V for 16 h, 400 V for 4 h), gels were equilibrated and the proteins separated in the second dimension on SDS-PAGE slab gels (50-15% linear acrylamide gradient, 25 cm height for running gel; stacking gel c. 8 cm, 3% acrylamide; gels run at 15-30 mA/gel until dye front was 8 cm from the bottom). Gels were fixed and stained with Coomassie brilliant blue R-250 (Sigma), impregnated with Autofluor (National Diagnostics, Somerville, NJ), dried under vacuum onto filter paper (Whatman No. 1) and exposed to presensitized X-ray film (Kodak, X-Omat AR).

Quantitative studies of systemic labeling in neonate hamsters

To study the time course of systemic labeling, 4 µCi of [^{35}S]-methionine in 1.0 µl PBS was injected into the right eye of P2 pups. In addition, the same animals received an i.p. injection of 4 µCi of [^{3}H]-methionine (DuPont/New England Nuclear) in 50 µl PBS. This amount of [^{3}H]-methionine was low enough not to significantly alter the diffusion or uptake of the intraocularly administered [^{35}S]-methionine. Two animals per time point were sacrificed at 15, 30, 60 and 120 minutes after the injection. The right eye, LSC, right cortex (RCtx, which included the entire right hemisphere and most of the right basal ganglia), and liver were rapidly dissected and stored at -70°C. Samples were homogenized in a 10-fold excess (w/v) of 10% trichloroacetic acid (TCA), allowed to stand for 45-120 min at room temperature, then centrifuged (1000 X g, 5 min). The pellet was resuspended in fresh 10% TCA and centrifuged again two more times. Samples of TCA-insoluble material were solubilized in 0.5 N NaOH, neutralized with HCl, and counted in Econofluor with 7% Protosol (DuPont/New England Nuclear).

Suppression of systemic labeling by competition with non-radioactive amino acids

Two methods for suppressing systemic labeling were investigated. In the first of

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these, an excess of non-radioactive methionine (Sigma; 1 mg/g body wt., raising estimated methionine concentration in soft tissue to 10mM) was injected i.p. a half hour after intraocular injection of 4 μCi of [^{35}S]-methionine. In the second method, an excess of non-radioactive leucine (Sigma; 1 mg/g body wt.) was injected i.p. at the same time as the intraocular [^{35}S]-methionine injection, raising the estimated concentration of leucine in soft tissue to about 15mM. P2 pups were sacrificed 60 min after intraocular labeling. Proteins transported from the eye would not yet reach the colliculus by 60 min (unpublished data), thus allowing us to examine systemic labeling in isolation. Eyes, SC, and RCtx were rapidly dissected out and homogenized in TCA as described above.

Enhanced visualization of rapidly transported proteins on 2-D gels by suppression of systemic labeling

Six P2, P5 and P6 hamsters were injected in the right eye with 100 μCi [^{35}S]-methionine as in the first experiment, and also received 1 mg/g body wt. of non-radioactive leucine i.p. immediately after eye injections. Protein labeling patterns in the ipsilateral and contralateral SC were examined by 2-D gel electrophoresis and fluorography, and were compared with labeling patterns in colliculi of control animals that did not receive systemic leucine injections.

RESULTS

Protein labeling in the SC following intraocular injections of [^{35}S]-methionine

Radioactive amino acid precursors injected into the eye are incorporated into proteins of retinal ganglion cells, some of which are conveyed via rapid axonal transport to central visual target structures, including the contralateral superior colliculus. The ipsilateral SC, which receives a relatively minor projection from the eye (Insausti et al., 1985), served as a control for systemic labeling in these studies.

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The profile of labeled proteins in the colliculus contralateral to the injected eye (i.e., the LSC) is compared for P2 and adult hamsters in Fig. 1 (A and C) and is representative of 3 separate experiments. Of the numerous differences seen in the two fluorograms, some are presumably related to developmental changes in the complement of proteins transported down the optic nerve. However, prominent among the proteins seen in gels from the P2 animals (Fig. 1A) are actin and tubulin (identified with reference to published 2-D gel data (Strocchi et al., 1981; Tytell et al., 1981), which are not normally present in the rapid phase of axoplasmic transport (Goodrum and Morrel, 1982; Hoffman and Lasek, 1975; Tytell et al., 1981; Willard et al., 1979). The labeling of these proteins suggests that in the P2 animal, radioactive precursor leaked from the eye into the circulation and across the blood-brain barrier to be incorporated into proteins synthesized in the SC and other brain structures. This is confirmed by the labeling pattern of the ipsilateral SC. Although the ipsilateral SC receives a relatively minor retinal projection (Insausti et al., 1985), and should thus show a relatively low density of labeling, its labeling pattern in the neonates is nearly identical to that of the contralateral SC (Fig. 1B). Thus, it can be concluded that the protein labeling pattern in the SC of neonate animals following intraocular injections of radiolabeled precursors is dominated by proteins that are labeled systemically. A comparison of Fig. 1A and Fig. 1B suggests that some components (*) may be more heavily labeled in the contralateral than in the ipsilateral SC, and these may reflect molecular species that are truly transported from the retina. However, under these experimental conditions, labeling differences between the two colliculi are extremely difficult to detect.

In contrast to neonates, the SC ipsilateral to the injected eye of adult hamsters shows almost no protein labeling (Fig. 1D); the small amount that does appear presumably corresponds to proteins that are intra-axonally transported by the sparser ipsilateral retinotectal pathway.

Quantitative studies of systemic labeling in neonate hamsters

In order to develop methods for minimizing systemic labeling in neonates, it was necessary to establish the time course at which radiolabeled precursors diffuse from the eye and become incorporated into brain proteins from the circulation. At the same time, it was also necessary to examine whether amino acids injected i.p. would have rapid access to CNS structures, so that these might be able to compete with labeled precursors which diffuse from the eye. The time course of protein labeling in the SC, cortex, and liver following intraocular injection of [^{35}S]-methionine and i.p. injection of [^{3}H]-methionine are presented in Fig. 2.

Variation in the amount of intraocular fluid that refluxed at the site of the eye injection resulted in considerable variation in the amount of protein labeling in individual retinae. This made it impossible to determine the time course of diffusion of radioactive precursors from the eye directly. Nevertheless, in the SC, cortex, and liver, protein labeling with [^{35}S]-methionine can be seen to rise continuously over the 2h measurement period (Fig. 2A, B). These data indicate then, that the radioactive precursor rapidly diffused from the eye and into the circulation, where some of it became available for local labeling of brain cells. With regard to amino acids that were administered i.p. these began to be incorporated into brain proteins soon after injection and the incorporation continued to increase over the time course examined (Fig. 2C).

Suppression of systemic labeling by competition with non-radioactive amino acids

Two methods for suppressing systemic labeling were investigated. In the first of these, an excess of non-radioactive methionine was injected i.p. one half hour after intraocular labeling. This permitted protein labeling in the retina to proceed uninterrupted for a period of time before the non-radioactive methionine "chase" reached the eye. Once

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injected, however, the non-radioactive methionine vastly diluted the systemic pool of labeled precursor available to brain structures for all but the first 30-45 min. The second method took advantage of the fact that large neutral amino acids compete with each other for the same facilitated diffusion mechanism across the blood-brain barrier as methionine (Pardridge, 1977; Pardridge and Oldendorf, 1977). Thus, an excess of non-radioactive leucine injected i.p. at the time of intraocular injection would be expected to decrease the diffusion of [^{35}S]-methionine from the blood stream, and to reduce the uptake into the brain of [^{35}S]-methionine from the circulation.

Both methods were found to be equally effective in suppressing systemic labeling of brain structures. Table I shows the amount of protein labeled in the retina, SC, and cerebral cortex 1 h after intraocular injections of [^{35}S]-methionine in the two experimental conditions. All values are based on at least two observations. Variations in labeling of retinal protein presumably reflect uncontrolled variations in injection size, leakage of the vitreous through the injection hole, and perhaps also the effect of the competitive amino acid injections (e.g., excess leucine preventing [^{35}S]-methionine from diffusing out of the eye). Because of the variations in the size of the initial injections, labeling of brain structures were all normalized by the radioactivity in the retina. The average ratios of protein labeling in the brain structures normalized by that of the retina reflect the amount of background labeling relative to specific labeling of retinal protein, and predict the signal-to-noise ratio for a given experimental procedure, i.e., the ratio of labeling of retinal proteins, some of which will become part of rapid axonal transport, relative to systemically labeled proteins. Due to greater variability in the dissection of the SC, a more reliable and conservative estimate of systemic labeling levels is indicated by the data for the RCtx. Using these data, both the injection of non-radioactive methionine a half hour after labeling and the injection of excess leucine at the time of retinal labeling decreased systemic labeling in brain structures by about 60%.

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Table 1.

Effects of systemic amino acid injections on background labeling of CNS structures following intraocular labeling

Tissue Protein labeling

	No systemic injection		I.p. methionine, 30min delay			I.p. leucine, no delay		
	cpm	Normalized activity	cpm	Normalized activity	%change	cpm	Normalized activity	%change
Retina	209,446	—	147,682	—		178,937	—	
Left SC	2,058	0.012	427	0.003	75	525	0.003	75
R Ctx	8,555	0.060	3,919	0.026	57	4,217	0.023	62

Cpm are corrected for background and quenching. Normalized activity is radioactivity in CNS tissue normalized by radioactivity in retina: corrects for variations in injection size. Counted 1 h after injecting 4 μCi [^{35}S]-methionine in retina. % Change is calculated relative to normalized labeling without systemic injections.

Enhanced visualization of rapidly transported proteins on 2-D gels by suppression of systemic labeling

To demonstrate that the methods described above can substantially enhance visualization of rapidly transported proteins in neonate animals, patterns of protein labeling in the two superior colliculi were examined on 2-D gels following intraocular labeling with [^{35}S]-methionine accompanied by i.p. injection of excess leucine. Postinjection survival times of 4 h were again used. Fig. 3 shows fluorograms of the contralateral and ipsilateral SC for P2 pups with and without systemically administered leucine. As described above, pups that received no leucine showed nearly identical patterns of protein labeling in the contralateral and ipsilateral SC, with actin and tubulin being labeled predominantly (Fig. 3A, B). In animals that received an excess of leucine at the time of eye injectin, the background of systemically labeled proteins is greatly diminished (Fig. 3C, D). Actin and tubulin, although still present, are considerably less

heavily labeled than in Fig. 3A, B. Comparison of the labeled proteins in the two colliculi now reveals several molecular species in the contralateral SC that are barely detectable in the ipsilateral SC. These presumably reflect proteins that are rapidly transported from the retinal ganglion cells to the axon terminals. Prominent among these is a protein with an apparent mol. wt. of 50 kDa and isoelectric point (pI) of 4.8. Also transported to the SC are proteins of 27 kDa, pI 5.0 and 32 kDa, pI 5.6. Similar results were obtained for experiments using P5 and P6 animals.

DISCUSSION

In the neonate hamster optic pathway, visualization of rapidly transported proteins by conventional means is obscured by the problem of systemic labeling. Within the first 60 min after intraocular labeling, much of the radioactive precursor diffuses out of the eye into the circulation. Some of the precursor then crosses the blood-brain barrier to become incorporated into proteins that are synthesized in central structures. Under normal circumstances, this labeling is of a magnitude that makes it impossible to distinguish the proteins that are synthesized in the retina and transported to optic nerve terminals from those that are synthesized locally in the colliculus. However, through use of large injections of competing non-radioactive amino acids, systemic labeling could be effectively blocked, enabling proteins transported from the retina to the SC to be identified unambiguously.

Insofar as systemic labeling of brain structures begins immediately after intraocular injections (Fig. 2A), it can be inferred that the intraocular pool of radioactive precursor starts to diffuse out of the eye soon after the initial labeling. Thus, much of the retinal labeling that contributes to the pattern of axonally transported proteins visualized in the SC had probably taken place by the time excess non-radioactive methionine was injected

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i.p. 30 min later. Raising systemic levels of methionine to 10 mM was effective, however, in competing with the radioactive precursor that had diffused from the eye to diminish the amount of brain protein labeling that occurred over the next several hours. Thus the delayed non-radioactive chase suppressed the 'noise' level in these studies (i.e., the level of background labeling), without significantly diminishing the signal (i.e., the labeling of retinal proteins that are destined for rapid transport to the SC).

The use of a competing injection of leucine afforded a somewhat less direct but equally effective means of suppressing systemic labeling. As shown by Pardridge (1977) and by Pardridge and Oldendorf (1977), the transport of several different large neutral amino acids utilizes the same facilitated diffusion mechanism. An excess of circulating leucine competes with the radioactive methionine for transport across the blood-brain barrier, and may also prevent radioactive precursor from diffusing out of the eye. Thus, whereas excess non-radioactive methionine competes with the labeled pool of methionine at the level of incorporation into newly synthesized protein, the excess leucine competes with the labeled methionine at the level of access to the brain. One hour after injection of radioactive precursor into the eye, either the i.p. injection of excess leucine (administered at the time of intraocular labeling) or the i.p. injection of excess non-radioactive methionine (administered after a 30 min delay) reduced the amount of systemic labeling by about 60%. Over the next few hours the signal-to-noise ratio would be expected to become even more favorable, as labeled proteins transported from the retina accumulate in target structures while systemic labeling continues to be suppressed by competition with the excess non-radioactive amino acids.

In contrast to the situation in neonates, injections of radioactive methionine into the eye of adult hamsters showed no systemic labeling of brain structures (Fig. 1D) and thus no change after i.p. injection of excess amino acids (data not shown). The only protein labeling seen in the colliculus ipsilateral to the injected eye of adult hamsters appeared to

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be a component of fast axonal transport, presumably conveyed by the small ipsilateral retino-collicular projection (Chalupa and Rhoades, 1979; Frost et al., 1979; Insausti et al., 1985; Woo et al., 1985). In a time course series, we have found that the blood-brain barrier reaches maturity some time between postnatal days 12 and 17 (in preparation).

In comparing the proteins of fast axonal transport in the neonate and adult optic pathway, a number of differences are apparent. A protein which migrates at 50 kDa, pI 4.8, is seen in the neonate but not in the adult, whereas proteins which migrate at 27 kDa, pI 4.8 and 64 kDa, pI 5.2, appear to be present at much higher levels in the adult. By mol. wt., pI, and presence in the membrane fraction (unpublished data), the 50-kDa, pI-4.8 protein appears to be similar to the acidic growth-associated phosphoprotein identified in other developing and regenerating neural pathways and in growth cones (Benowitz and Lewis, 1983; Bock et al., 1984; De Graan et al., 1985; Katz et al., 1985; Skene and Kalil, 1984; Skene and Willard, 1981a,b). The detailed time course of the molecular changes in rapid axonal transport and the correlation of these with known events in the development of the hamster visual pathway will be the subject of subsequent studies.

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FIGURE LEGENDS

Fig 1. Protein labeling in the SC following unilateral intraocular injection of [^{35}S]-methionine. Right eyes of P2 and adult hamsters were injected with 10 μCi [^{35}S]-methionine. Proteins labeled after a 4h survival time in the contralateral SC (which is the principle target of optic axons) and the ipsilateral colliculus (which receives a sparser optic input) were visualized by 2-D gel electrophoresis and fluorography. Neonate hamsters show nearly identical incorporation patterns in the contralateral and ipsilateral colliculi (A and B, respectively), including heavy labeling of actin (A) and α- and ß-tubulin (αT and ßT), indicative of high levels of background labeling. Proteins that show some left-right differences are noted (*, A) and may represent components of fast axonal transport. In the adult, the ipsilateral SC (D) shows minimal radioactivity, demonstrating an absence of systemic labeling. Thus, proteins visualized in the adult LSC (C) represent components of fast axonal transport exclusively.

Fig. 2. Time course of protein labeling following intraocular or i.p. injections of radiolabeled methionine in neonate hamsters. Following intraocular injection of [^{35}S]-methionine, the labeling of protein in the LSC and RCtx (A) and liver (B) rises continuously over the 2h sampling period as radioactive precursor that has diffused from the eye and into the general circulation crosses the blood-brain barrier and is incorporated into proteins synthesized locally in these structures. C: amino acid injected i.p. crosses the blood-brain barrier and is incorporated into protein in the LSC and RCtx. Levels of labeled protein continue to increase linearly.

Fig. 3. Enhanced visualization of rapidly transported proteins in the neonate hamster by suppression of systemic labeling. Proteins labeled in the LSC and RSC 4 h after injecting the right eye with [^{35}S]-methionine were examined by 2D gel electrophoresis as in Fig. 1. In addition, some animals (C,D) also received i.p. injections of leucine (1 mg/g body weight) at the same time as the intraocular injection. Protein labeling patterns of the contralateral (C, left) and ipsilateral (D, right) colliculi are strikingly different from each other in animals that received i.p. injections of leucine, indicating that systemic labeling has been almost entirely suppressed (c.f. A, B, control labeling without i.p. leucine from Fig. 1): actin and tubulin show only low levels of labeling on the two sides, while components that are rapidly transported to the LSC can be identified unambiguously as those proteins that are labeled much more heavily in the LSC (C) than the RSC (D).
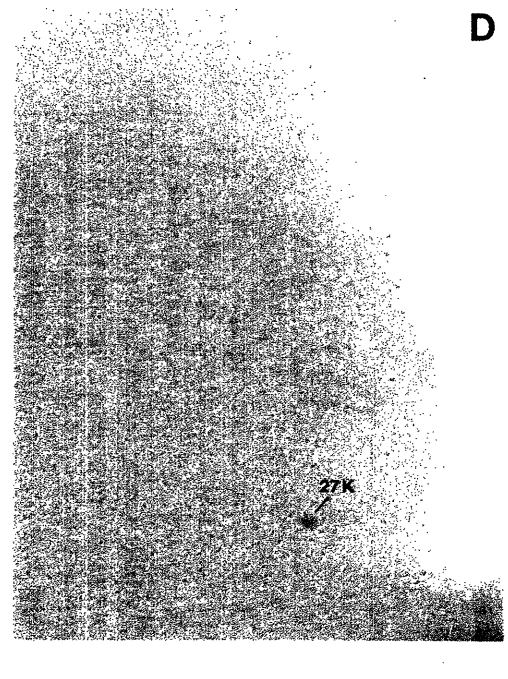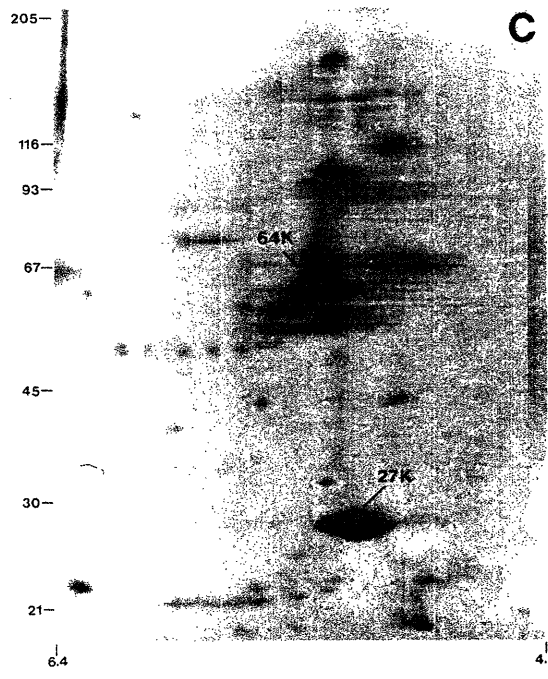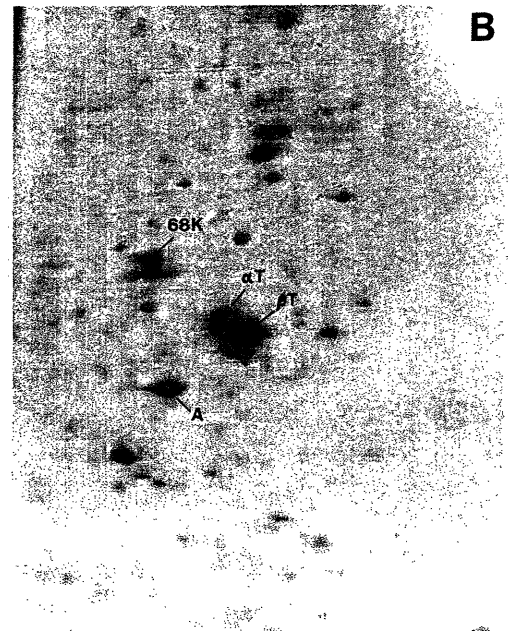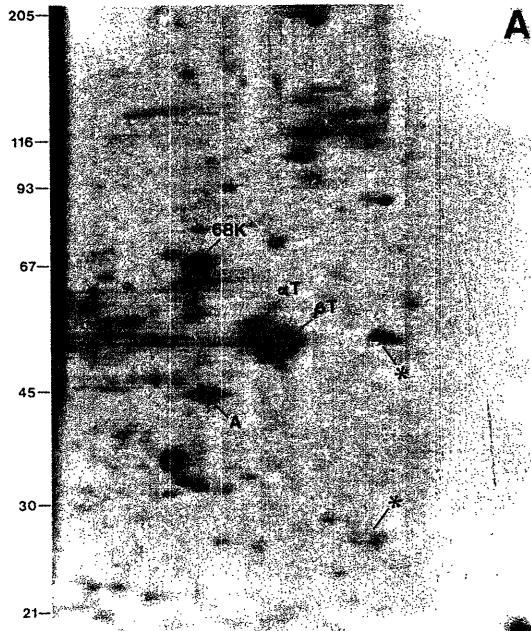
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A

B

C

D

205—
116—
93—
67—
68K
αT
βT
*
45—
A
*
30—
21—

205—
116—
93—
67—
68K
αT
βT
A

205—
116—
93—
67—
64K
45—
27K
30—
21—
6.4

27K
4.3

D

FIGURE 2

A   68K   B
          αT βT
      *   A

*

50K 4.8   C   βT   D
                   A
32K 5.6

27K 4.8
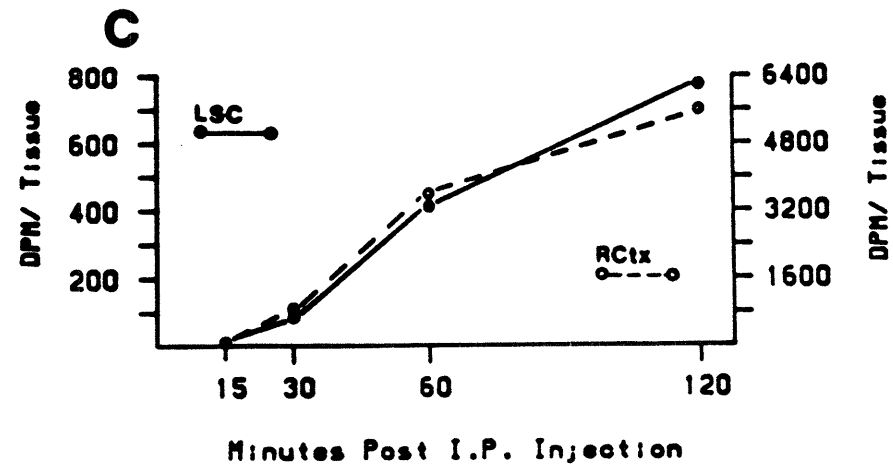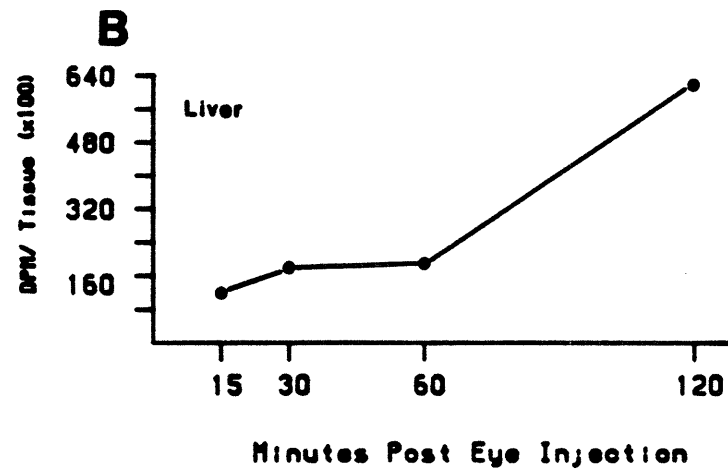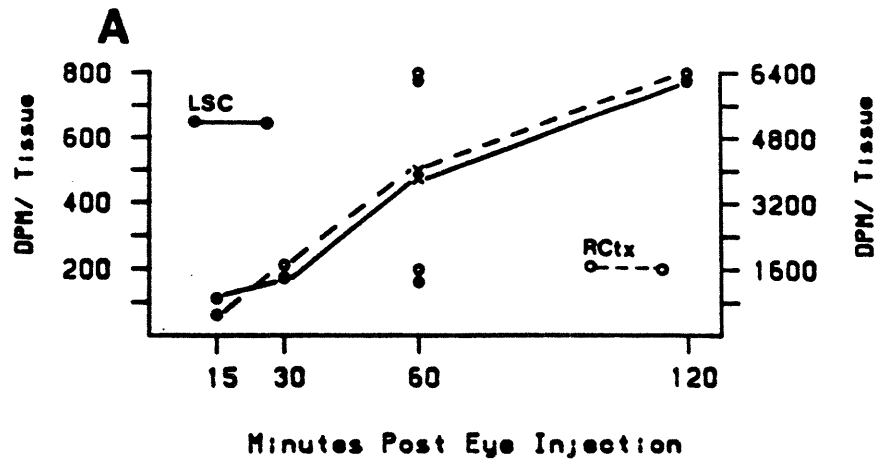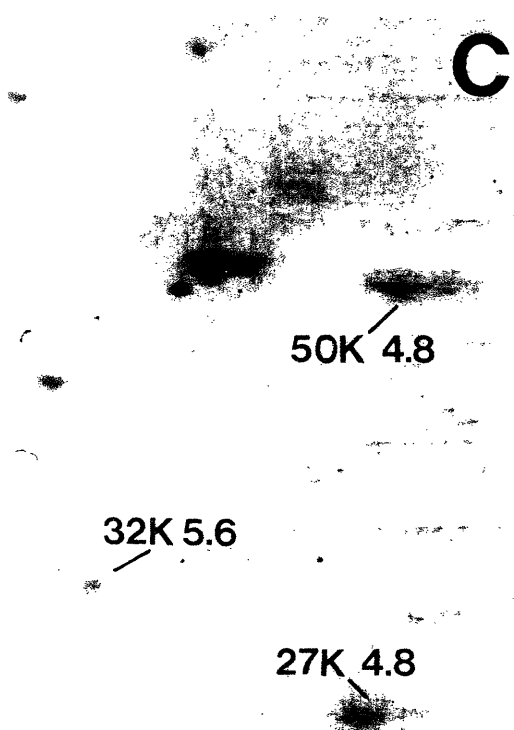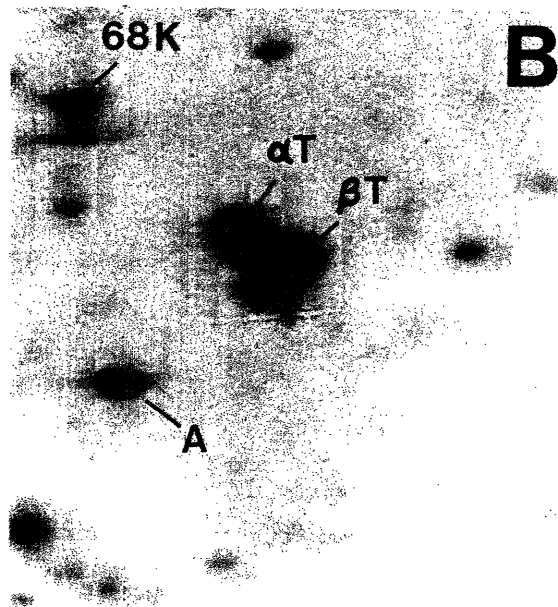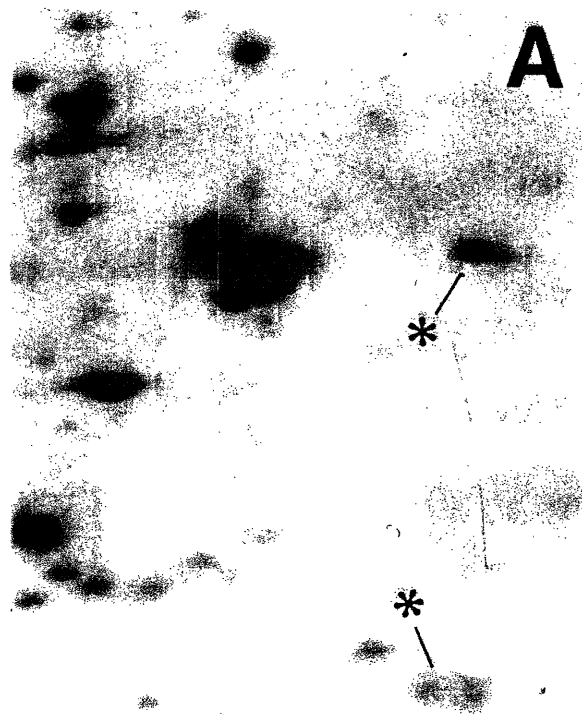
CHAPTER 3

CHANGES IN RAPIDLY TRANSPORTED PROTEINS IN DEVELOPING HAMSTER RETINOFUGAL AXONS.

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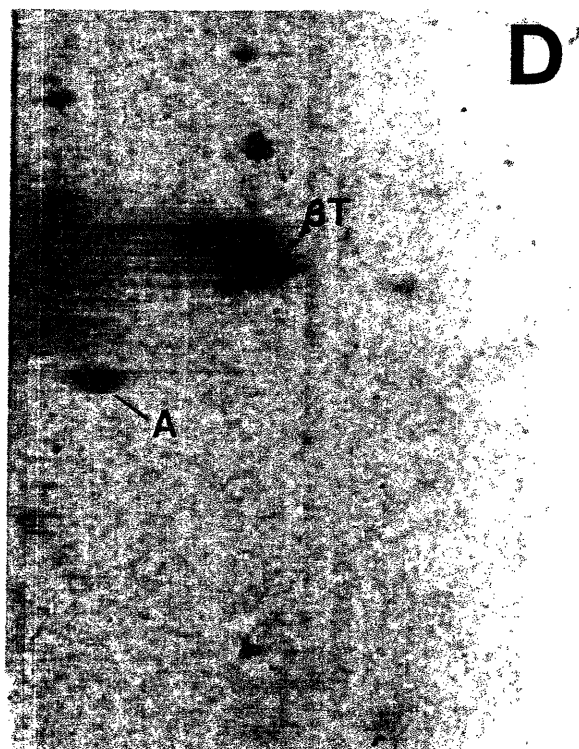[1]Department of Brain and Cognitive Sciences, Whitaker College,

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ABSTRACT

Proteins synthesized in retinal ganglion cells and conveyed to the terminals of optic tract axons in the rapid phase of axonal transport were analyzed at different developmental stages in the hamster. Animals between 2 days of age and adulthood were labeled intraocularly with [^{35}S]-methionine, and after a 4 hour survival time, the superior colliculus was dissected out, subjected to subcellular fractionation, and radiolabeled proteins in the particulate fraction analyzed by 2-dimensional gel electrophoresis and fluorography. The previously identified growth-associated phosphoprotein, GAP-43 (GAP-48, B-50, F1, pp46), was synthesized and transported at high levels in the neonate, but these levels declined precipitously after the second postnatal week. Immunohistochemical studies using a monospecific antibody showed that GAP-43 was localized along the entire length of retinal axons in the optic tract and target areas in P2 animals, but was virtually absent in the adult visual pathway. By metabolic labeling, two proteins with molecular weights of about 230kD also showed a sharp decrease during development. In contrast, acidic proteins of 27kD and 64kD, which were barely detectable in the neonate, increased steadily to become the most heavily labeled proteins of rapid axonal transport by the second postnatal week. Another group of proteins, of about 94-110kD, also rose to peak levels after birth but then declined. Temporal correlations between the molecular changes described here and the known anatomical events in optic tract development suggest that the synthesis and transport of particular membrane proteins may be directly related to the sequence of morphological changes.

The development of the nervous system is marked by the formation of precise connections among subsets of neurons that are often separated by considerable distances. Molecular constituents of the nerve terminal plasma membrane are likely to play a major role in mediating many events that take place during this process, including axon guidance, target recognition, and the formation of end-arbors and synapses. Thus, the identification of specific proteins delivered to the growing tips of axons during critical stages of development is likely to contribute to our understanding of the biological mechanisms that underlie the formation and modification of synaptic relationships.

Many proteins destined for the nerve terminal membrane are synthesized in the perikaryon and conveyed down the axon in the rapid phase of axonal transport (Grafstein and Forman, 1980; Hammerschlag and Stone, 1982; Lorenz and Willard, 1978; Willard et al., 1974). Changes in this group of proteins have been described in a variety of developing and regenerating systems. Studies in regenerating optic nerves of lower vertebrates (Benowitz and Lewis, 1983; Perry and Grafstein, 1987; Skene and Willard, 1981a), and in regenerating mammalian peripheral nerves (Redshaw and Bisby, 1984; Skene and Willard, 1981b) have revealed the existence of rapidly transported proteins whose rates of synthesis are associated with the regrowth of nerve fibers. One of these proteins, GAP-43, has also been found in the normally developing central nervous system of rabbit (Skene and Willard, 1981b), rat (Jacobson et al, 1986) and hamster (Kalil and Skene, 1987; Moya et al, 1987), suggesting a possibly universal role in the formation of neuronal pathways.

In the present studies we have examined the time-course of expression of a number of rapidly transported proteins in the developing hamster retinofugal pathway. Since many of the morphogenetic events have been previously characterized in this system, we

are in a position to suggest correlations between protein changes and specific anatomical phenomena. In addition, the short gestation period (15.5 days) of the hamster makes earlier stages of neuronal development accessible in the neonate. Our results demonstrate a number of striking developmental changes, some which have not been previously described. Each of the major rapidly transported proteins appears to have a characteristic ontogenetic sequence, with some being expressed at high levels early in development but then diminishing, others increasing continuously over the first two weeks of postnatal life to achieve adult levels, and still others showing a rise and fall over a relatively brief period. Results of preliminary studies using a monospecific antibody to the previously identified growth-associated protein, GAP-43, parallel the results of our metabolic labeling studies, and show striking developmental changes in both the level and localization of this protein in the primary visual system.

MATERIALS AND METHODS

Labeling of proteins rapidly transported to the SC

Syrian hamsters (*Mesocricetus auratus*) were bred in our colony. The specific ages used in this study were selected because of their correspondence with major events in optic tract development (see Fig 2J). Neonates (P2 and P5, where P0 = day of birth) were anesthetized by hypothermia and injected in one eye with 100 μCi [^{35}S]-methionine (1000 Ci/mmol, NEN, Boston MA; or Tran [^{35}S]-label, 1100 Ci/mmol, ICN, Irvine, CA) in 1 μl 0.05M phosphate-buffered saline (PBS), pH 7.4. Earlier results have shown that in neonates the high degree of non-specific background labeling due to the immaturity of the blood-brain barrier can be minimized by giving large systemic injections of non-radioactive amino acid to compete with labeled precursor that diffuses out of the eye and into the

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circulation (Moya et al, 1987). Animals at P2, P5, and P12 were therefore injected intraperitoneally with non-radioactive leucine (Sigma, 1 mg/g body wt) dissolved in PBS, administered at the same time as the intraocular injection. Labeling in P12, P17, and adult animals was achieved by anesthetizing hamsters with Chloropent (0.35 ml/100 g body wt, Fort Dodge Laboratories) and injecting 100 μCi of [^{35}S]-methionine dissolved in 2 μl PBS into the eye. All animals recovered under a heat lamp. After allowing 4 hours for the labeled methionine to be incorporated into newly synthesized proteins within retinal ganglion cells and rapidly transported to central targets, the ipsilateral and contralateral superior colliculi (SC) were rapidly dissected out, frozen on dry ice, and stored separately at -70°C.

Tissues from 4-6 animals per age group were pooled and homogenized in ice-cold sucrose buffer [0.32 M sucrose (Schwarz-Mann, Cambridge, MA), 50 mM Tris-HCl, pH 7.4, 10 nM RNase and 10 nM DNase (Sigma)] and then centrifuged at 100,000 x g for 60 min at 4°C to yield a total particulate fraction. This was lyophilized to remove water, then solubilized in isoelectric focusing buffer [O'Farrell, 1975; 1 μg of protein/μl of 9.5 M urea (Schwarz-Mann), 5% ß-mercaptoethanol (BioRad), 6% ampholines in a ratio of 2:2:1 of pH 3.5-5.0, pH 5.0-8.0, pH 3.5-10.0 (LKB, Gaithersburg, MD), 2% NP-40 (Nonidet, Particle Data Laboratories, Elmhurst, IL)] for 30 minutes at 30°C. Between 200 and 400 μg of total protein was subjected to isoelectric focusing (16 hr at 300v, 4 hr at 400v). After completion of the run, gels were equilibrated in SDS-gel buffer (pH 6.8: Solution "O", O'Farrell, 1975) for at least 30 minutes at room temperature and the proteins separated in the second dimension by SDS-polyacrylamide gel electrophoresis on a 5-15% linear acrylamide gradient. Gels were fixed with acetic acid/methanol, stained with Coomassie brilliant blue (Sigma), impregnated with a radiographic enhancer, Autofluor (National Diagnostics, Somerville, NJ), dried under vacuum onto filter paper (Whatman No. 1), and

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exposed to presensitized X-ray film (Kodak, X-Omat AR) for times ranging from 40 to 60 days. Gel electrophoresis of tissue from each age group was replicated in 6 separate experiments; three 2-D gels from each age group were quantitatively analyzed as described below.

Quantitation of rapidly transported proteins

A tracing of each fluorogram was prepared on acetate sheets and used as a template to cut labeled proteins from the gels. Excised gel pieces were solubilized in 6% Protosol in Econofluor (New England Nuclear/ Dupont) and the amount of radioactivity determined by liquid scintillation counting. In the P2 and P5 neonates, some systemic background labeling could still be detected on the fluorograms, as indicated by the presence of certain labeled proteins known not to be conveyed in the rapid phase of axonal transport (e.g., actin and tubulin: see Fig 1A) in both the ipsi- and contralateral colliculi. The ipsilateral SC, which receives only a relatively minor retinal projection in the hamster, shows a labeling pattern that predominantly reflects systemic labeling due to diffusion of [^{35}S]-methionine out of the eye (Moya et al, 1987). In order to eliminate the contribution of locally synthesized proteins from the quantitation of transported proteins, the radioactivity for each protein observed in the ipsilateral SC (i.e., background) was subtracted from that in the contralateral SC. The adjusted radioactivity for each protein was then normalized to the total radioactivity in transported proteins on each fluorogram. This normalization allowed us to express the labeling of each protein as a fraction of total rapidly transported protein, and also helped to minimize the effects of possible age-dependent differences in protein synthesis in the retina, rate of axonal transport, or number of labeled proteins transported.

Immunohistochemical localization of GAP-43 in the developing visual system

The preparation of the immune serum used to visualize one identified growth-associated protein, GAP-43, has been described previously (Benowitz et al, 1988; Neve et al, 1987). To test this immune serum for cross-reactivity and specificity for hamster GAP-43, 400 μg of particulate brain protein from neonatal hamsters was prepared by subcellular fractionation and subjected to 2-dimensional gel electrophoresis as described above. In other experiments, separation on SDS-slab gels was done under nonreducing conditions (i.e., without ß-mercaptoethanol in the SDS-gel buffer: buffer "O", O'Farrell, 1975) in order to minimize a staining artifact in the Western blots (see below). Proteins were transferred electrophoretically to nitrocellulose as described by Phelps (1984, with the modifications of Meiri et al, 1986) and the resulting blot was reacted with the antibody to GAP-43 at a dilution of 1:1000. Immunoreactivity was visualized using a peroxidase-conjugated secondary antibody. To enhance the contrast of the reaction product, a double chromogen method was used in which peroxidase was visualized with 0.05% 4-chloro-napthol in 20% methanol and 0.05% H_2O_2 in tris-buffered saline (TBS), followed by 0.05% diaminobenzidine (DAB) in TBS with 0.01% H_2O_2.

For immunohistochemistry, P2 and adult hamsters were deeply anesthetized with Choloropent and perfused transcardially with a brief saline rinse, followed by 4% paraformaldehyde in 0.1 M PBS. Brains were removed, post-fixed for 2 hrs at 4°C, then cryoprotected in buffered sucrose. Frozen 40μm sections were cut in the transverse plane on a sliding microtome. Sections were placed in 0.3% H_2O_2 in methanol for 30 min to neutralize endogenous peroxidases, washed in PBS containing 0.4% Triton X-100 (Sigma) and 0.5% $NaNH_3$ (PBSTN) and incubated in a blocking solution (PBSTN with 20% normal rabbit serum) for 30 min. Sections were then incubated for 3 days with the primary antibody, or with non-immune serum as a control, at a dilution of 1:2000, washed in

PBSTN, incubated for 1 hr with biotinylated rabbit anti- sheep IgG (Vector Labs), washed again in PBSTN, and incubated for 1 hr with avidin-biotin conjugated to horseradish peroxidase prepared according to the manufacturer's directions (Vector Labs). Tissue was washed once in PBSTN followed by 2 washes in TBS. Peroxidase was visualized histochemically using DAB as the chromogen. Sections were mounted onto alum-gelatin coated slides, air-dried, and covered.

RESULTS

Labeled proteins rapidly transported to the SC

The pattern of proteins synthesized in the retina and rapidly transported to central targets was found to change markedly during development (Fig. 1A-E). Table 1 presents quantitative data for 3 representative ages and shows the degree of systemic labeling in the P2 and P5 neonates. By P12, systemic labeling became insignificant, diminishing to less that 0.3% of the total label incorporated into the SC (data not shown).

Prominent among the proteins transported to the SC in P2 and P5 neonates was one with an apparent molecular weight of 50kD and pI of 4.8 (Fig 1A and B). This protein is similar in its electrophoretic mobility, shape, and presence in the rapid phase of transport to the identified growth-associated protein, GAP-43 (Jacobson et al, 1986; Meiri et al, 1986; Moya et al 1987; Skene and Willard, 1981a,b). The results of the 2-D Western blot reacted with an immune serum generated against GAP-43 from rat show that the 50kD, pI 4.8 protein in hamster is immunologically similar to the rat protein (see below) and hence is referred to hereafter as GAP-43. By P12 labeling of this protein had declined, and was nearly undetectable in the P17 and adult SC (Fig 1C,D and E). Quantitative analysis showed that GAP-43 accounted for 12.5 and 10.8% of the total

radioactivity transported to the SC at P2 and P5, respectively; at P12 it was about one third this level, while at P17 and in the adult it was about 1% (Fig 2A).

Two high molecular weight proteins of about 230kD, pI 4.9 and 5.1, also showed a striking decline during development. The more acidic of these (Fig 1A), contained over 30% of the label in rapidly transported proteins at P2, decreased to less than 13% at P5, and to about 2% in the adult (Fig 2C). The more basic protein declined somewhat more gradually, going from about 17% at P2 and P5, to a third of this level at P12 and then diminishing further (Fig 2B). By contrast, proteins Y and Z (Fig 1B) showed only a minimal decline with increasing age (Table 1).

A 27kD, pI 4.8 protein showed a complementary time course of expression (Fig 1A-E). Its synthesis and transport increased steadily throughout the first 3 weeks of postnatal life and remained high in the adult. Quantitative analysis showed that at P2, the 27kD protein accounted for 13.4% of the total label in rapidly transported components in the SC, whereas by P17 it was about 50% (Fig 2D). Two proteins with molecular weights of 64kD, pI 5.5 and 67kD, pI 4.7, also increased sharply, but with a delayed onset. The 67kD protein was not observed on P2 but increased abruptly at P5. The 64kD protein could not be detected at P2 and P5, but by P12 (Fig 1C-E) this protein comprised about 10% of the total labeling, and about 20% in the adult (Fig 2E).

Table 1. Quantitation of rapidly-transported proteins.

	P2			P5			Adult		
	contra (cpm)	ipsi (cpm)	% of transport	contra (cpm)	ipsi (cpm)	% of transport	contra (cpm)	ipsi (cpm)	%of transport
GAP-43	240.6	82.9	10.3	131.2	34.0	13.6	37.8	--	2.4
230kD b	280.4	96.9	12.0	60.2	0.0	8.4	3.9	--	0.2
230kD a	605.4	0.0	39.5	66.8	0.0	9.3	27.5	--	1.7
27kD	199.5	41.4	10.3	214.6	16.8	27.7	889.2	--	55.4
64kD	0.0	0.0	0.0	0.0	0.0	0.0	358.1	--	22.3
94kD	88.0	68.3	1.3	0.0	0.0	0.0	85.9	--	5.4
100kD	85.0	35.4	3.2	55.6	0.0	7.8	29.3	--	1.8
110kD	133.7	69.8	4.2	65.2	8.4	7.9	34.0	--	2.1
67kD	0.0	0.0	0.0	43.2	0.0	6.0	53.4	--	3.3
X	73.3	52.1	1.4	21.8	3.4	2.6	0.0	--	0.0
Y	174.0	77.7	6.3	67.8	8.4	8.3	45.0	--	2.8
Z	261.0	82.0	11.7	62.4	2.8	8.3	38.4	--	2.4
Total	1534.4			715.0			1602.5		

Table 1. Quantitation of rapidly-transported proteins. Labeling of proteins in the contralateral SC in P2 and P5 neonates represents both transport and systemic labeling whereas labeling in the ipsilateral SC represents systemic labeling almost exclusively. The latter values were used to correct the values in the contralateral SC to estimate labeling due to axonal transport alone. By P12 virtually no systemic labeling occurs.

Proteins with apparent molecular weights of about 100kD and 110kD showed a distinctive pattern of expression, increasing rapidly and then decreasing again during later stages (Fig 1A-E). Labeling of these two proteins was low in the SC on P2, increased by P5, and then declined after P12. Another protein, 94kD, showed a more gradual rise and fall, as did the protein labeled X in Fig 1B. In general, the mature pattern of protein

labeling appears to be achieved by P17.

Immunohistochemical localization of GAP-43 in the developing visual system

The sheep antiserum raised against rat GAP-43 reacted specifically with the apparently homologous protein on a 2-D gel Western blot containing all hamster brain particulate proteins (Fig 1G and H). Under nonreducing conditions, 2 spots with identical isoelectric points and apparent molecular weights of 50kD and 100kD were visualized (Fig 1G). However, in the presence of ß-mercaptoethanol, only a single spot of 50kD, pI 4.8 was seen (Fig 1H). The 100kD, pI 4.8 species recognized by the antibody to GAP-43 in the nonreducing conditions is likely to be a polymeric form of GAP-43, as suggested by results from size-exclusion chromatography experiments (Benowitz et al, 1987).

Use of this antibody in immunohistochemical studies revealed marked differences in the abundance and distribution of GAP-43 between neonatal and adult brain tissue. In general, the neonatal brain sections were more darkly stained than the adult (Fig 3B,C and E). This staining was specific for the antigen, since sections reacted with non-immune serum exhibited virtually no staining (Fig 3A). Moreover, marked regional variations in staining patterns were observed in both the neonate and the adult. In the immature brain, considerable amounts of GAP-43 were detected along axon bundles, whereas in more mature tissue, immunoreactivity was restricted to specific central nuclear groups and was virtually absent in most fiber tracts.

The optic tract was densely stained in the retinofugal system of P2 neonates, as were fiber fascicles within the dorsal nucleus of the lateral geniculate body (LGBd) and axons in the brachium of the SC (Fig 3E). In the SC, positively stained fiber bundles were observed coursing longitudinally throughout the superficial layers in a distribution similar to that of fascicles of retinal axons at this age (Fig 3B and C). In contrast to this

neonatal pattern, the optic tract of adult hamsters was virtually devoid of staining, while the neuropil of the lateral geniculate and the SC were only lightly stained (Fig 3D and F). The detailed time course of immunohistochemical staining for GAP-43 is currently being investigated (see Moya et al., 1987b).

DISCUSSION

During development, proteins conveyed to nerve terminal membranes in the rapid phase of axonal transport are likely to contribute to processes that underlie axon elongation and the establishment of functional synapses, including growth cone motility, target recognition, membrane turnover, cell-cell adhesion, control of specific ion fluxes, and the development of synaptic specializations. The results of the present study demonstrate striking changes in the synthesis of many of these proteins over time, some of which appear to be temporally correlated with specific changes in the development of the retinofugal projection.

Technical considerations.

Variability between fluorograms, due to differences in amount of radioactivity and in the exposure of the photographic emulsion, limits the interpretations of qualitative comparisons to the most obvious changes in labeling patterns. To overcome this limitation, we have quantified the radioactivity in each rapidly-transported protein, eliminated background labeling, and used a normalization procedure to allow comparisons to be made over different ages. Background labeling was eliminated by subtracting the counts for each protein in the ipsilateral SC from those in the contralateral SC, assuming that the ipsilateral pattern is primarily a reflection of systemic labeling (see Table 1). The

existence of a minor ipsilateral retinotectal projection results in a slight underestimation of the levels of contralaterally transported protein using this method. However, the ipsilateral retinotectal projection in adult hamsters derives from only 1.2% to 1.7% of all retinal ganglion cells (Hsiao et al., 1984). While this projection is somewhat larger in the neonates, estimates derived from other rodent species suggest that in P2 and P5 animals, the subtraction procedure would decrease actual values by less than 6%, and probably would not affect normalized values at all.

The normalization of background-corrected radioactivity for each protein to the total rapidly transported radioactivity was done in order to take into consideration possible developmental changes in rates of protein synthesis and axonal transport, and in the number of transported proteins. Such developmental changes made it impossible to identify a single unchanging, labeled protein to use as a reference. However, as a result of applying this normalization procedure, we cannot rule out the possibility, for example, that reported changes in the level of a protein could result from a second molecular species dominating the labeling pattern at a particular age rather than the level of the first protein actually decreasing. While a comparison of absolute levels of specific proteins would be interesting, this is technically difficult and our data should be viewed as showing changes in the expression of each protein relative to the entire pattern of rapidly transported proteins during development. The reliability of this method is confirmed by the multiple replications for each age and by additional quantitative data on the pattern of rapidly transported proteins in the lateral geniculate body of these same cases, which is entirely consistent with the present results (unpublished data). For one particular protein, GAP-43, the validity of our results is also underscored by the high correlation between the quantitative analysis and the immunohistochemical data on this protein (see also Moya et al, 1987b). Finally it should be noted that the data were derived with the

use of a single amino acid to label proteins. Use of another amino acid (e.g., proline) would undoubtedly change the relative levels of labeling in various proteins with respect to one another, but would not be expected to significantly affect the temporal pattern of change for a particular protein during development.

Development of the hamster retinofugal pathway

Anatomical studies in the hamster have provided considerable data on the major developmental events in the primary visual pathway (Frost et al, 1979; Jhaveri et al, 1983; Sachs and Schneider, 1984; Schneider et al, 1985; cf. Sachs et al, 1986 for a comparison of development in the mouse). In the hamster, the first retinal axons leave the eye at embryonic day 11.5 (E11.5; day of mating = E0) and elongate rapidly, growing at a rate of 60-100 μm/hr over the surface of the lateral geniculate and reaching the caudal end of the SC by E13.5. At these early stages, retinofugal axons have a simple morphology with numerous varicosities and short filopodial extensions. Around the time of birth (E15.5), collateral branches from these axons begin to penetrate target zones. By P5, a single terminal arbor is being elaborated on each axon in the SC, as extraneous branches are being eliminated. Also by P5, an adult-like precision in retinotectal topography is nearly established. During the next two weeks arbors elaborate extensively and increasing numbers of synapses develop with target cells in the brain. Eye opening occurs on P14. Myelination of optic tract axons begins around the same time and is accompanied by a dramatic increase in the diameter of the axons.

Paralleling the normal progression of developmental changes is a progressive decline in the plasticity of the optic axons in response to injury. Prior to the time of retinotectal arbor formation, transection of the optic tract just anterior to the SC results in retinal axon regeneration, whereas axons cut after P3 fail to regrow across the region of

transection (So et al, 1981). On the other hand, the critical period for collateral sprouting of retinal arbors into nearby vacated terminal space persists for at least 2 weeks after birth (Schneider et al, 1985).

The timing of these anatomical phenomena is summarized in Fig 2J. The temporal changes in the expression of certain molecular species appear to be correlated with major events of axonal growth (compare Figs 2A-I with 2J). For example, the time at which retinal axons cease fasciculated elongation and begin to arborize in target areas (i.e., between P2 and P5) coincides with the decline in synthesis and transport of the 230kD proteins and the relative increase in the 67kD and 110kD proteins. This transition also coincides with the loss in the ability of retinal axons to regenerate across a transection of the tract. During the early period of active terminal arbor elaboration (P5), two proteins that were low in the P2 neonate (e.g., 100kD and 110kD) reach high levels; both proteins subsequently decline at the time when the adult morphology is achieved. As retinal axons arborize extensively in the SC from P5 to P12, the 50kD (GAP-43) and the basic 230kD protein diminish greatly. Between P12 and P17, when retinal axons have eliminated extraneous collateral branches and more mature terminal arbors are formed, the transport of the 27kD and 64kD proteins essentially reaches mature levels. This period also coincides with the time at which retinal axons lose their ability to extend collateral branches into newly available target space.

GAP-43 in the developing optic pathway

One protein that shows a striking developmental regulation is identical to the growth-associated phosphoprotein, GAP-43, in terms of its apparent molecular weight, isoelectric point, developmental regulation, presence in the rapid phase of axonal transport, and immunological characteristics (Jacobson et al, 1986; Skene and Willard,

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1981 a,b). GAP-43 in rat is also similar to a protein described in regenerating goldfish optic nerve (48K 4.8, GAP-48 or protein 4: Benowitz and Lewis, 1983; Perrone-Bizzozero and Benowitz, 1987; Perry et al, 1987), and is identical to the membrane phosphoprotein described as pp46, B-50 or F1 (Jacobson et al, 1986; Katz et al, 1985; Meiri et al, 1986; Nelson and Routtenberg, 1985; Perrone-Bizzozero et al, 1986; Snipes et al, in press; Zwiers et al, 1980). Convergent studies from several laboratories show that GAP-43 is a membrane-bound substrate of protein kinase C that is enriched in growth cones and immature synapses (DeGraan et al, 1985; Katz et al, 1985; Meiri et al 1986; Skene et al, 1986). In addition to its presence in the developing or regenerating optic pathway (Benowitz et al, 1981, 1983; Skene and Willard, 1981a,b; Snipes et al, 1986; also preliminary studies in the hamster visual system: Moya et al, 1987), high levels of synthesis and transport of GAP-43 have been observed in other immature neuronal systems (Jacobson et al, 1986; Kalil and Skene, 1986), cultured embryonic CNS neurons (Perrone-Bizzozero et al, 1987), and pheochromocytoma cells undergoing NGF-induced differentiation (Basi et al, 1987; Karns et al, 1987; van Hooff et al, 1986).

The present studies show that synthesis and transport of this protein are high in the hamster retinofugal pathway during early stages of arbor formation. Similarly, synthesis and transport of GAP-43 are highest in the hamster pyramidal tract (Kalil and Skene, 1986) at a time when corticospinal axons initiate collateral penetration into thalamic and pontine nuclei (Reh and Kalil, 1981, Fig 8). In the developing rat cerebral cortex as a whole, overall levels are greatest during the first week after birth (Jacobson et al, 1986), a time at which most axons have initiated terminal arbor formation.

Use of a monospecific antibody also shows that at P2, high levels of GAP-43 are present in the known position of the retinal fibers in the optic tract, lateral geniculate nucleus, and SC. At this time, corticotectal axons have not yet invaded the SC (Ramirez

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et al, 1986), and hence the staining observed in the SC most likely represents the distribution of this protein along retinal fibers. Thus, high levels of GAP-43 in nerve terminals and along retinal axons, are seen at the time at which the specific target is reached. While synthesis of GAP-43 relative to other rapidly-transported proteins declines after P5, absolute amounts of the protein in nerve terminals may remain high throughout the period of elaboration of end arbors. In the regenerating goldfish optic pathway, the expression of this protein remains above baseline levels during the entire period in which activity-dependent tuning of the retinotectal visual map occurs (Benowitz and Schmidt, 1987). Conceivably, the presence of GAP-43 at least during the initial period of arborization in the developing hamster optic pathway may likewise be permissive for activity-dependent tuning processes in this system.

While levels of GAP-43 become vanishingly low in the developing retinofugal pathway after the second postnatal week, the protein continues to be synthesized at high levels in certain other neurons (Neve et al, 1987) and is enriched in particular regions of the adult brain (see LP in Fig 3E; Oestreicher and Gispen,1986; Benowitz et al, 1988). In addition, changes in this protein's phosphorylation are highly correlated with long-term potentiation in rat hippocampus (Lovinger et al, 1985; Nelson and Routtenberg, 1986). Thus, it is possible that in those neural systems in which GAP-43 synthesis remains elevated into maturity, the protein may play an ongoing role in the modulation of synaptic function.

Other protein changes

Levels of the rapidly-transported acidic 230kD protein declined even earlier than GAP-43. The protein resembles the polysialated form of the neural cell adhesion molecule, N-CAM, in terms of its apparent molecular weight (200 to 250k), and pI (4.4 to 4.6;

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Hoffman, et al 1982).   Should further studies demonstrate, e.g., by immunological or sequence criteria, that the 230kD species is indeed a form of N-CAM, this would suggest that the synthesis or post-translational modification of this molecule may be precisely timed to regulate adhesive interactions between cells during specific developmental events (e.g., the end of fasciculated axon elongation; cf. Schlosshauer et al, 1984).

Three other constituents of fast axonal transport were found to increase markedly during development.   Two of these, the acidic 27kD and 94kD proteins, increased continuously after birth and reached mature levels by P17.   The 27kD protein appears to be identical to a protein described as S1 which, in damaged peripheral nerve, decreases sharply in synthesis and transport after nerve damage, but then returns to high levels at about the time of synaptic contact (Redshaw and Bisby, 1985).   The 94kD protein, which shows a more gradual increase, may be homologous to protein S14 described by Shirao and Obata (1985) which shows a similar increase during maturation of the chick optic tectum.   The developmental increase in the 27kD and 94kD proteins observed in the present study coincides with changes in retinal axon size and the extent of terminal maturation (see Fig 2J).   The third protein in this group, 64kD,   showed an abrupt increase, being undetectable in the P2 and P5 neonates but achieving high levels by P12. Thus, maximal synthesis and transport of the 64kD protein begin at a time when retinal axons are actively elaborating terminal arbors and remain high in the adult.   A protein with a similar shape, apparent molecular weight, and pI to the 64kD species is rapidly transported in the intact hypoglossal nerve of rat, but diminishes markedly in peripheral nerves during axonal regeneration (see Redshaw and Bisby, 1984, Fig 5).   The 27kD, 64kD and 94kD proteins thus appear to be associated with later stages of neural development and may be components of presynaptic receptors, transmitter vesicles, membrane channels, or other synaptic specializations, or could serve to stabilize the

membrane or induce myelination.

Two acidic proteins with apparent molecular weights of 100kD and110kD were low in the neonate, increased to peak levels by P5, and then declined to low levels in the adult. Hence, although these proteins may be required for events such as target recognition, they appear to be less essential once stable connections have been established. In the goldfish retinotectal projection, several acidic proteins of similar apparent molecular weights showed increased levels in synthesis and transport only during the time of initial contact with the appropriate target (Benowitz et al, 1983). Experimental verification of such target regulation in the hamster retinotectal projection is also possible by surgical manipulation that deprive axons of their appropriate targets (see Schneider, 1973; So and Schneider, 1978). Studies combining metabolic labeling with such manipulations may further show whether the macromolecular regenerative response to injury is a complete re-induction of the protein synthesis and transport patterns seen in the immature developing animal, or whether the regenerative response reflects a distinct growth state.

It is interesting to note that in the present studies, essentially every protein conveyed to the nerve terminal membranes in the rapid phase of axonal transport underwent a marked change during development so that between P2 and P17 the labeling profile changed almost completely. Thus, in addition to the identity and physiological significance of the individual proteins visualized here, another question of interest concerns the nature of the signals that orchestrate this complex pattern of metabolic changes. In particular, it will be of importance to identify interactive events in which the pattern of molecular changes determines the morphological sequence of development, and those in which dynamically changing interactions with extrinsic constituents induce the molecular changes observed. Recent reports have made it clear that the neuronal as well

as the glial and end-organ environments can significantly alter the growth characteristics (Hantaz-Ambroise et al, 1987; Lance-Jones and Landmesser, 1981; Liuzzi and Lasek, 1987; So and Aguayo, 1985) and underlying pattern of gene expression in neurons (Benowitz et al, 1983; Baizer and Fishman, 1987; Redshaw and Bisby, 1985; Yoon et al, 1986).

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FIGURE LEGENDS

Figure 1. Rapidly transported proteins in the developing hamster retinofugal pathway. Proteins were labeled by intraocular injection of [^{35}S]-methionine; after allowing 4 hrs for proteins synthesized in retinal ganglion cells and conveyed by rapid axonal transport to accumulate in the nerve endings, particulate proteins in the SC were analyzed by 2-dimensional gel electrophoresis and fluorography. Fluorograms are shown with apparent molecular weight in kilodaltons along the ordinate and isoelectric point (pI) along the abscissa. In the P2 and P5 neonates (A and B) several proteins not conveyed in rapid axonal transport were systemically labeled due to the immaturity of the blood-brain barrier (e.g., actin, A and ß-tubulin, ßT). Other proteins that show marked developmentally-regulated changes are indicated with arrows. (A) P2, (B) P5, (C) P12, (D) P17 and (E) adult. (F-H): Two-dimensional Western blot of total particulate brain proteins from neonatal hamsters reacted with an antiserum to GAP-43. Proteins were separated by 2-dimensional gel electrophoresis under nonreducing conditions (F and G) or reducing conditions (H) and then transferred electrophoretically to a nitrocellulose membrane. The resulting blots of proteins separated under non-reducing conditions were reacted with preimmune serum (F), or with an antiserum made against the GAP-43 protein of rat, which recognized hamster brain proteins (arrowheads, G) with the identical pI as GAP-43 (4.8) and apparent molecular weights of 50kD and 100kD. When the blot of proteins separated under normal reducing conditions was reacted with the anti-GAP-43 antiserum, a single protein with apparent molecular weight 50kD and pI 4.8 was visualized (H).

Figure 2. Quantitative analysis of rapidly transported proteins during development. Rapidly transported proteins were cut from the gels and radioactivity determined by liquid

scintillation counting. The radioactivity for each protein is expressed as a percentage of the total rapidly transported radioactivity at each age. Each data point is the mean ($\pm$ s.e.m.) from 3 different experiments. In some cases the error bars are less than the size of the symbol (•). Top row: proteins that decline in synthesis and transport during development include (A) GAP-43 and two (B,C) 230kD proteins, pI 4.9 and 5.0. Upper middle row: proteins that increase during development and remain high include proteins with apparent molecular weights of (D) 27kD, (E) 64kD and (F) 67kD. Lower middle row: proteins that initially increase in synthesis and transport and subsequently decrease include (G-I) the 100kD, 110kD and 94kD proteins. Bottom: schematic representation showing the time course of morphological changes in the developing retinofugal pathway.

Figure 3. Immunohistochemical localization of GAP-43 in transverse sections through P2 (a, b, c and e) and adult (d and f) hamster brain. (a) Control section through midbrain of P2 animal incubated with non-immune serum. (b) Midbrain section reacted with monospecific antibody to GAP-43. Note intense staining of axon bundles and fiber tracts. (c) Higher power view of superior colliculus from (b). Thin black line delineates ventral border of the retinal-recipient zone (SGS+SO). Staining is restricted to axon bundles in the medial and superficial regions of this zone. (d) Similar section through SGS and SO of an adult brain. Only light staining in neuropil is evident. (e and f) Staining pattern seen in lateral thalamus of P2 and adult animals. Note positive staining in axons of the optic tract and eml in neonates, whereas retinal axons in mature animal are devoid of reaction product. Abbreviations: Aq = Aqueduct of Sylvius; BIC = brachium of the inferior colliculus; CG = central grey; CP = cerebral peduncle; eml = external medullary lamina; LGBd, LGBv = dorsal and ventral nuclei of the lateral geniculate body; LP = lateral posterior thalamic nucleus; OT = optic tract; SC = superior colliculus; SGS = superficial

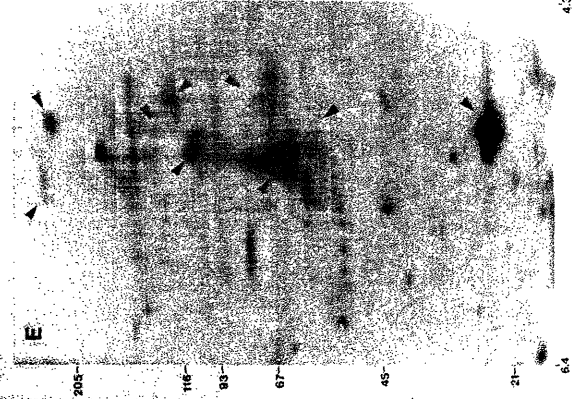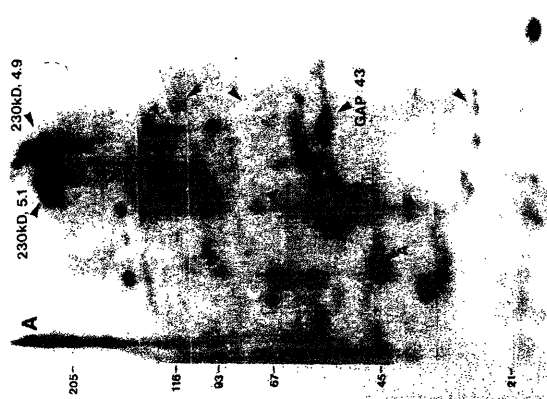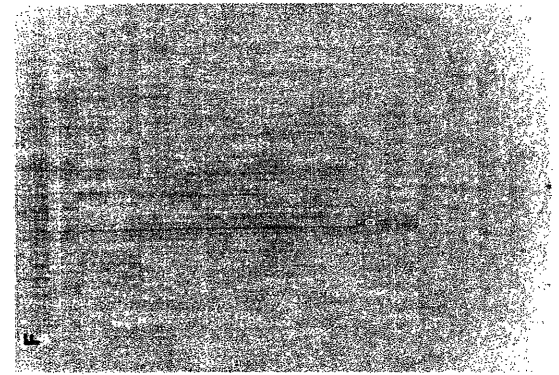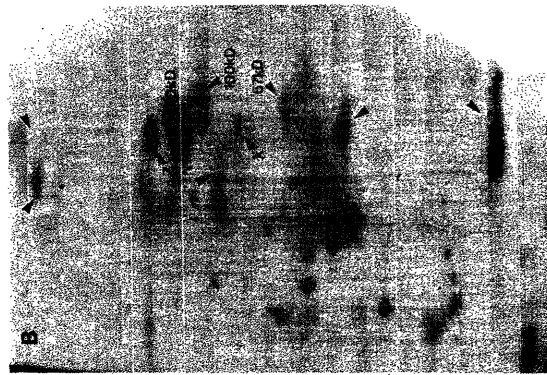grey layer; SO = optic fiber layer. Scale bar: 100μm in a, b and f; 200μm in c, d and e.

D

274kD

H

C

G

B

230kD

180kD
97kD

F

A

230kD, 5.1   230kD, 4.9

GAP 43

205—

116—
93—

67—

45—

21—

E

205—

116—
93—

67—

45—

21—

6.4   4.3

FIGURE 2

a

b

SC

Aq

CG

c

SGS+
SO

P2

d

SGS

SO

Adult

P2 control

OT

LGBd

LP

OT

LGBv

LGBd

Cp

Adult
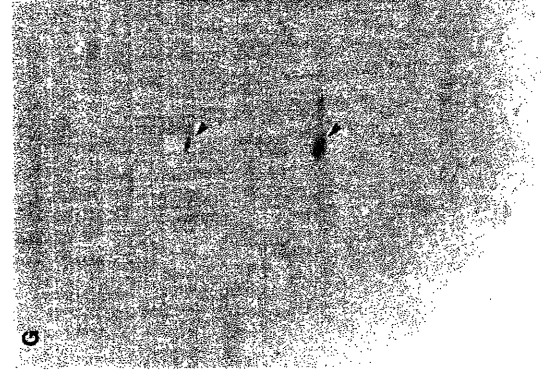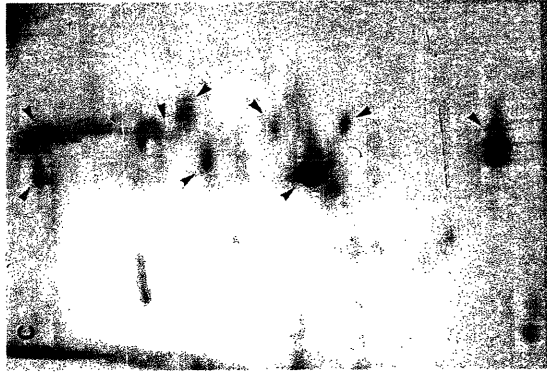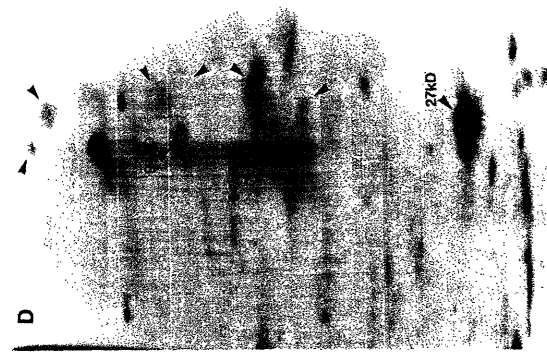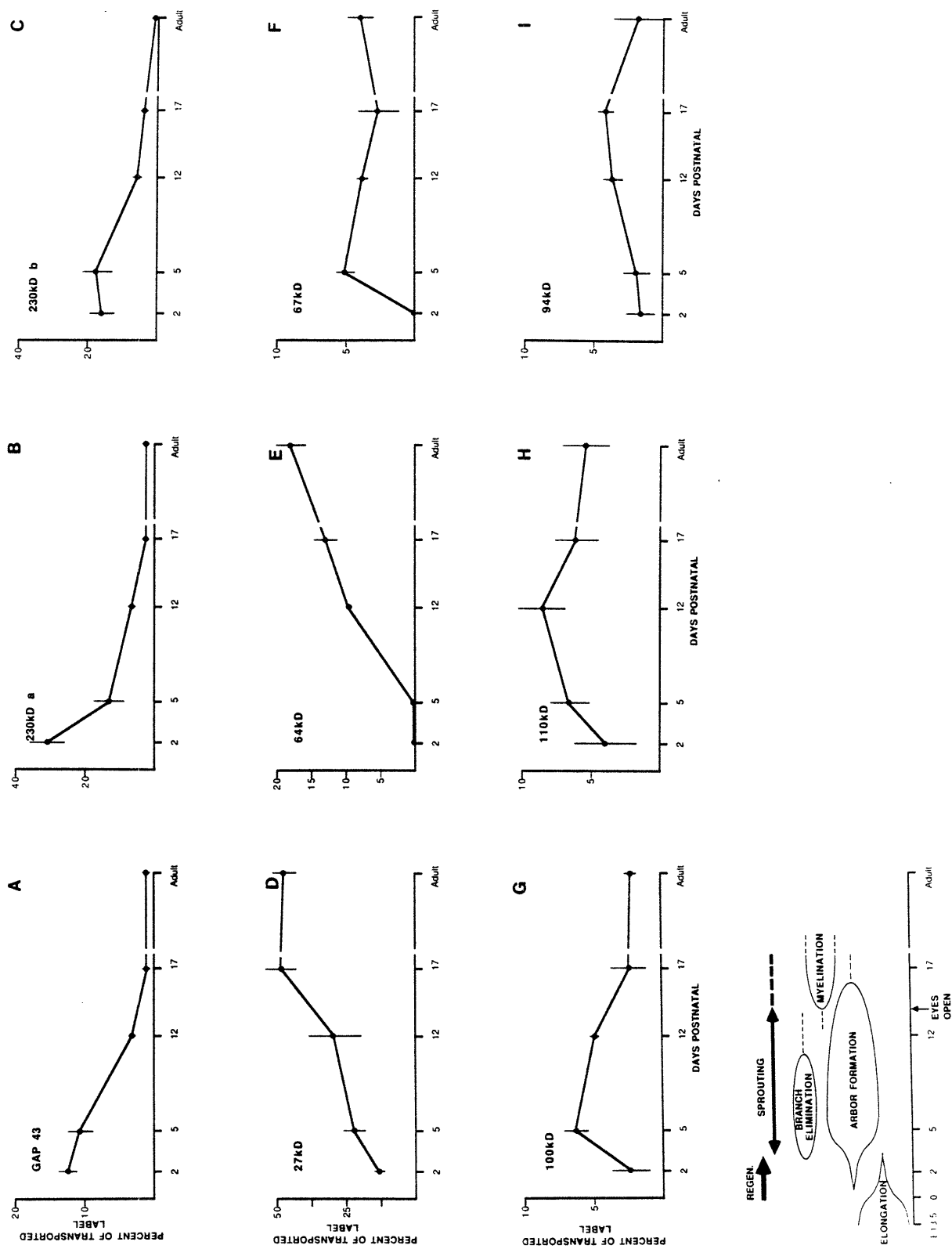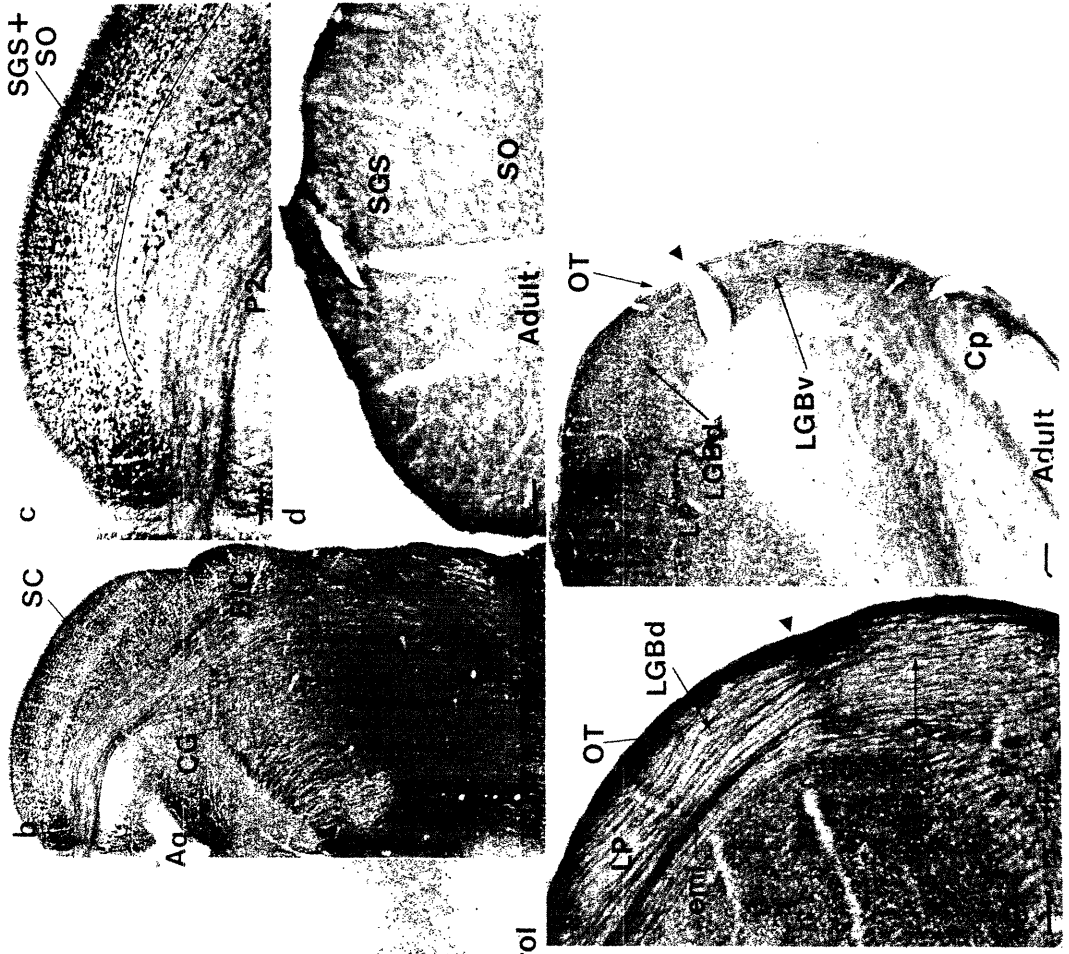
CHAPTER 4

**IMMUNOHISTOCHEMICAL LOCALIZATION OF GAP-43 IN THE
DEVELOPING HAMSTER RETINOFUGAL PATHWAY.**

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ABSTRACT

The hamster retinofugal pathway, like other developing neural systems, is marked by a high level of synthesis and axonal transport of the neuron-specific phosphoprotein, GAP-43, at early stages of axon outgrowth and synaptogenesis. Synthesis of this protein then declines sharply during the maturation of terminal arbors in central target areas. To better understand the relationship of GAP-43 to specific developmental events, we used a monospecific antibody to examine the localization of the protein in the optic tract and retinal target areas at various stages. In late embryonic and neonatal hamsters, dense staining of GAP-43 was preferentially localized in fibers the entire extent of the optic tract, including fascicles coursing over and through the lateral geniculate nucleus (LGN) and within the upper layers of the superior colliculus (SC). The retinal origin of these fascicles was confirmed by their rapid disappearance after removal of the contralateral eye. During the first postnatal week, the fiber fascicles showed a marked decline in immunoreactivity, but the protein was still found throughout the neuropil of the LGN and SC. In the second postnatal week, GAP-43 levels in the neuropil of the LGN and SC had diminished, and by 12 days after birth these structures showed only light immunoreactivity. In terms of the known sequence of developmental events, the high levels of GAP-43 in embryonic and neonatal optic tract axons coincide with axon elongation, initial target contact and collateral formation, whereas the subsequent concentration of the protein in the neuropil coincides with the maturation of terminal arbors and synaptic relationships.

The developmental events of the hamster retinofugal pathway have been characterized in some detail. Retinal ganglion cell axons emerge from the eye and elongate rapidly in fascicles through the optic nerve, many reaching their central targets before the day of birth. During the first postnatal weeks, these axons send branches into the terminal neuropil and begin to elaborate end arbors. By the beginning of the third postnatal week, the eyes have opened and the morphology of retinal axon arbors resemble the adult (Jhaveri et al., 1983; Sachs and Schneider, 1984; Schneider et al., 1985).

In order to understand the biological mechanisms that underlie these morphogenetic events, we have investigated developmental changes in the synthesis and transport of membrane proteins that are destined for retinal terminals, including molecular species likely to be important for membrane addition, growth cone motility, responsiveness to extrinsic signals, and cell-cell adhesion (Moya et al., 1988). Among the prominent changes that occur during development is a marked decline in the synthesis and axonal transport of the growth-associated protein, GAP-43, a neuron-specific phosphoprotein of the terminal membrane that is also associated with the development and regeneration of axons in other systems (Benowitz and Lewis, 1983; Katz et al., 1985; Kalil and Skene, 1986; Meiri et al., 1986, 1988; Moya et al., 1987, 1988; Perry et al., 1987; Redshaw and Bisby, 1984; Skene and Willard, 1981a,b; Zwiers, et al., 1980). In most neuronal populations, synthesis of GAP-43 decreases markedly with maturation, although the protein persists in synapses of certain brain areas throughout life (Benowitz et al., 1988a,b; Jacobson et al., 1986). In one such region, the hippocampus, changes in the phosphorylation of GAP-43 have been correlated with the alterations in synaptic function or structure that are associated with long-term potentiation (Lovinger et al., 1985;

Nelson and Routtenberg, 1985). It is possible that GAP-43 may likewise play a role in mediating activity-dependent changes in synaptic organization during development and regeneration (Benowitz and Schmidt, 1987; Meiri et al., 1988).

In the hamster retinofugal pathway, the synthesis and axonal transport of GAP-43 are high during the first postnatal week, a period when axons shift from elongation to the initiation of terminal arborization, then decline sharply in the second postnatal week as mature terminal arbors form (Moya et al., 1988). However, it is quite possible that the actual concentration of the protein in terminals could follow a somewhat different time course, depending upon its rate of turnover. In this study, we used a monospecific antibody against GAP-43 to determine the availability of the protein in optic nerve terminal areas and to analyze developmental changes in the distributional pattern of the protein. Our results demonstrate that the overall levels of GAP-43 closely follow the time course of synthesis, although the intracellular localization of the protein shows a striking shift with development. The protein is abundant throughout retinal axons during elongation, shifts to the target neuropil as the axons begin to arborize in terminal zones, and then greatly diminishes to low levels as mature terminal arbors become established.

METHODS

The selectivity and specificity of the monospecific antibody for GAP-43 used in these studies have been described (Benowitz et al., 1988a; Moya et al., 1988). For the present studies, 2-3 hamsters at embryonic day 15 (E15; day of conception = E0), postnatal day 2 (P2; day of birth = E16 = P0), P5, P12, P19 and in adulthood were deeply anesthetized with an overdose of Chloropent (Fort Dodge Laboratories) or Nembutal (Abbott Laboratories) and transcardially perfused with buffered 4% paraformaldehyde. To determine the origin of GAP-43 positive fibers in the superior colliculus, P1 and P4

animals were anesthetized by hypothermia and the right eye removed. Animals were allowed to recover under a heat lamp and returned to the nest. After 24 hrs, an interval adequate to allow considerable retinal axon degeneration in young hamsters (Schneider, 1973), they were anesthetized and perfused as described above.

Brains were removed, postfixed for 2-3 hrs, and cryoprotected in buffered 30% sucrose. Frozen 40μm transverse or sagittal sections were cut and stored in phosphate-buffered saline with 0.5% NaN_3 (PBSN, pH 7.4). Endogenous peroxidase activity was neutralized by incubating sections in 0.3% H_2O_2 in methanol for 30 min. Sections were washed in 3 changes of PBSN for 30 min, nonspecific binding was blocked by incubation in 20% normal rabbit serum, washed again, and then were incubated for 3 days at 4°C in immune serum, or nonimmune serum (control sections), at a dilution of 1:2000 in PBSN with 0.4% TX-100 (Triton X-100, Sigma). Sections were washed in PBS TX-100 and incubated with biotinylated secondary antibody (Vector Labs) at a dilution of 1:250 for 1 hr, rinsed and incubated with avidin biotin complex conjugated to horseradish peroxidase (Vector Labs) prepared as directed by the manufacturer. After 2 rinses in 50mM Tris buffer (pH 7.5), the peroxidase was visualized using DAB as the chromogen.

RESULTS

The LGN and SC showed striking developmental changes in the localization and levels of GAP-43. At E15, the protein could be visualized along the entire extent of optic tract (OT) fibers coursing over the surface of the thalamus (Fig 1A). At this time, the first retinal axons have reached the SC, although later-arriving axons are still elongating in the OT and no major axon collateralization has occurred in the LGN and SC (Jhaveri et al., 1983; Schneider et al., 1985). Darkly stained fiber bundles were also evident coursing transversely within the LGN, where the deeper running retinal axons of the internal optic

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tract are located. A similar pattern of staining was seen on P2 and P5 (Fig 1B and C).

By P8, densely stained fiber fascicles were less prominent, instead GAP-43 immunoreactivity was predominantly distributed throughout the target neuropil, which had mottled appearance due to the absence of staining of postsynaptic cell bodies (Fig 1 D). During the second postnatal week, GAP-43 diminished such that the LGN was only lightly stained on P12 and later ages (Fig 1E and F). A band of dark immunoreactivity differentiating the intergeniculate leaflet was apparent on P2 and became more prominent at P5 and P8.

In the SC, the time course and pattern of GAP-43 immunoreactivity was similar to that observed in the LGN. Densely stained fiber fascicles were observed in the superficial layers of the SC at E15, P2 and P5, appearing as dark, punctate clusters (Fig 2A-C). Sagittal sections through the neonatal SC verified that this punctate staining was comprised of longitudinal, GAP-43-positive fascicles coursing rostrocaudally through the SC (data not shown). In perinatal animals, these darkly staining axons were distributed throughout the upper layers of the SC, in a pattern similar to the known distribution of retinal fibers at this time (Woo et al., 1985; see Edwards et al., 1986 for studies in the mouse). By P5, the optic fiber layer (SO) has differentiated and immunoreactive axons were now restricted primarily to this stratum. On P8 the fascicles could be only faintly visualized in the SO (Fig 2D). GAP-43 immunoreactivity throughout the neuropil of the SC was apparent at P8 but declined markedly by P12 (Fig 2E). Control sections reacted with nonimmune serum were devoid of staining (Fig 2G).

During the first postnatal week, the staining pattern of the fiber fascicles in the SC showed a striking medio-lateral gradient (Fig 2B and C). At P2, staining of fiber fascicles was greater in medial portions of the SC than in lateral regions, and this gradient became even more pronounced at P5, at which time the densest GAP-43 positive fascicles were

observed in the medial SO. By P8, fascicles in the lateral part of the SO showed virtually no staining, but a few lightly stained bundles could still be observed in the medial SC (Fig 2D). This staining gradient was most obvious in the caudal half of the SC, indicating that the axons in the caudolateral colliculus are the first to lose GAP-43 immunoreactivity.

In order to demonstrate that the darkly stained fiber fascicles in the neonatal SC were of retinal origin, GAP-43 immunoreactivity was examined 24 hrs after unilateral eye enucleation in P1 and P4 animals. In the SGS and SO of the SC contralateral to the eye removal, densely stained fiber fascicles were eliminated, whereas in the ipsilateral SC, which receives most of its retinal projection from the remaining intact eye, fiber bundles were still apparent (Fig 3). Note, however, that GAP-43-positive fiber fascicles in deeper layers of the SC, which do not receive retinal projections, were unaffected on both sides. Over the LGN, staining of the OT contralateral to the removed eye was lighter than on the other side, although a number of densely stained fibers remained, which presumably include ipsilaterally projecting fibers from the remaining eye, or other growing axons of non-retinal origin (data not shown).

DISCUSSION

The present studies show that GAP-43 is present at high levels along axons in the hamster primary visual pathway during early stages of retinal axon development, but then declines sharply as this pathway matures. The availability of this protein in axons and terminals closely follows the time course of its synthesis and transport (Moya et al., 1988). Thus, considerable amounts of GAP-43 are present in retinal axons and immature synapses during early morphogenetic events such as axon elongation, target contact, establishment of retinotectal topography, and the initiation of end-arbor elaboration. The subsequent disappearance of GAP-43 in the hamster primary visual pathway coincides

with the formation of adult-like terminal arbors, increased number of retinofugal synapses and eye opening (Sachs and Schneider, 1984; Schneider and Jhaveri, 1984; c.f. Lund and Lund, 1976 for studies of synaptogenesis in the rat and Sachs et al, 1986 for studies in the mouse), and is similar to the time course observed in developing rat (McGuire et al., 1988).

The dense GAP-43 immunoreactivity along the entire extent of axon fascicles in neonatal animals may simply reflect high levels of the protein being rapidly transported to nerve terminals. However, in this case the protein would be associated with the membranous vescicles conveyed down the axon in the rapid phase of transport, and hence should show a punctate pattern of staining (cf. Meiri et al., 1988 for data on cell culture) rather than the continuous, dense distribution seen in here and in studies in the developing rat (McGuire et al., 1988). Alternatively, GAP-43 may be deposited along the entire axon with other membrane components as part of the early axonal growth process. Although axons reportedly elongate by membrane addition in terminal areas (Bray and Chapman, 1985), axon maturation in vivo must also involve membrane addition in more proximal segments to support the marked increase in axon diameter (Black and Waxman, 1986), as well as length. This suggests that the contribution of this molecule to axonal growth and neuronal interactions may not be confined to terminals. Rather, its distribution throughout the membrane of the entire central axon trunk, would enable this protein to participate in the expansion of axons or the sprouting of collaterals.

GAP-43 immunoreactivity along fiber fascicles is heaviest between P2 and P5, but diminishes by P8. This decline coincides with the shift of retinal axons from the rapid, fasciculated, elongation mode of growth to the arborization of terminals in target areas (Jhaveri et al., 1983; Sachs et al., 1986; Schneider et al,. 1985) and begins at about the time when topographic relationships have become established (Schneider and Jhaveri,

1984). Furthermore, during this time there is also a decrease in the plastic response of retinofugal axons to injury. Retinal axons will regenerate and functionally reinnervate their normal target in the SC if the brachium of the SC is cut before P4, but if axons are severed after this time, they fail to regenerate across the site of the cut. However, significant sprouting of retinofugal axons proximal to the lesion can be observed for up to 2 weeks after birth (Frost et al., 1979; Schneider, 1973; Schneider et al., 1985; So and Schneider, 1978; So et al., 1981). While it is clear from the eye enucleation cases that the darkly stained fascicles in the SC are of retinal origin, immunoreactivity observed at later ages in the SGS and SO may also include GAP-43 originating in cortical axons which first enter the SO on P5 (Ramirez et al., 1986). We did not observe dark fascicles at P5 in the upper layers of the SC after eye enucleation (data not shown), suggesting that the later arriving cortical axons may not be as fascicluated or form bundles large enough to be seen in the present studies.

The observed gradient of GAP-43-positive fascicles in the upper layers of the SC corresponds with a maturational gradient of retinofugal projections. Axons in the medial portion of the SC retain their immunoreactivity longer than those in the lateral colliculus, suggesting that the latter fiber population (originating in the upper retina) matures earlier than axons from the lower retina. Although a mediolateral gradient in the maturation of retinotectal axon arbors has not been reported, there is considerable evidence that the ventral retina lags behind the dorsal during embryonic development (Edwards et al., 1986; Silver and Robb, 1979). Furthermore, fiber tracing studies have shown that ganglion cell axons from the upper (and temporal) retina elongate and mature before those origniating from the lower retina (Edwards et al., 1986; Wikler et al., 1985). These findings are consistent with the results of the present study, in which GAP-43 immunoreactivity decreases in retinal axon fascicles in the lateral SC before those in medial regions.

The immunoreactivity seen in the target neuropil most likely represents GAP-43 within presynaptic membranes (Gispen, et al., 1985; Verhaagen et al., 1988). The apparent shift in localization of the protein from the axon to terminals occurs after retinal topography has been established, and coincides with terminal arborization in target areas and the formation of large numbers of synaptic connections. GAP-43 declines in the neuropil of retinal targets during the second postnatal week when mature-looking retinal arbors form. Similarly in the rat SC, GAP-43 immunoreactivity was seen to shift to neuropil during the time of synaptogenesis, before diminishing (McGuire et al., 1988). When the eyes open at P14 in the hamster, pupillary responses to light can be evoked, indicating that at least some retinal axons are capable of conveying visual information at this time. In the regenerating goldfish optic pathway, the correlation between GAP-43 expression and the time course of activity-dependent tuning suggests that the presence of the protein in nerve terminals may contribute to the process whereby patterned visual stimulation influences the organization of synaptic relationships (Benowitz and Schmidt, 1987).

The distribution of GAP-43 throughout axons early in development suggests that this protein has the potential for widespread influence on neuronal functions which become confined to the terminal region at later stages of development. Since GAP-43 is associated with the internal face of the plasma membrane (Basi et al., 1987; De Graan et al., 1985), this molecule is well situated to exert its influence throughout the axon via the protein kinase C cascade and phosphoinositide metabolism (Aloyo et al., 1983; Meiri et al., 1988; Van Hoff et al., 1988; Zwiers et al., 1980; see Nishizuka, 1986 for review). It may play a universal role in the growth process per se early in development, an idea supported by the fact that it is enriched in growth cones (DeGraan et al., 1985; Katz et al., 1985; Meiri et al., 1986; Skene et al., 1986), has been observed throughout virtually all

growing axons which have been studied (Kalil and Skene, 1986; our unpublished data), and transfection of PC12 cells with the cloned gene enhance process outgrowth (Neve et al., 1988). Later, when synaptic relationships are being established or modified, GAP-43 may contribute to the mechanism whereby the pattern of physiological activity influences synaptic structure or function (Benowitz and Schmidt, 1987; Lovinger et al., 1985; Nelson and Routtenberg, 1985; Snipes et al., 1987).

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FIGURE LEGENDS

Figure 1. Immunohistochemical localization of GAP-43 in the LGN during development. Densely stained fiber fascicles are observed in the OT and coursing through the neuropil of the LGN at E15, P2 and P5 (A-C). At P5 and P8 the staining appears mottled as immunoreactivity is absent from cell bodies (C and D). At P12 (E), staining has diminished and by P19 (F) it has taken on the adult pattern (not shown), with low levels in the OT and LGN. Note that dark staining of the LP differentiates this nucleus at later ages. Scale bar=100μm.

Abbreviations: LGBd, LGBv = dorsal and ventral nuclei of the lateral geniculate body; LP = lateral posterior thalamic nucleus; OT = optic tract.

Figure 2. Immunohistochemical localization of GAP-43 in the superior colliculus. Darkly stained fiber fascicles which appear as punctate clusters in the superficial layers of the SC where retinal fibers course are prominent at E15, P2 and P5 (A-C). At P8 fewer fascicles are visualized with the antibody (D). Note that a medio-lateral gradient of these fascicles is apparent in the neonate and has diminished greatly by P8. At P12 and P19 the staining of the neuropil has also diminished to near adult levels (E and F). Control sections from P2 were reacted with nonimmune serum and are virtually devoid of staining (G). Scale bar=100μm.

Abbreviations: Aq = Aqueduct of Sylvius; BIC = brachium of the inferior colliculus; CG = central grey; CP = cerebral peduncle; eml = external medullary lamina; SC = superior colliculus; SGS = superficial grey layer; SO = optic fiber layer.

Figure 3. Densely stained fascicles in the superficial SC are of retinal origin. P1 pups had their right eye enucleated and were perfused on P2 and prepared for

immunohistochemistry. On the side contralateral to the enucleation, fiber fascicles in the superficial SC had been eliminated, while the SC receiving projections from the remaining eye appeared comparable to normal, intact animals.
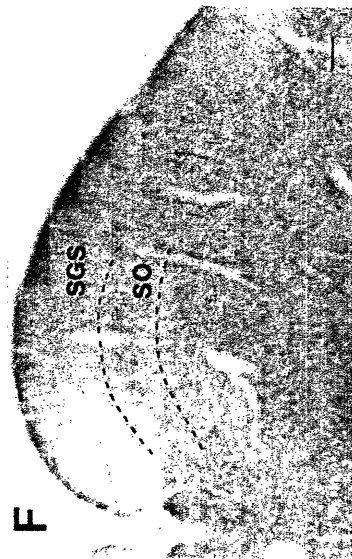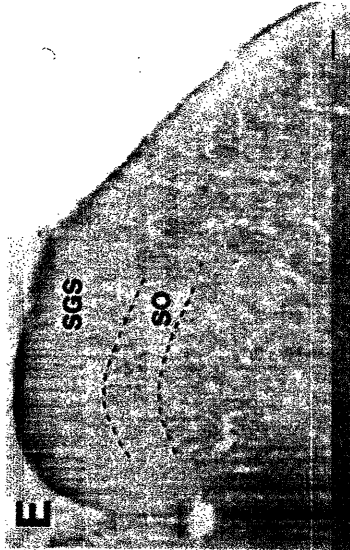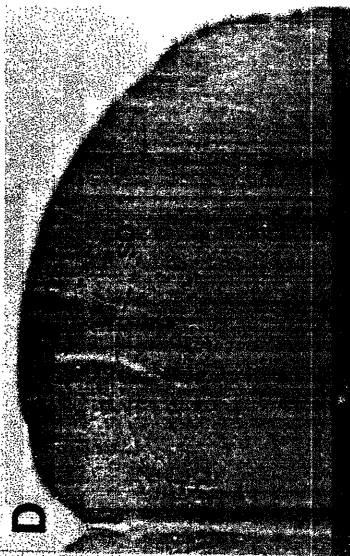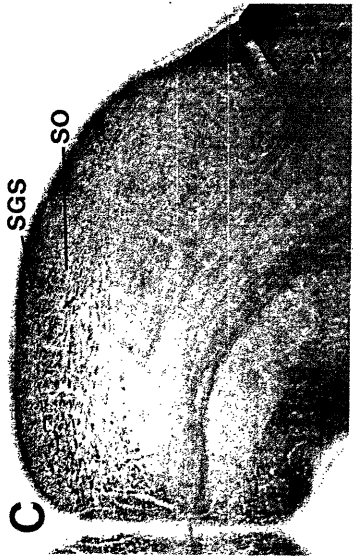
Intact

SGS+SO

SGS+SO

Enucleated

A SGS+SO

B SGS+SO

C SGS SO

D

E SGS SO

F SGS SO

G
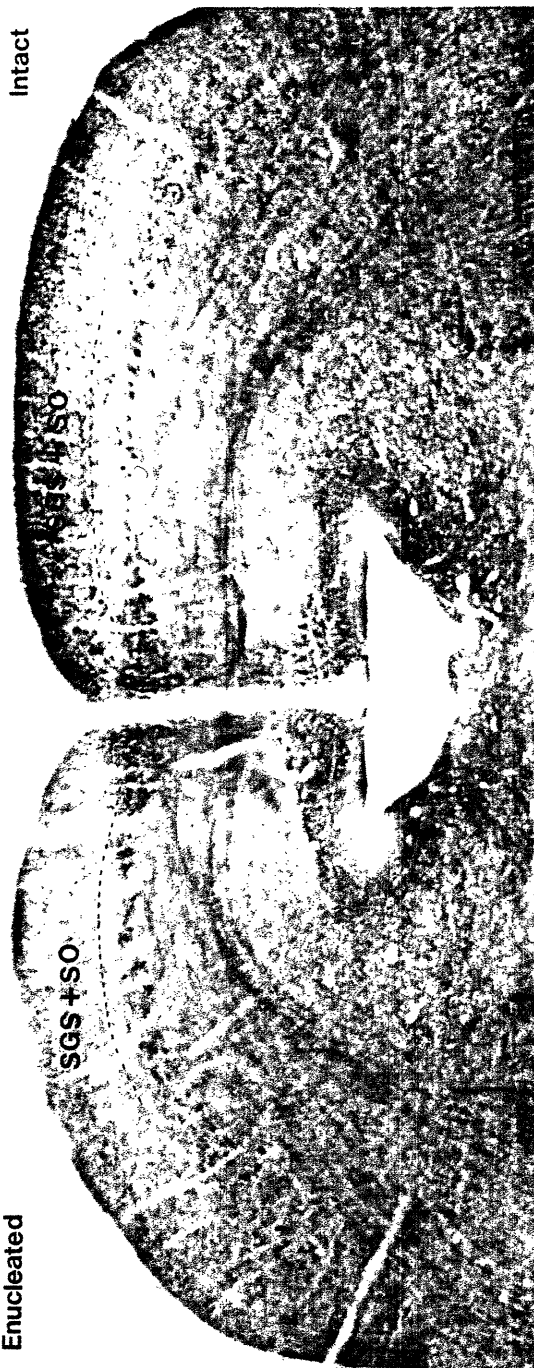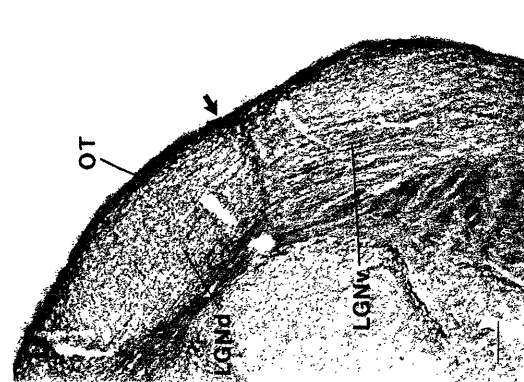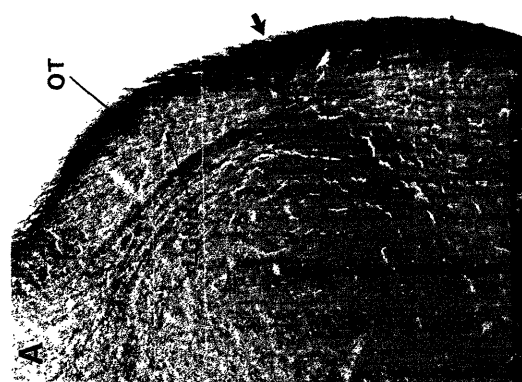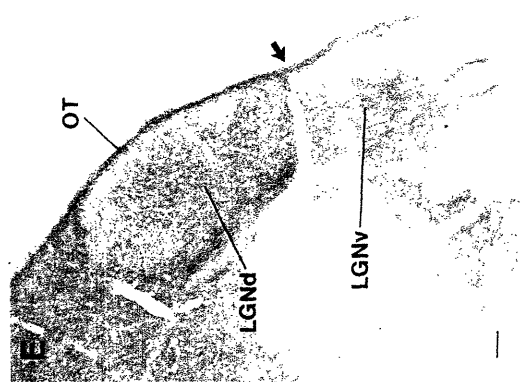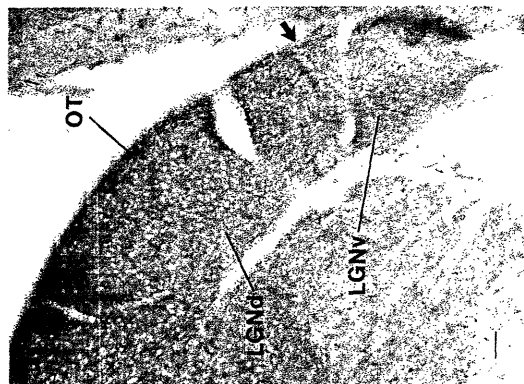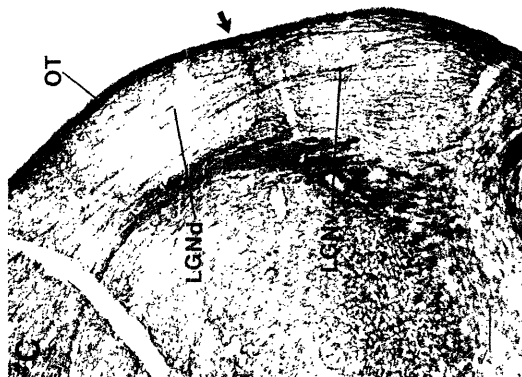
CHAPTER 5

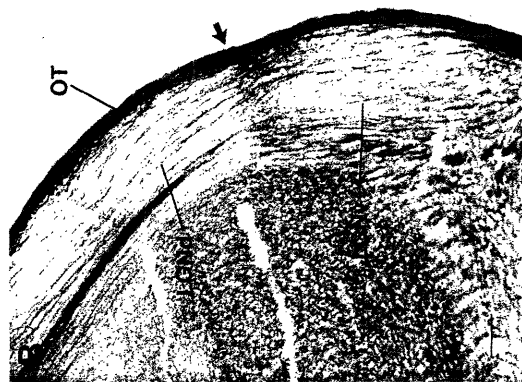# ALTERATION OF RETINAL PROJECTIONS TO THE DIENCEPHALON IS ACCOMPANIED BY CHANGES IN RAPIDLY TRANSPORTED PROTEINS

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ABSTRACT

In the developing hamster visual system, the pattern of proteins synthesized in retinal ganglion cells and rapidly transported to nerve terminal membranes changes markedly during the course of axon elongation, target penetration and terminal arbor formation (Moya et al., 1988). In order to determine which of these protein changes might be regulated by interactions between developing nerve endings and the cells which they contact, we examined the pattern of membrane proteins that results when retinal fibers fail to encounter one of their principal targets, the superior colliculus (SC). Previous studies have shown that early lesions of the SC result in the formation of abnormally dense retinal projections to the lateral geniculate nucleus (LGN) and lateral posterior (LP) nucleus of the thalamus (Sabel and Schneider, 1988; Schneider, 1973). Under these experimental conditions, the time course of several, but not all rapidly transported proteins was altered. Proteins which showed a delayed developmental change after tectal lesions included a 64kD species, whose normal increase during the second postnatal week did not appear until P17; and species of 67kD and 230kD which normally decline over the first two weeks, remained elevated for at least a week after the SC lesion. In contrast, the growth-associated phosphoprotein, GAP-43, was abnormally low in thalamic retinofugal terminals 4 days after the surgery. By maturity, the synthesis and transport of all rapidly transported proteins examined attained normal levels, suggesting that retinal ganglion cells induced to form abnormal connections nevertheless become committed to a program of protein expression that may restrict their potential for regeneration or synaptic remodeling.

The orderly progression of events in the developing nervous system is manifest in the stereotypy of the growth process and the specificity of the connections that are formed. In the hamster retinofugal pathway, the formation of connections is characterized by two modes of axonal growth which can be readily distinguished by their rate of elongation, state of fasciculation, frequency and location of branching, and the number and location of swellings or boutons. Before birth, retinal axons rapidly elongate in fascicles through the optic nerve, emerge from the optic chiasm to form the optic tract, and by the day of birth, they have extended over the surface of the diencephalon and midbrain tectum. After a brief delay, retinal axons begin to extend branches which penetrate into the appropriate target neuropil and initiate the formation of terminal arbors and synaptic contacts (Jhaveri et al., 1983; Sachs and Schneider, 1984; Schneider et al., 1985). In previous studies, we have described the time course of expression of nerve terminal membrane proteins during these developmental events (Moya et al., 1988). Our results suggested that some molecular species may play a role specific to early axon outgrowth, while others appeared to be related to terminal arborization and the development or maintenance of mature connections.

Some of the changes in rapidly transported proteins occurred at the time retinal axons encountered cells in target areas, raising the question of what role do cell-cell interactions play in regulating protein changes. The hamster retinofugal pathway provides a unique opportunity to experimentally manipulate the growth and target selection of developing axons, and to examine the effects of these manipulations on the expression of nerve terminal proteins. The normal developmental sequence can be altered by early lesions that destroy the upper layers of the superior colliculus (SC) and eliminate a significant portion of the normal retinal axon terminal space. These axons can regenerate over the damaged area and cross into the medial portions of the remaining intact SC (Schneider, 1973; Schneider et al.,

1985; So and Schneider, 1978). However, when the extent of the recrossing pathway is minimized, retinal axons form abnormally dense patches of hyperinnervation in the lateral geniculate nucleus (LGN) and the lateral posterior nucleus (LP) of the thalamus which normally receives little or no direct visual input in the adult rodent (Crain and Hall, 1980; Sabel and Schneider, 1988; see Perry and Cowey, 1982 for studies in the rat). In the present studies, we have examined the changes in nerve terminal proteins when developing retinal axons fail to encounter their normal target complement and are induced to form abnormal connections in the diencephalon. Our results demonstrate that the formation of abnormal projections does indeed alter the pattern of proteins in developing nerve terminals, and point to specific proteins that may directly participate in interactions with target cells.

METHODS

Early lesions of the SC

Syrian hamsters were bred in the laboratory, anesthetized on postnatal day 1 (P1; day of birth = P0) by hypothermia, and subjected to surgery as described previously (Sabel and Schneider, 1988). Briefly, the scalp was opened and the SC was visualized through the as yet uncalcified skull. The lesion was made by superimposing the head of a pin attached to a soldering iron over the right SC for 3 sec. Pin temperature was controlled by means of a variable AC rheostat in series with the soldering iron. The scalp wound was sutured closed and pups were allowed to recover under a heat lamp before being returned to the nest.

In order to verify the extent of the lesions and the resulting abnormal growth, 2-3 hamsters from several different operated litters were anesthetized and injected in the left eye with HRP (1.0mg in 2µl of 2% DMSO/saline) at P16 or in adulthood. Animals were allowed to survive 24 hrs, deeply anesthetized with chloropent (0.35ml/100g body weight,

Fort Dodge Laboratories) and perfused with phosphate buffered 4% paraformaldehyde, or 2% paraformaldehyde plus 0.5% glutaraldehyde. Brains were removed, postfixed 2-3 hours, and cryoprotected in 30% sucrose. Frozen $40\mu m$ transverse sections were cut and reacted to visualize anterogradely transported HRP using the TMB-AHM method (Jhaveri et al., 1988), then counterstained with neutral red.

Labeling of proteins transported to the diencephalon

The methods for labeling rapidly transported proteins, 2 dimensional gel electrophoresis, and quantitation of labeled proteins have been described in detail (Moya et al., 1988). Neonate animals (P2 and P5) were anesthetized by hypothermia and injected intraocularly with $100\mu Ci$ of [^{35}S]-methionine (New England Nuclear, Boston, MA) or Tran [^{35}S]-label (ICN, Irvine, CA) dissolved in $1\mu l$ of phosphate buffered saline (PBS). P12, P17 and adult animals were anesthetized with chloropent (0.35 ml/100g body weight, Fort Dodge Laboratories) and injected intraocularly with $100\mu Ci$ of radiolabeled amino acids dissolved in $2\mu l$ PBS. Injections were made into the eye contralateral to the lesion (i.e., left eye). In neonatal hamsters, radioactive amino acids that are injected into the eye diffuse into the circulation, readily cross an immature blood-brain barrier and are incorporated into locally-synthesized proteins resulting in significant background labeling (Moya et al., 1987). We have shown that this problem can be minimized by giving large systemic injections of non-radioactive amino acids which compete for access to the brain with the label that has diffused from the eye (Moya et al., 1987). Therefore P2 and P5 animals were also injected i.p. with non-radioactive leucine (1mg/g body weight) at the time of retinal labeling to reduce the background.

After allowing 4 hours for newly synthesized retinal ganglion cell proteins to be rapidly transported to optic nerve terminals in the diencelphalon, animals were deeply

anesthetized with chloropent, and an area including the LGN and LP contralateral to the injected eye was rapidly dissected out under visual guidance. One hemisphere from the neonatal animals was also removed for analysis of systemic labeling. Samples were immediately frozen on dry ice and stored individually at -70°C.

Tissue from 3-6 animals at each age was pooled and homogenized in ice-cold sucrose buffer [0.32M sucrose (Schwarz-Mann, Cambridge, MA), 50mM Tris, pH 7.4 (Sigma)] with 10 strokes in a teflon-glass homogenizer at high speed. Homogenized samples were centrifuged at 100,000 x g for 60 min and the total particulate pellets were resuspended in 50mM Tris for protein determination. After lyophilization, 400μg of protein was solubilized for 30 min at 30°C in 9.5 M urea (Bio-Rad) containing 2% NP-40 (Nonidet, Particle Data Laboratories, Elmhurst, IL), 6% ampholines in a ratio of 2:2:1 (pH 3.5-5.0, pH 5.0-8.0, 3.5-10.0; LKB, Gaithersburg, MD), and 5% ß-mercaptoethanol. Isoelectric focusing was carried out for 16 hr at 300v, then 4 hr at 400v. Gels were equilibrated and proteins separated in the second dimension on linear gradient (5-15%) polyacrylamide gels (O'Farrell, 1975), then fixed in acetic acid/methanol and stained with Coomassie Brilliant Blue. Gels were prepared for fluorography by impregnation with Autofluor (National Diagnostics, Somerville, NJ), dried onto filter paper (Whatman No. 1) and exposed to preflashed X-ray film (Kodak X-Omat, AR) for 30-50 days. Three to 4 gels from each time point were quantified as described below.

Quantitation of rapidly transported proteins

Templates were prepared from each fluorogram and used as a guide to excise labeled proteins from the gels. Gel pieces were solubilized in 6% Protosol in Econofluor (NEN, Boston, MA) and radioactivity determined by liquid scintillation counting. Some background labeling was observed in neonatal animals, as evidenced by the presence of

labeled proteins in the cortex. The cortex, which does not receive a direct retinal input and in which the profile of systemic labeling was similar to that in thalamic tissue (unpublished data), provides a good index of systemic labeling and was therefore used to quantitatively eliminate the contribution of such labeling. Virtually no systemic labeling could be detected by P12 (Moya et al., 1988). The background-corrected values for the neonatal ages and the directly measured values for the older time points were used in subsequent calculations.

Since the time course of virtually all transported proteins changed during development, it was not possible to normalize the labeling of a given protein to a particular reference protein. Therefore, the radioactivity in each protein was normalized to the total radioactivity transported to the LGN and LP and the data were expressed as a percentage of the total transported label. A more detailed discussion of this procedure and its limitations has appeared previously (Moya et al., 1988).

RESULTS

Verification of early lesions

The surgical method used here has been shown to produce reliable and replicable lesions of the SC (Sabel and Schneider, 1988). Figure 1 shows a case that received an SC lesion on P1, then was injected intraocularly with HRP on P16 and perfused on P17 (A and B) to visualize the extent of retinofugal projections. The lesion destroyed the superficial layers of the SC (Fig 1A), and is similar in extent and location to the other cases verified histologically (3 cases from 2 litters verified at P17 and 2 cases from 2 litters verified as adults). A minor recrossing retinal projection can be observed along the medial edge of the intact SC. Dense patches of retinal hyperinnervation in the LGN and an abnormal projection to the LP can be seen in sections through the appropriate levels of

the thalamus (Fig 1B). Similarly, a case from a different litter that was allowed to survive more than 4 months shows a small recrossing retinal projection at the level of the SC (C), along with dense patches of retinal projections in the LP and LGN (D). Littermates of these animals were used for the labeling of rapidly transported proteins as described above.

Alterations in the pattern of rapidly transported proteins

The pattern of proteins labeled in the retina and rapidly transported to terminals in the midbrain changes during development. As we have shown previously for the SC (Moya et al., 1988), GAP-43 is one of the prominently labeled proteins in the neonatal LGN of normal animals. Also clearly evident at this time are proteins of 230kD, 110kD, 100kD and 27kD (Fig 2A). GAP-43 and the 230kD species decrease markedly during development, whereas the 27kD and 64kD show large increases (Fig 2B-D). In the P5 thalamus containing altered retinal terminals, GAP-43 is noticeably reduced (Fig 2E); at P17, the 64kD protein appears to be diminished in the fluorograms from early lesioned animals (Fig 2G).

Quantitation of labeled proteins shows that in normal animals, the levels of nerve terminal proteins in axons projecting to the thalamus were virtually identical to that reported previously for axons projecting to the SC (Moya et al., 1988). However, after early lesions, the time course of protein labeling is altered in terminals of retinal axons in the LGN and LP. Compared with controls, levels of GAP-43 in SC-lesioned animals were markedly diminished at P5. Whereas this protein normally comprises nearly 10% of the transported label at P5, it represented less than 5% of the totally transported label in lesioned animals. At P12, this protein had diminished further, and at later ages, levels of this protein were very low, similar to the levels observed in normal animals (Fig 3A).

Several proteins were significantly delayed in their time course of labeling during the formation of abnormal retinal terminals. The 64kD (pI 5.2) protein, which normally shows a marked increase from P5 to P12, had a delayed rise in animals with abnormal connections, and did not increase until P17 (Fig 3E). An acidic (pI 4.7), 67kD protein was also delayed. This protein normally increases in the neonate between P2 and P5, and is maintained at high levels into the second postnatal week before declining by P17. However, in animals with early lesions, levels of this protein were high even at P17 (Fig 3I). A 230kD protein, pI 5.1 was elevated at P12 (12.1% of the total transported label in the lesioned cases versus 6.8% in normals) and at P17 (5.5% versus 2.2%), before diminishing (Fig 3B). To a lesser extent, the acidic 230kD protein also was elevated at P17, but was at levels comparable to normal at all other time points (Fig 3C).

All proteins which showed a change in lesioned animals had attained normal levels in the adult. Levels of the 27kD, 94kD, 100kD, and 110kD proteins in the lesioned group were similar to those observed in normal animals at every age examined.

DISCUSSION

These results demonstrate that early lesions of the hamster visual system which lead to the formation of abnormal connections, alter the pattern of nerve terminal membrane proteins. Proteins of the terminal membrane are likely to participate in such developmental events as target recognition, terminal arborization and synaptogenesis through the regulation of growth cone motility, extension of microspikes, cell-cell adhesion, axonal branching, sprouting of collaterals, and responses to ion fluxes or other extracellular cues. Early SC lesions deprive retinal axons of a significant portion of their normal target area while at the same time eliminating projections from the SC to the LP and LGN. By "pruning" distal branches and preventing the formation of terminals in the

SC, the growth vigor of retinal axons is enhanced, allowing retinal terminals to occupy dennervated regions of the LGN and LP (Sabel and Schneider, 1988; for discussion see Schneider, 1981). In this regard, the pattern of proteins in the nerve terminal may be viewed as the molecular manifestation of the rules of axonal growth that contribute towards determining retinal connections, while at the same time, the progression of developmental events can regulate changes in membrane proteins.

The abnormally dense patches in the LGN, and perhaps in the LP as well, do not represent an entirely novel departure from the normal repertoire of retinal axon growth. Rather, it has been suggested that the enhanced growth vigor of retinal axons, the availability of terminal space and the lack of competition from SC efferents may contribute to the maintenance and augmentation of a dense, exuberant retinal projection in the LGN and LP that normally exists at early stages of development (Perry and Cowey, 1982; Sabel and Schneider, 1988). The SC lesion on P1 is also likely to alter the timing of retinal axon growth. Since in normal animals, retinal axons begin to arborize in the LGN at about the day of birth, the SC lesion on P1 might be expected to delay subsequent arborization. Similarly, preliminary evidence indicates that the arborization of retinal terminals in the LP of SC-lesioned hamsters is not observed until about P4 (Carman and Schneider, unpublished results).

It should be kept in mind that the labeled proteins in retinal fibers terminating in the LGN and LP represent molecular species destined not only for fibers that form abnormal connections, but also for terminals in normal zones of the LGN. Since the pattern of labeled proteins observed here is determined by a multiplicity of target interactions and terminal signals, some normal and some abnormal, we can only describe our results as the outcome of a complex aggregate situation, and cannot yet relate specific molecular changes to individual growth events.

Previous studies have shown that target manipulations influence the pattern of neuronal protein synthesis and transport in other neural systems. In the regenerating goldfish optic nerve, removal of the optic tectum greatly alters the expression of several axonally transported proteins, prolonging the synthesis of some and delaying the appearance of others (Benowitz et al, 1983; Grafstein et al, 1986; Yoon et al, 1986). The pattern of rapidly transported proteins in regenerating rat peripheral nerve has similarly been shown to be dependent on the availability of target muscle tissue (Bisby, 1988; Redshaw and Bisby, 1985), while in vitro experiments have demonstrated molecular changes associated with the contact of specific target neurons in culture (Bazier and Fishman, 1987). Our studies differ from previous ones, however, in that we have examined changes in neuronal proteins associated with the development of abnormal connections, rather than the growth of axons in the presence or absence of an available target.

Our previous studies had shown that the levels of several proteins changed at the time of terminal arbor formation, or synaptogenesis, suggesting the possibility that their expression might be regulated by target interactions. Among these, a 67kD species, which appears to be highly correlated with the arborization of terminals in normal retinal axons but not with earlier or later morphogenetic events, is delayed in animals with early SC lesions, with its peak levels not observed until P17, before diminishing to normal levels in the adult. Similarly, a 64kD protein normally increases after the first postnatal week and reaches the mature high levels by P17, but in lesioned animals, does not increase until P17. An apparently similar 64kD protein has likewise been shown to be at low levels during rapid axon growth in regenerating peripheral nerve, but increases and is maintained at high levels when target tissue is contacted and terminals are elaborated (see Fig 5, Redshaw and Bisby, 1984). Since the appearance of this protein seems to

correlate with the establishment of mature connections, it may serve as a reliable marker for the formation of adult-like terminals.

Two proteins whose synthesis and transport appear to be associated primarily with early stages of axon development, also showed a delay in their time course in abnormal retinal terminals. The 230kD, pI 5.5 protein was elevated at P12 and P17, while the more acidic high molecular weight protein was slightly elevated only at P17. Both proteins declined to normal levels in the adult. Whether the delay in specific proteins coincides with a delay in terminal arborization is not yet directly known, and awaits the completion of a detailed time course of the morphological events following early SC lesions.

GAP-43, a membrane phosphoprotein whose synthesis and transport are related to the development and plasticity of neural connections (De Graan et al., 1985; Kalil and Skene, 1986; Moya et al, 1988; Nelson and Routtenberg, 1985; Skene and Willard, 1981b; Zweirs et al., 1980), showed a marked and unanticipated decrease in retinal axons during the formation of abnormal connections. This decrease may reflect a significant down regulation of synthesis in retinal ganglion cells that no longer need to support axon growth in tectal branches lost after the lesion, which have been estimated to account for about two-thirds of the optic tract extending beyond the LP (Schneider, 1979; Udin and Schneider, 1981). Since no other protein showed a similar decrease, such an interpretation would indicate that GAP-43 is selectively regulated by the amount of axon growth. It is also possible that decreased synthesis could be mediated by an inhibitory retrograde signal from retinal terminals that have prematurely established synaptic contacts in the absence of competition from other terminals. While other studies have reported a decrease in this protein during nerve regeneration when physiological activity has been blocked (Antonian et al., 1987; Benowitz and Schmidt, 1987), whether its synthesis is in fact target dependent, has not been resolved (Baizer and Fishman, 1987;

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Benowitz et al., 1983; Bisby, 1988; Kalil and Skene, 1986).

Alternatively, the decrease in GAP-43 observed here could be mediated by protein regulation at the local level. Recent evidence for this has been reported in which levels of GAP-43 increase markedly in the neuromuscular junction within a day after nerve crush (Verhaagen et al., 1988). Since the terminals have been physically separated from the cell body, new protein synthesis cannot account for this increase. Rather, the increase may represent an accumulation of GAP-43 previously synthesized and already being conveyed by rapid axonal transport at the time of nerve crush. Its accumulation in nerve terminals that are isolated from the cell bodies could arise from the loss of a normal proteolytic degradation of the protein that may occur in intact terminals. The marked decrease in this protein observed in the present studies then, may stem from the activation of such a degradatory mechanism by the early lesions.

While the labeling of several nerve terminal membrane proteins was altered, it is important to note that not all rapidly transported proteins examined were significantly affected by the experimental manipulation. This would suggest that while early manipulations are effective in inducing abnormal terminals to form, this does not involve a global alteration in retinal ganglion cells' program of gene expression. Proteins which are not altered by target manipulations may participate in developmental events that are independent of target interactions. These studies clearly demonstrate that target manipulations can alter the pattern of nerve terminal proteins, which may in turn determine further interactions with the target. It is conceivable then, that the proteins which are regulated by target interactions are those that directly participate in these interactional events. Furthermore, it is clear that as abnormal connections mature, a normal pattern of nerve terminal proteins eventually emerges, suggesting that retinal ganglion cells become committed to their pattern of connections and may not preserve the

molecular potential for further growth and modification.

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FIGURE LEGENDS

Figure 1. Early lesions of the superior colliculus result in the formation of abnormal retinal projections. The right SC was lesioned on P1 and HRP was injected into the eye contralateral to the damaged SC on P16 (A and B) or in adult animals (C and D), and the animals allowed to survive for 24hrs. The early lesions extensively damaged the right SC, while the left SC remained undisturbed (A and C). At the level of the SC, densely labeled retinal fibers course near the surface of the damaged area and a small projection can be observed crossing the midline into the intact SC, ipsilateral to the injected eye (A and C). In the diencephalon, dense patches of retinal terminals are seen in the dorsal LGN, and an anomalous retinal projection has formed in the LP, a nucleus which in the normal animals, receives little if any direct retinal input (B and D). Abbreviations: LGNd=dorsal lateral geniculate nucleus; LP=lateral posterior nucleus of the thalamus; SC=superior colliculus. Scale=100μm.

Figure 2. Retinal proteins rapidly transported to the diencephalon of normal and early lesioned hamsters. Proteins were labeled by intraocular injections of [^{35}S]-methionine, and after 4 hrs to allow newly synthesized proteins to be rapidly transported in ganglion cell axons, particulate proteins in the diencephalon were analyzed by 2-dimensional gel electrophoresis and fluorography. Apparent molecular weight in kiloDaltons is indicated to the left of A and E, and pI is indicated along the bottom of E. Fluorograms of labeled proteins from normal (upper row) and lesioned (lower row) hamsters at P5 (A and E), P12 (B and F), P17 (C and G) and adult ages (D and H) show a number of changes in rapidly transported proteins identified in previous studies (Moya et al., 1987 and 1988), which are indicated here by arrow points.

Figure 3. Quantitation of rapidly transported proteins in hamster retinal axons during normal development and after early lesions. Proteins were excised from the gels and radioactivity determined by liquid scintillation counting. The radioactivity in each protein is expressed as a percentage of the total radioactivity in the rapid phase of axonal transport at each age. Each data point represents the mean ($\pm$ s.e.m.) from 3-4 different experiments. In some cases error bars are less than the size of the symbol (•). Error bars have been omitted between the curves of the two groups where they overlap. Data from normal animals are depicted by solid lines and broken lines represent data from animals with early SC lesions. Top row: Among the proteins which normally show a decline from high levels in the neonate to low levels in adults are GAP-43, and 230kD species with pI 5.1 and 5.3 (A-C). After early lesions, GAP-43 shows a marked reduction at P5, while its time course at later ages is comparable to normal animals. Levels of the 230kD, pI 5.3, and to a lesser extent, the more acidic high molecular weight protein, are prolonged after early lesions. Middle row: Proteins which show a general increase during normal development include the 27kD protein and a protein of about 64kD which is delayed in its increase after the experimental manipulations (D-F). Bottom row: Among the proteins which show an increase in levels of labeling followed by a subsequent decrease include a 67kD species which is delayed in its time course (G-I). Note that not every protein was altered by the early lesions, and that in adult animals, all proteins had achieved the normal adult levels.

SC

A

LP

B

LGNd

SC

C

LP

D

LGNd
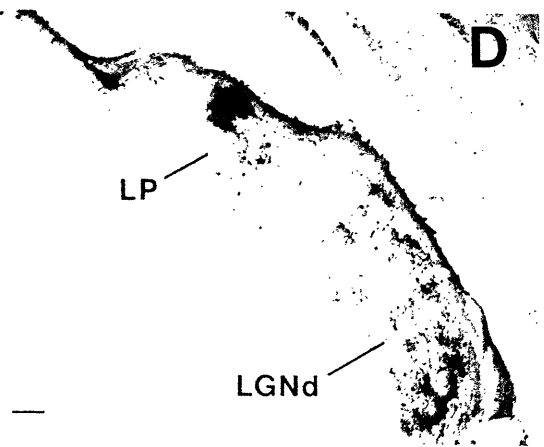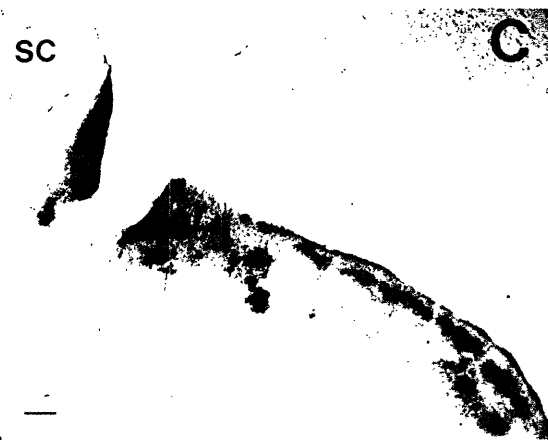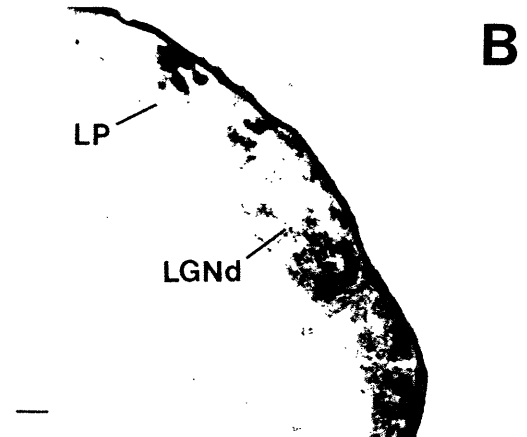
LESION   INTACT
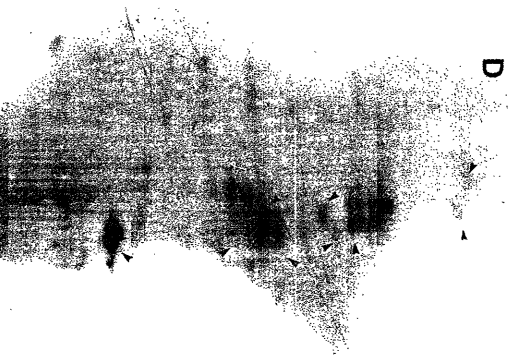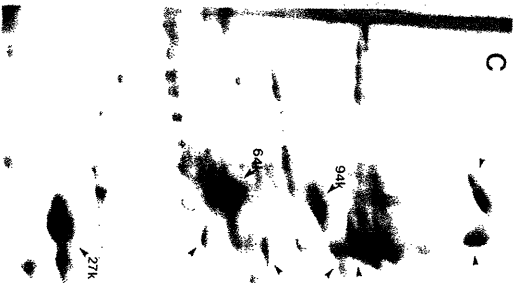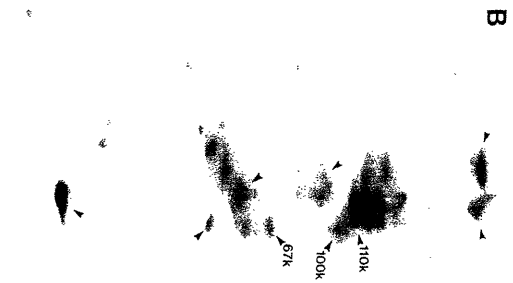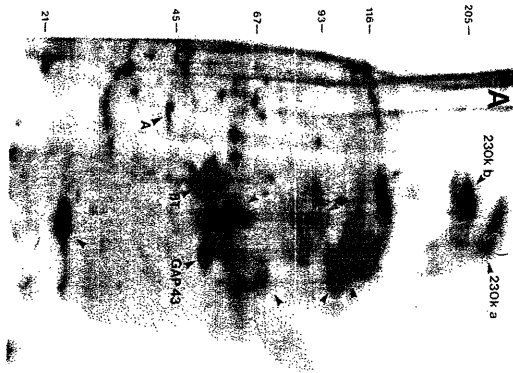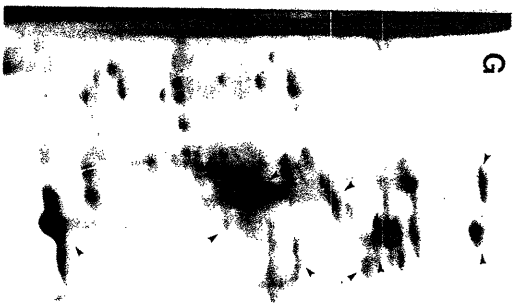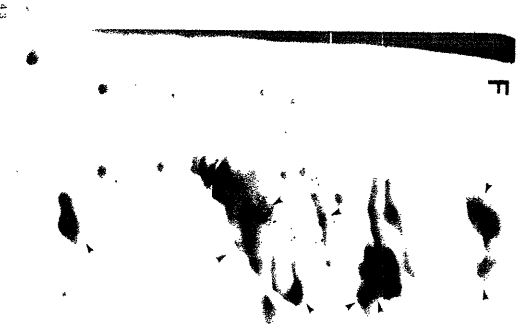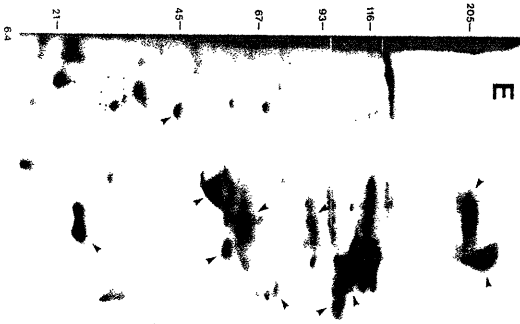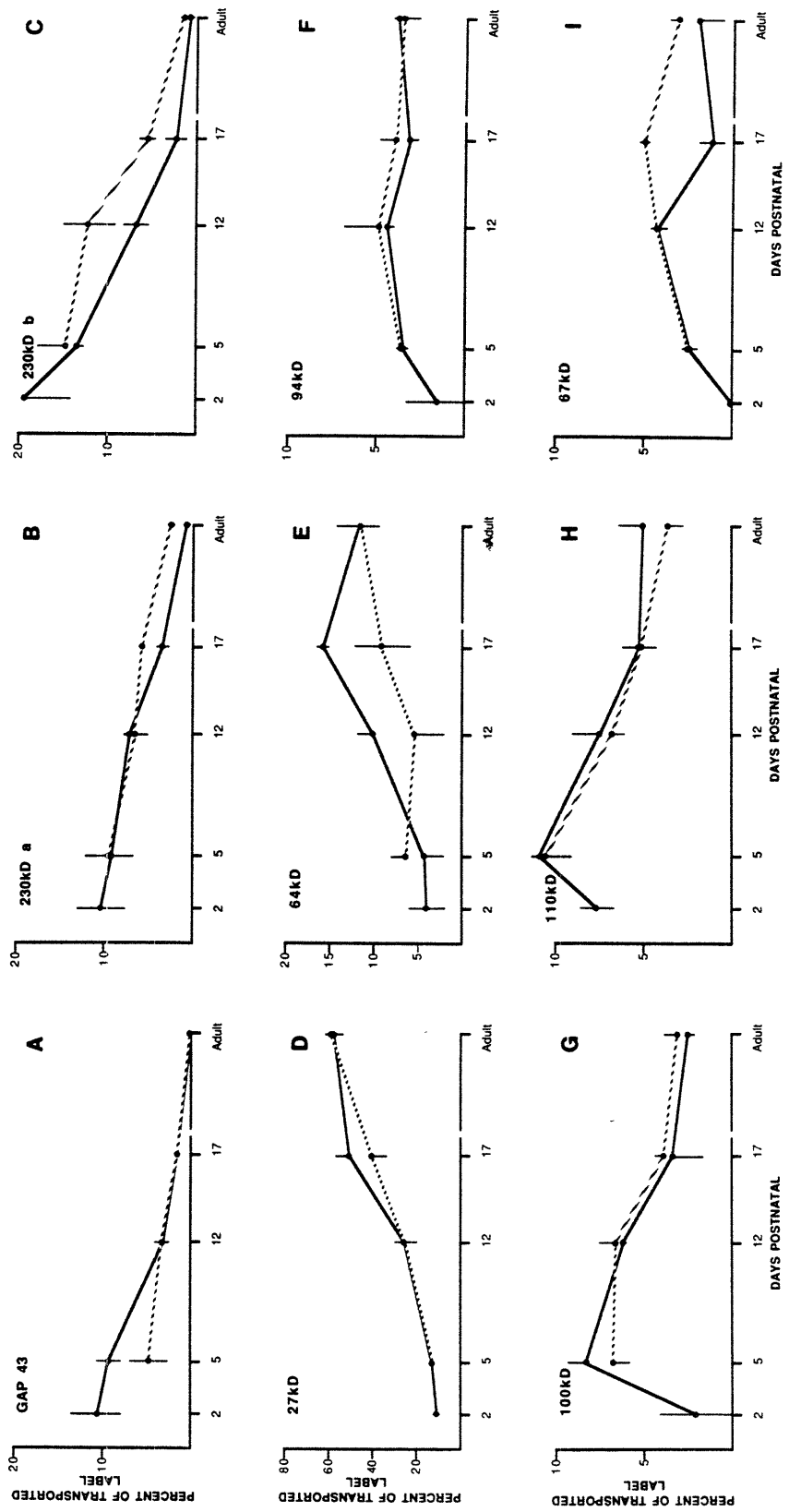
FIGURE 3

CHAPTER 6

ABNORMAL RETINAL PROJECTIONS SUPPRESS GAP-43 IN THE DIENCEPHALON

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ABSTRACT

In neonatal hamsters, lesions of the superior colliculus result in the formation of dense patches of retinal projections to the lateral posterior nucleus (LP) of the thalamus, which normally receives little if any direct retinal input. Our previous studies have shown that the presynaptic, growth-associated protein, GAP-43 is expressed at low levels in mature retinal axons, whereas in the LP, levels of this protein are high. In the present studies we examined whether retinal projections induced to form in the LP would retain high levels of GAP-43 similar to the levels observed throughout the LP, perhaps reflecting an instability of these connections. Using a monospecific antibody to GAP-43, we found that this protein does not persist in retinal axon terminals even after the formation of abnormal LP connections. The normally dark immunoreactivity seen in the LP was absent from areas corresponding to the dense patches of abnormal retinal terminals, suggesting that retinal axons had displaced GAP-43-containing terminals in the LP. Elimination of the axonal projections from the superior colliculus or from the posterior neocortex, two regions that provide major afferents to the LP, had no observable effect on GAP-43 staining in the LP, indicating that cells in these areas did not contribute GAP-43-rich terminals to this thalamic cell group. However, ibotenic acid injections in the LP, which eliminate local interneurons but do not damage fibers of passage, showed local interneurons to be the primary source of GAP-43 in the LP. The terminals of these local interneurons appear to be displaced by the abnormal retinal projections. These results show that, with respect to GAP-43, retinal axons are not induced to assume a different phenotype when abnormal projections are formed in an area containing abundant GAP-43-positive terminals. These findings also indicate that these axons express a competitive advantage which allows them to occupy regions of an anomalous target to the exclusion of terminals from local interneurons.

As in other mammals, the hamster visual system is marked by a highly organized projection from the retina to its primary targets, the lateral geniculate nucleus (LGN) and the superior colliculus (SC) (Frost et al., 1979). Visual information from the LGN is relayed to the posterior neocortex which has decending projections to the SC, LGN and lateral posterior nucleus (LP) of the thalamus (Crain and Hall, 1980a; Hahm et al., 1986; Sefton et al., 1981; Ramirez et al., 1986), while ascending secondary projections from the SC synapse in the LP, LGN and pretectum (Crain and Hall, 1980a; Perry, 1980). Although the hamster LP normally receives little if any direct retinal input, early lesions of the SC which destroy the retino-recipient zones lead to the formation of a significant retinal projection to the LP (Crain and Hall, 1980b; Frost et al., 1979; Kalil and Schneider, 1975; Sabel and Schneider, 1988; Schneider, 1973). This anomalous projection is characterized by dense patches of retinal terminals (Sabel and Schneider, 1988; Chapter 5).

In terms of the molecular events that underlie the development of retinal projections, our previous studies have described a number of dynamic changes in specific nerve terminal membrane proteins that are associated with such events as axon elongation, the formation of collateral branches and synaptogenesis (Moya et al., 1987,1988; Chapters 3 and 4). One of the proteins which showed a striking developmental shift was identified as the presynaptic membrane phosphoprotein GAP-43, which has been implicated in phosphoinositide metabolism and regulation of Ca^{2+}-mediated neurotransmitter release (Freeman et al., 1988; Gispen et al., 1985; Katz et al., 1985; Schrama et al., 1988; Van Hooff et al., 1988; Zweirs et al., 1980). In accord with findings in other neural systems, synthesis and transport of GAP-43 are high at early stages of axonal outgrowth, then decline sharply when stable synaptic relationships are established (Benowitz and Lewis,

1983; Kalil and Skene, 1986; Meiri et al., 1986; Perry et al., 1987; Redshaw and Bisby, 1984; Skene and Willard, 1981a, b; Verhaagen et al., 1988). Consistent with our molecular studies, immunohistochemical analysis revealed that developing retinal axons contained high levels of this protein which declined after the establishment of topography and the initiation of terminal arbors (Moya et al., 1988; Chapter 4). Furthermore, in mature animals, virtually all areas which received direct retinal input exhibit low GAP-43 immunoreactivity. In contrast, the LP was one of the few regions of the thalamus which contains high levels of the protein (Benowitz et al., 1988; Moya et al., 1988; Chapters 4 and 5; unpublished results). We were thus interested in determining whether retinal axons which were experimentally induced to project to the LP would themselves contain high levels of GAP-43, perhaps reflecting the formation of metastable connections still capable of being restructured, or if instead, they would still become committed to their normal adult program of protein expression marked by low levels of GAP-43.

Our results show that in adult retinal terminals, GAP-43 does not persist even after the formation of an abnormal projection to the LP. In addition, regions of the LP which receive dense patches of retinal projections showed a marked reduction in immunoreactivity, suggesting that retinal axons had displaced GAP-43-positive terminals from other sources. Elimination of major afferents to the LP did not reduce immunoreactivity in the LP, however, ibotenate injected into the LP showed that local interneurons are likely to be the primary source of GAP-43 in this nucleus. Hence, it is likely that it is the terminals of these latter cells which are excluded from the dense patches of retinal terminals.

METHODS

Lesions of the SC in neonates

Syrian hamster pups were anesthetized by hypothermia on postnatal day 1 (P1; day of birth=P0) and the skull over the SC exposed. The SC was visualized through the as yet uncalcified skull and lesions were made by superimposing the head of a pin connected to a modified soldering iron over the right SC as described previously (Sabel and Schneider, 1988; Chapter 4). Temperature was controlled by means of a variable AC power source. The scalp was sutured closed and the pups were allowed to regain normal body temperature under a heating lamp before being returned to the nest.

Labeling of retinal projections

To visualize the pattern of retinal projections resulting from the lesions, three P16 animals and three adults from different operated litters were anesthetized and injected in the eye contralateral to the lesion (i.e., left eye) with 1mg of HRP dissolved in 2µl of 2% DMSO in normal saline. Animals were allowed to survive for 24 hours and then deeply anesthetized with an overdose of Chloropent (Fort Dodge Laboratories, Fort Dodge, IA). After a brief saline rinse, animals were transcardially perfused with 4% paraformaldehyde in phosphate buffer. Brains were removed and postfixed for 2-3 hours, then cryoprotected in buffered 30% sucrose. Frozen 40µm transverse sections were cut and stored in ice-cold phosphate buffer, pH 6.0, and anterogradely transported HRP was visualized using the TMB-AHM method (Jhaveri et al, 1988). Immunohistochemistry for GAP-43 was carried out on alternate sections using a monospecific antibody as described elsewhere (Benowitz et al, 1988; Moya et al, 1988; Chapter 3).

Identification of GAP-43 containing projections to the LP

The LP receives major input from the SC and posterior areas of the neocortex (Crain and Hall, 1980a; Perry, 1980; Perry and Cowey, 1982). In order to determine if corticofugal or tectofugal projections were the source of the high levels of GAP-43 observed in the LP, we performed either large posterior cortical ablations or transections of the ascending projections from the SC. Normal adult animals were anesthetized with Nembutal/Valium (0.08ml : 0.16ml/ 100g body weight) and, in the case of the cortical ablation, the posterior third of the neocortex was aspirated under visual guidance. The cavity was filled with sterile gelfoam soaked in saline and the scalp was sutured closed. For the elimination of the tectofugal projection, a small cavity in the overlying hemisphere was made to visualize the surface of the midbrain and diencephalon, and a gap about 3mm wide and 1-2mm deep, extending from the pretectal/tectal border into the SC, was aspirated. This gap was made from the midline to the most lateral aspects of the colliculus. Sterile saline-soaked gelfoam was placed in the cavity and the scalp sutured. Animals were allowed to survive for 15 days and were then deeply anesthetized and perfused as above.

To examine the possibility that local interneurons were the source of GAP-43 in the LP, we injected ibotenic acid (Sigma; 10μg/μl in phosphate buffered saline, pH 7.4) into the LP of normal, adult animals. The lateral and medial geniculate nuclei, superior colliculus, and pretectum were visualized through a small cavity made by aspiration of the overlying hemisphere, and a series of pressure injections were made aimed at the center of the LP at 3 rostro-caudal levels. Sterile, saline-soaked gelfoam was placed in the cavity and the scalp sutured closed. Animals were allowed to survive for 3, 10, or 15 days and then perfused as above. In all cases, GAP-43 immunohistochemistry was performed as indicated above.

RESULTS

Early lesions of the SC result in abnormal retinal projections to the LP which are characterized by dense patches of innervation. These projections are prominent in adult animals, and are apparent by P17, just over 2 weeks after surgery (Fig 1 A and C). Reaction of tissue sections with a monospecific antibody to GAP-43 shows that all retinal axons, including the abnormal projections in the LP, are devoid of immunoreactivity. Furthermore, the pattern of GAP-43 immunoreactivity in the LP is altered after the formation of abnormal retinal projections. In contrast to the dark uniform staining in the LP of normal animals, hamsters with early lesions of the SC show prominent regions of reduced immunoreactivity (Fig 1B and D). Comparison with the adjacent sections used to visualize anterogradely transported HRP shows that the areas of diminshed GAP-43 immunoreactivity correspond to the dense patches of abnormal retinal terminals (Fig 1A-D).

Elimination of projections to the LP from posterior cortex or the superior colliculus did not alter the normal pattern of GAP-43 immunoreactivity in the LP (not shown). However, elimination of local interneurons by ibotenic acid injections into the LP did diminish GAP-43 immunoreactivity. Three days after ibotenic acid injections, regions of reduced GAP-43 could be seen in the LP, while the intact side shows the normal pattern of dark, uniform staining (Fig 2A and B). Viewed under high magnification, the reaction product in the region of diminished GAP-43 staining has a granular appearance, consistent with GAP-43-containing terminal debris (Fig 2C and D). Ten days after the ibotenic acid injections, regions in the LP that were virtually devoid of GAP-43 immunoreactivity were observed (Fig 3B); these regions correspond with areas of dense gliosis, indicating cell loss (Fig 3A). Areas of enhanced GAP-43 immunoreactivity could be seen in surrounding regions that had been mechanically damaged by the pipet tip. Comparable areas on

sections incubated with non-immune serum show that this enhanced immunoreactivity is not likely to be a staining artifact (Fig 3C).

DISCUSSION

Our previous studies have shown that the synthesis and transport of GAP-43 declines markedly during retinal axon development, and that this protein is virtually absent from adult optic fibers (Moya et al., 1987, 1988; Chapters 4 and 5). Moreover, by immunohistochemistry, levels of this protein are found to be low in all areas of the adult hamster brain which receive direct retinal input including the two nuclei of the LGN, the SC, the posterior pretectal and olivary pretectal nuclei, the nucleus of the optic tract, the medial terminal nucleus of the accessory optic tract and the superchiasmatic nucleus (Moya et al., 1988; Chapters 4 and 5; unpublished observations; see Frost et al., 1979 for summary of retinal projections in the hamster). However, the LP, which receives secondary visual inputs, is one of the few regions of the adult thalamus in which levels of GAP-43 are high (Benowitz et al., 1988; Moya et al., 1988; Chapters 4 and 5). The present studies examined the question of whether retinal axons would assume a molecular phenotype consistent with their target, or if they would retain a normal molecular identity, and in the process alter the phenotypic pattern of the target.

Our results show that the growth-associated protein, GAP-43, does not persist in retinofugal axons even after the formation of abnormal connections. This would suggest that the retinal ganglion cells follow a program of protein expression that may restrict their potential for further growth or plasticity. Unlike the normal adult, the LP of hamsters does not show a uniform distribution of GAP-43 after early tectal lesions, but has markedly diminished levels in those areas that receive the abnormal retinal terminals. The loss of GAP-43 immunoreactivity in the LP is not an artifact of the anterograde tracer,

since HRP does not interfere with GAP-43 staining in immature retinal projections to the normal LGN and SC (unpublished observations).

Our lesion studies indicate that local interneurons are the primary source of GAP-43 in the LP, since the loss of the two major extrinsic projections to this nucleus does not diminish levels of the protein, while ibotenic acid injections which eliminate local neurons without damaging fibers of passage (Kohler and Schwarcz,1983), markedly reduce GAP-43 in the regions that show gliosis and cell loss. Additional support for this comes from the presence of GAP-43-positive degeneration debris in the area of injections with a time course consistent with the effects of the excitotoxin.

It might be argued that retinal axons are excluded from GAP-43-positive terminal areas and that it is only after the loss of such terminals in the early SC lesion cases that retinal projections may form in the LP. However, it is clear that lesions of the SC in neonates or adults by themselves do not diminish GAP-43 in the LP, indicating that the SC contributes little or none of this protein to the LP. Hence, the lesion is not providing GAP-43-poor areas for occupation by the retinal terminals, and the formation of abnormal retinal terminals is not secondary to the loss of a GAP-43-positive input. Rather, it is likely that the formation of the abnormal retinal projection alters the distributional pattern of GAP-43 in the LP.

These studies show that with respect to GAP-43, retinal axons are not induced to assume a different molecular identity by the formation of abnormal connections with an anomalous target, however, electron microscopical studies have revealed that retinal axons in the LP do assume a heterotypic morphology. Retinal fibers which project to the LP after early SC lesions form axo-dendritic synapses, with a morpholgy consistent with that of tectofugal projections to the LP (Crain and Hall, 1980a; Kalil and Schneider, 1975). Although tectal and retinal projections to the LP are similar in morphology and GAP-43

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expression, these two projections have very different effects on the LP. While the normal tectofugal projection is compatible with the dense, uniform distribution of GAP-43 immunoreactivity throughout the LP, the formation of retinal projections exclude this protein from the areas of abnormal retinal terminals.

The formation of the dense patches of retinal terminals in the LP which exclude GAP-43-positive terminals of local interneurons may arise from competitive interactions mediated by synchronous physiological activity. During early stages of development, retinal axons contain high levels of GAP-43 which has been associated with activity-dependent tuning of retinal connections and synaptic plasticity (Benowitz and Schmidt, 1987; Lovinger et al., 1985; Nelson and Routtenberg, 1985). The abnormal retinal projection in the hamster has been shown to be more topographically organized than the tectofugal projection, providing an anatomical basis for synchronous firing by neighboring retinal ganglion cells. In addition, physiological studies have shown that visually evoked responses in LP neurons are more likely in cases with an abnormal, direct retinal input than in cases with the normal tectofugal circuitry (Sabolis, 1977). Thus, the retinal projection to the LP has an appropriate anatomical substrate and physiological capability to utilize synchronous activity in the formation of segregated terminal regions. Synchronous activation of postsynaptic cells has likewise been implicated in the segregation of visual input in supernumary-eyed frogs, the formation of experimentally induced ocular dominance organization in the goldfish and the activity-dependent formation of ocular dominance columns in the developing primate visual cortex (Law and Constantine-Paton, 1981; LeVay et al., 1980; Meyer, 1979). It is not yet known, however, whether the competitive advantage suggested here results in the displacement of GAP-43-positive terminals in the LP, or the inhibition of GAP-43 synthesis. The latter possibility can be addressed now by in situ hybridization experiments using the RNA

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probe for GAP-43 expression (Neve et al., 1987).

While the role that GAP-43 may play in mature LP terminals is unknown, previous studies have shown this neuron-specific, presynaptic membrane phosphoprotein to be associated with axonal development and regeneration, which regulates phosphoinositide metabolism (Benowitz and Lewis, 1983; Gispen et al., 1985; Moya et al., 1987 and 1988; Redshaw and Bisby, 1984; Skene and Willard, 1981a,b; Van Hooff et al., 1988; Verhaagen et al, 1988). In the hippocampus, another region of the adult brain where levels of this protein are high (Benowtiz et al, 1988; McGuire et al, 1988), changes in GAP-43 phosphorylation are associated with changes in synaptic structure or function that underlie long term potentiation (Lovinger et al, 1985; Nelson and Routtenberg, 1985). It is tempting to speculate upon a similar role for GAP-43 in the LP, which receives converging input from the tectum, visual cortical association areas and temporal neocortex (Crain and Hall, 1980a; Mason and Groos, 1981; Perry and Cowey, 1982; Takahashi, 1985), suggesting a potential integrative function for this nucleus. If this were the case, high levels of GAP-43 in the LP would also be consistent with elevated expression of this protein in associative areas of cortex (Benowitz et al., 1988; Neve et al., 1987).

The studies presented here show that the formation of abnormal connections alter the distributional pattern of molecular markers. When retinal axons are induced to form abnormal connections they do not persist in the expression of the presynaptic growth and plasticity-associated protein, GAP-43, suggesting that they are commited to their pattern of connections, perhaps with a restricted potential for further growth or synaptic modification. This commitment to a phenotype, marked by low levels of GAP-43, takes place in the midst of forming terminals in a region which normally contains abundant GAP-43-positive terminals. Furthermore, retinal axons appear to have a competitive advantage over other axons, since they exclude GAP-43-containing terminals of local

interneurons from the area of dense retinal projections and alter the pattern of immunoreactivity in the LP. Finally it is important to note that the present studies have also provided evidence that GAP-43-positive neurons in the LP appear to enhance GAP-43 expression in response to injury, a response that may include sprouting of fibers as well. Whether this ability to sprout is directly related to their high constitutive levels of GAP-43 expression, will be the subject of future studies.

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FIGURE LEGENDS

Figure 1. Abnormal retinal projections to the LP exclude GAP-43 immunoreactivity. The right SC was lesioned on P1 and animals were allowed to survive for 16 days (C and D) or 4 months (A and B), when HRP was injected into the eye contralateral to the lesion. Brains were processed to visualize anterogradely transported HRP (A and C), and for GAP-43 immunohistochemistry (B and D). At the level of the diencephalon, dense patches of an abnormal retinal projection have formed in the LP (A and C) which correspond to areas of diminished GAP-43 immunoreactivity (B and D). Abbreviations: LGNd= dorsal nucleus of the lateral geniculate body; LP=lateral posterior nucleus of the thalamus.

Figure 2. Ibotenic acid injected into the LP diminishes GAP-43. Pressure injections of ibotenic acid into the LP show reduced GAP-43 immunoreactivity after 72 hrs (B), while the intact side of the same section, shows the normal pattern of uniform, dark staining (A). At high magnification, granular deposits of reaction product can be seen in the neuropil of the injected side (D). The intact side show no such deposits (D). Scale in A and B is 100μm. Scale in C and D is 50μm.

Figure 3. Long term survival after ibotenic acid injection into the LP show marked gliosis and diminished GAP-43 immunoreactivity. Ibotenic acid was injected into the LP and animals were allowed to survive for 10 days. Sections stained with cresyl violet show dense gliosis in the LP and deeper regions of the thalamus (arrows in A). Adjacent section stained for GAP-43 shows diminished areas of immunoreactivity (arrows in B) which correspond to areas of gliosis. Sections reacted with non-immune serum (C) show that the enhanced immunoreactivity seen subjacent to an area of mechanical damage in

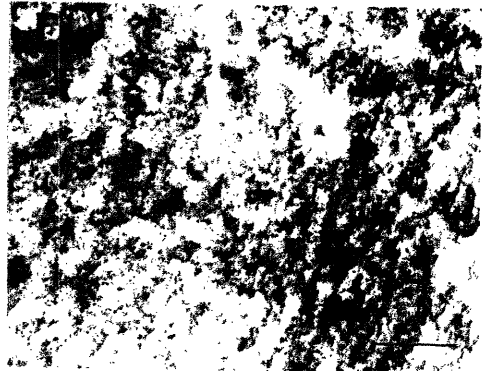119

the LP (B) is not a staining artifact. Scale: 100μm.

A

B

LP

LGNd

C

D

LP
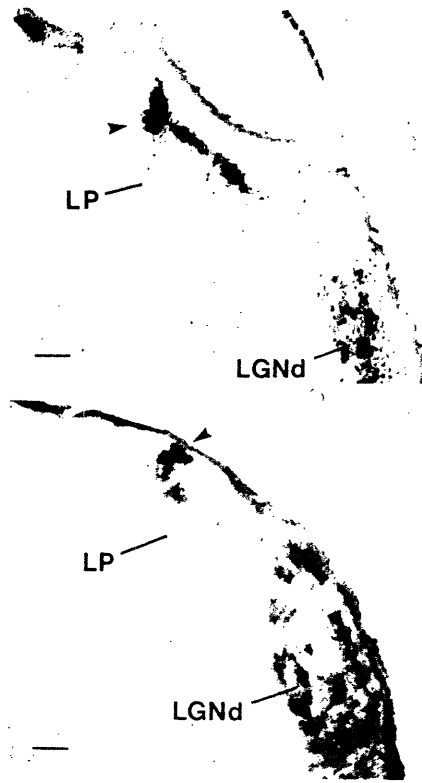
LGNd
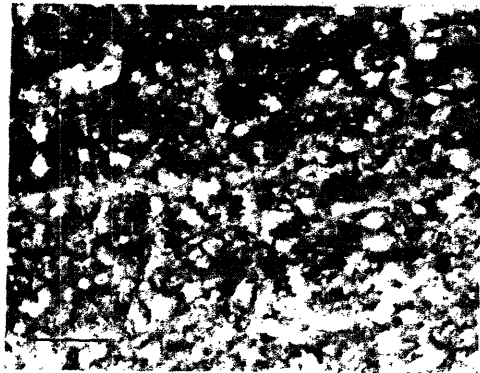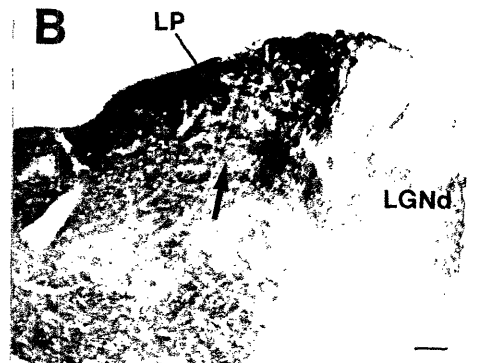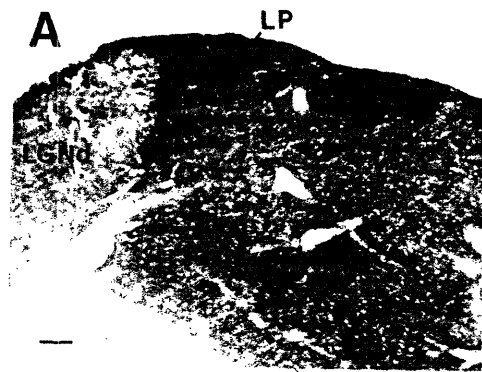
CHAPTER 7

CHARACTERIZATION AND IDENTIFICATION OF DEVELOPMENTALLY REGULATED, RAPIDLY TRANSPORTED PROTEINS IN HAMSTER RETINAL AXONS.

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ABSTRACT

Previous studies in the developing hamster visual system have pointed to a number of rapidly transported nerve terminal membrane proteins whose expression is temporally correlated with such events as axon elongation, terminal arborization, and the establishment of mature synaptic connections (Moya et al., 1988; Chapter 3). The present studies were directed at characterizing and identifying some of these proteins using biochemical and immunologic techniques. Our results show that one of the proteins which exhibits a marked decline when retinal fibers shift from axon elongation to terminal arborization is identical to the neural cell adhesion molecule, N-CAM. Four other developmentally regulated species also appeared to be glycoproteins, including a 230kD species which is present at early stages of axonal growth and is similar to the neuron-glia cell adhesion molecule. Another acidic glycoprotein, Mr=94kD, which is temporally associated with the formation of terminal arbors, exhibits biochemical properties similar to the ß-subunit of the integrin family of receptors for extracellular matrix components. In addition, preliminary evidence suggests that a 67kD, pI 4.7 protein, whose expression coincides with target contact and differentiation of terminal morphology, is a membrane-associated, myristoylated phosphoprotein, which may be identical to the proto-oncogene product pp60[src]. These findings suggest mechanisms by which specific proteins may contribute to the events that underlie the development of neuronal connections in the optic pathway, and perhaps in other parts of the nervous system as well.

Knowledge of the mechanisms whereby axons elongate rapidly, recognize specific target cells, and establish synaptic relationships is essential in order to understand how the organization of the nervous system arises. Previous studies have examined the pattern of rapidly transported proteins in developing retinal axons and have shown that specific proteins are correlated with axon elongation, terminal arborization or the formation of mature connections (Moya et al., 1988). Moreover, changes in several of these proteins appear to be regulated by target interactions (Chapter 5). Among the proteins associated with early stages of axonal growth, one was identified as GAP-43, a membrane-associated, kinase C substrate phosphoprotein which is involved with phosphoinositide metabolism, calcium-mediated neurotransmitter release, neural development and plasticity associated changes (Benowitz and Lewis, 1983; Gispen et al., 1985; Kalil and Skene, 1986; Katz et al., 1985; Meiri et al., 1986; Perry et al., 1987; Redshaw and Bisby, 1984; Skene and Willard, 1981a,b; Van Hooff et al., 1988; Verhaagen et al., 1988; Zweirs et al., 1980). However, in order to understand the contribution of nerve terminal proteins to the orchestration of developmental events, it is important to characterize many of the membrane constituents and identify those that may be functionally characterized in other systems.

In the present studies we have examined the glycosylation, phosphorylation, electrophoretic migration properties and cellular compartmentalization of rapidly transported proteins. In addition, we used a monospecifc antibody to the neural cell adhesion molecule, N-CAM, to examine the possibility that one of the rapidly transported proteins was identical to this well-characterized molecule and the results show that a high molecular weight protein which is associated with early stages of axonal growth, is in fact N-CAM. Several other proteins have been characterized as glycoproteins,

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including one that exhibits biochemical properties similar to members of the integrin family of extracellular matrix receptors. In addition, a rapidly transported protein associated with terminal arborization and whose time course is altered during the formation of abnormal connections, appears to be similar to the proto-oncogene product, pp60^{src}, which has been implicated in cell transformation and neuronal differentiation. Combined with our data on the time course of these proteins in developing retinal axons, these results suggest specific molecular mechanisms by which nerve terminal proteins contribute to fasciculated axon growth, the elaboration of terminals within target structures, and the formation of synaptic contacts.

METHODS

Identification of glycoproteins

Concanavilin A (Con A) binding proteins were identified on 2-D Western blots according to the method of Gordon-Weeks and Harding (1983). In order to visualize rapidly transported proteins in the retinofugal pathway, hamsters were given intraocular injections of [^{35}S]-methionine on postnatal day 5 (P5; day of birth = P0) as described previously (Moya et al., 1987, 1988). After a four hour survival period hamsters were deeply anesthetized and the superior colliculus was dissected out and frozen at -70°C. Labeled tissue from 8 animals was pooled, homogenized in ice cold buffer [0.32M sucrose (Schwarz-Mann, Cambridge, MA), 50mM Tris, pH 7.4 (Sigma, St. Louis, MO)], and centrifuged at 100,000 x g for 60min. 400µg of the particulate fraction was lyophilized, solubilized in isoelectric gel focusing buffer (O'Farrell, 1975), then subjected to 2-dimensional gel electophoresis as described previously (Moya et al, 1987, 1988). At the completion of the run, proteins were electrophoretically transferred to nitrocellulose (Phelps, 1984) at 4°C overnight at 250mA using a transfer buffer consisting of 0.025M

Tris, pH 8.8, 0.192M glycine, 0.1% (w/v) SDS, and 20% methanol. After the transfer, the nitrocellulose was fixed in methanol/acetic acid for 10 min, allowed to dry, and then was exposed to X-ray film (X-Omat AR, Kodak) for 30 days.

To identify glycoproteins after autoradiography, blots were blocked in 50mM Tris, pH 7.4, 0.15M NaCl, 3% BSA, then incubated for 60 min with biotinylated concanavilin A (35μg/ml in blocking buffer; Sigma). After rinsing 3 times in TBS, the nitrocellulose was placed in HRP-conjugated avidin (10μg/ml in blocking buffer; Sigma) for 60 min, rinsed 3 times and the HRP visualized using 4-chloro-napthol as a chromogen.

For concanavilin A lectin-affinity chromotography, rapidly transported proteins from P5, P12, P17 and adult hamsters were labeled as described above and a total particulate fraction was prepared from the lateral geniculate nucleus and superior colliculus. From 8.75mg of total particulate protein, detergent extract was prepared using 1% Triton X-100 in 25ml of 50mM Tris, pH 7.4 for 30 min at 4°C. Following centrifugation at 14,000 x g for 10 min, the detergent-soluble fraction was passed twice through a concanavilin A-Sepharose column [5.0 ml packed bed volume (Sigma)] that had been equilibrated with 10 volumes of column buffer (50mM Tris, pH 7.4, 0.1% Triton-X100). Non-specifically bound proteins were eluted with 2 column volumes of 0.2M D-galactose in column buffer. We took advantage of the fact that concanavilin A has affinities for terminal D-glucose and D-mannose residues (Reeke et al., 1974) to examine the presence of each of these saccharides on membrane proteins. Following the elution of non-specifically-bound proteins, the column was eluted with 2 volumes of 0.2M N-acetyl-glucosamine in order to identify proteins containing D-glucose residues, and then it was eluted with 2 volumes of 0.2M a-D-methyl-mannoside to identify proteins with D-mannose residues. Each eluate and an aliquot of the starting material was dialyzed 3 times against 4l of 0.4% CH_3COONH_4, lyophilized and proteins separated by 2-dimensional gel electrophoresis.

Gels were fixed and stained with Coomassie Brilliant Blue in methanol/acetic acid, impregnated with Autofluor (National Diagnostics, Somerville, NJ), dried onto Whatman No. 1 filter paper, and exposed to preflashed X-ray film (X-Omat AR, Kodak) for 40 days.

Identification of N-CAM

Western blots of P3 hamster brain were prepared as above, rinsed in 2 changes of distilled water for 1 min, and then washed in 50mM Tris, pH 7.4 (Sigma), 0.15M NaCl, 0.5% Tween 20 (Sigma) [TBST] for 30 min. The blots were blocked in 3% BSA (Fraction V, Sigma) in TBST for 60 min, washed 3 times for 10 min, and incubated overnight at 4°C with an affinity-purified antibody raised against N-CAM (gift of Dr. U. Rutishauser) at a concentration of 50μg/ml in TBST containing 1% BSA. After rinsing in 3 changes of TBST, the blots were incubated for 60 min with biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA; 1:250 in TBST plus 1% BSA), rinsed again, and placed in avidin-biotin complex in TBST prepared as directed (Vector Labs). The blots were rinsed once in TBST, followed by 2 changes in buffer without the detergent. HRP was visualized using 4-chloro-napthol as the chromogen. All steps were carried out with extensive shaking and, except for the overnight incubation with the primary antibody, were performed at room temperature.

Examination of changes in protein cellular compartmentalization

In order to examine the possibility that some rapidly transported proteins could also be associated with cytoskeletal components, we compared the pattern of labeled proteins in the particulate and soluble fractions in neonates and adults. P5 and adult animals were labeled as described and the LGN was rapidly dissected out and stored separately at -70°C. Tissue from 3-5 animals per age was pooled and homogenized in buffer (0.32M

sucrose in 50mM Tris, pH 7.4). After centrifugation at 100,000 x g for 60 min, the supernatant was dialyzed as above. 400μg of the particulate fraction and the soluble fraction were lyophilized and then separated by 2-dimensional gel electrophoresis as described. Gels were stained, fixed and prepared for fluorography and exposed to X-ray film, as described above.

Examination of electrophoretic migration under reducing and non-reducing conditions

Cortex from P5 and P12 hamsters was homogenized and fractionated as above, and 400μg aliquots of the total particulate fraction were separated by 2-dimensional gel electrophoresis. An additional 400μg aliquot of each sample was separated under non-reducing conditions in which ß-mercaptoethanol was omitted from all steps, but in all other respects, the conditions were identical to those described above. Gels were fixed and silver stained.

Protein phosphorylation

Synaptosomal plasma membranes were purified from neonatal hamster and rat brain as described (Benowitz and Lewis, 1983) and 100μg of protein was incubated with 20μCi $[^{35}S]$-PO_3S^*-ATP in 20mM Tris (pH 7.0), 0.4mM EGTA, 10mM $MgCl_2$, 1mM dithiothreitol in the presence or absence of 7mM $CaCl_2$ for 2 min at 30°C. The reaction was stopped by freezing the reaction mixtures at -70°C. Samples were lyophilized and separated by 2-dimensional gel electrophoresis as described above.

RESULTS

Identification of glycoproteins

Several rapidly transported proteins exhibit affinity for the lectin concanavilin A,

which binds terminal a-D-glucosyl and a-D-mannosyl residues (Reeke et al., 1974). 2-dimensional nitrocellulose blots reacted with biotinylated concanavilin A (Fig 1) showed previously identified rapidly transported proteins of 230kD (pI 5.3), 130kD and 94kD exhibited affinity for the lectin. Known rapidly transported proteins which were not detected by the labeled Con A included species of 27kD, 50kD (GAP-43), 64kD, 110kD, 120kD and 230kD (pI 5.1). Lectin-affinity chromotography allowed us to differentiate between proteins containing the two terminal monosaccharides. Proteins eluted with N-acetyl-glucosamine included one of two species having a Mr of 230kD (pI 5.1, subsequently identified as N-CAM), 110kD, 100kD and 130kD, indicating the presence of terminal a-D-glucose saccharides (Fig 2B). Proteins that eluted with a-D-methyl-mannoside included both 230kD species and the 100kD rapidly transported protein (Fig 2C).

Identification of N-CAM

A monospecific antibody raised against purified N-CAM which has been shown to recognize all major forms of this molecule (Hall and Rutishauser, 1985), uniquely recognizes one rapidly transported protein in neonatal hamster brain (Fig 3). On a 2-dimensional gel Western blot, this protein migrates with an apparent molecular weight of 230kD, pI 5.1, and is one of the developmentally regulated, rapidly transported proteins visualized here and in our previous studies (Moya et al., 1988; Chapter 3).

Changes in protein migration properties under reducing and non-reducing conditions

The overall pattern of proteins separated by 2-dimensional gel electrophoresis was very similar in the presence or absence of ß-mercaptoethanol. Of the several differences that were observed, the 94kD species was the only rapidly transported protein which

showed an altered migration under the two conditions, having an apparent molecular weight of 94kD, and a pI of 5.1 in the presence of ß-mercaptoethanol, but an increased apparent molecular weight (96kD) and a slightly more basic pI under non-reducing conditions (Fig 4). In addition, gels run under non-reducing conditions also showed a smearing in the tubulin region, a novel doublet in the range of about 70kD and an apparent decrease in molecular weight for an acidic protein of about 64kD. Similar results were observed in material from P12 cortex (not shown).

Proteins associated with both the particulate and cytosolic fractions

The pattern of labeled proteins in the fluorograms of particulate and cytosolic proteins from P5 hamsters show few proteins in common. However, a protein similar in apparent molecular weight, pI and shape to the rapidly transported 100kD protein is prominently labeled in the fluorogram of soluble proteins (Fig 5). In addition, trace amounts of a 94kD protein can be detected in the soluble fraction as well. In adult animals, the 100kD protein remained prominently labeled, although the 94kD was not visible (not shown). No other known rapidly transported proteins were seen in the soluble fraction. The presence of the 100kD protein in the soluble fractions of neonate and adult animals in approximately constant levels indicates that there were no major developmentally dependent changes in the cellular compartmentalization of this protein.

Phosphorylated rapidly transported proteins.

Prominent among several calcium-dependent phosphoproteins from P1 hamster brain, is one, which by 2-dimensional electrophoretic properties, corresponds to the rapidly transported protein GAP-43 (Fig 6). This protein is also similar to phosphoprotein B-50, isolated from neonatal rat. In the absence of calcium,

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phosphorylation of GAP-43 could not be detected (not shown).

DISCUSSION

Proteins conveyed in the rapid phase of axonal transport are destined for the nerve terminal membrane (Grafstein and Forman, 1980; Hammerschlag and Stone, 1982; Lorenz and Willard, 1978; Willard et al., 1974) and are thus situated to influence or mediate such developmental events as axon elongation, cell-cell adhesion, target recognition and synaptogenesis. Our previous studies have shown that specific molecular species are temporally associated with early stages of development such as axon elongation, while others appeared to be associated with the formation and maintenance of mature connections (Moya et al., 1988; Chapter 3). In addition, the expression of several of these molecular species has been shown to be altered when axons fail to encounter their normal target territory (Chapter 5).

The present results show that a number of rapidly transported proteins are glycosylated, including species of 230kD, pI 5.1 and pI 5.3, 110kD, 100kD and 94kD (Figs 1 and 2). It is unclear why the two methods used to identify glycoproteins were not entirely consistent with one another. The 2-dimensional nitrocellulose blot probed with biotinylated concanavilin A might be expected to be less sensitive than lectin affinity chromotography. If this were the case then all proteins detected by the former method should have also appeared in the results from the latter. Two of the three labeled, rapidly transported proteins detected on the nitrocellulose blot (230kD, pI 5.3 and the 130kD proteins) were also detected by lectin affinity chromotography, while the 94kD protein was only visualized on the nitrocellulose blot. The results from the affinity column chromotography showed that the 230kD, pI 5.1 (N-CAM), 110kD, 100kD and the 130kD proteins were eluted from the concanavilin A column with N-acetyl-glucosamine,

indicating the presence of a terminal glucose on these molecules. Both of the 230kD proteins and the 100kD protein were eluted with a-D-methyl-mannose as well as a-D-methyl-mannoside, suggesting that these proteins contain both terminal glucose and mannose. Carbohydrate heterogeneity within molecules of N-CAM and between N-CAM isolated from different neural tissues has been reported (Rothbard et al., 1982), and it is conceivable that similar properties could account for the elution of the 230kD (pI 5.3) and 100kD proteins by both sugars as well.

Use of a monospecific antibody showed that the acidic 230kD (pI 5.1) protein is identical to the neural cell adhesion molecule, N-CAM (Fig 3). This integral membrane glycoprotein has been shown to be involved in neuronal adhesion, neurite fasciculation, axon guidance and junctional communication (Busrick et al., 1980; Hoffman et al., 1982; Rutishauser et al., 1988; Thanos et al., 1984). Through changes in the protein backbone and levels of glycosylation, which have been shown to be developmentally regulated (Cunningham et al., 1983; Schlosshauer et al., 1984), a mechanism for the pleiotropic effects of this molecule has been suggested in which high sialic acid content early in axonal growth allows homophilic interactions between molecules on adjacent neurites to meditate fasciculation. At later stages, a decrease in sialic acid content allows the junctional apposition of neuronal membranes, which may then be permissive for the interactions of other membrane molecules (Rutishauser et al., 1988). Our previous findings have shown that the protein identified here as N-CAM is synthesized and transported at high levels during early stages of retinal axon growth, then declines as retinal fibers extend individual branches into target areas and elaborate terminal arbors (Moya et al., 1988). This time course is consistent with its role in the fasciculation of elongating axons.

The 230kD, pI 5.3 glycoprotein also has been shown to be associated with early

stages of axonal growth (Moya et al., 1988; Chapter 3). Manipulations which deprive growing retinal axons of their normal target territories, and result in the formation of abnormal connections, delay the normal developmental decline for this protein, suggesting that it is regulated by target interactions (Chapter 5). By apparent molecular weight, glycosylation and presence during axonal growth and target innervation, this rapidly transported protein is similar to a neuron-glia cell adhesion molecule (Ng-CAM) also known as NILE and L1. This protein facilitates neuron-glia binding and is induced in PC-12 cells by NGF-mediated differentiation (Bock et al., 1985; Friedlander, et al., 1986; Grumet et al., 1984; Grumet and Edelman, 1984; Hoffman et al., 1986; Salton et al., 1983; Sajovic et al., 1986). While the reported pI of Ng-CAM is in the range of 6.1-6.4 (Salton et al., 1983), the 230kD protein in the present studies migrates to a pI of 5.3. A discrepancy in pI could arise from tissue specificity, glycosylation or species differences, all of which have been shown to effect electrophoretic characteristics of Ng-CAM. Definitive identification of this protein as Ng-CAM, however, will require further studies using monospecific antibodies, or the determination of the primary sequence.

The 94kD protein exhibits several biochemical properties similar to the ß subunit of integrin. The 94kD glycoprotein with a pI of 5.1 (Fig 1), shows a decreased apparent molecular weight when separated under non-reducing conditions (Fig 4). The ß subunit of the integrin family has likewise been reported to be an acidic glycoprotein (pI 5.3-6.1), ranging from 90-140kD (depending on cell type), with a decrease in apparent molecular weight when separated under non-reducing conditions (Hasegawa et al., 1985; Kishimoto et al., 1987; Pytela et al., 1985 and b; Tamkun et al., 1986; for review see Hynes, 1987 and Ruoslathi and Piershbacher, 1987). The higher apparent molecular weight in the presence of reducing agents is due to the loss of a compact molecular structure maintained by extensive disulfide interactions within a cysteine-rich domain of the protein. The

integrin family of proteins includes receptors for extracellular matrix proteins such as fibronectin, vitronectin, and laminin, and is thought to mediate cellular adhesion and migration. In contrast to N-CAM, the 94kD protein is at low levels in elongating retinal axons, but increases during the formation of terminal arbors and remains elevated in the adult (Fig 6 and Moya et al., 1988). While integrin has been shown to be an integral membrane protein, the presence of small quantities of the 94kD protein among cytosolic proteins (Fig 5) is consistent with the known association of integrin with specific cytoskeletal proteins (Horwitz et al., 1986; Tamkun et al., 1986). Preliminary attempts at immunoprecipitation of the 94kD protein with an antibody raised against integrin purified from fibroblasts have not been successsful, but additional experiments are currently underway.

Hamster GAP-43 is phosphorylated under conditions similar to those reported for rat GAP-43 (Fig 6), also known as B-50, pp60 and F1, all considered to be identical (Benowitz and Routtenberg, 1987; Katz et al., 1985; Nelson and Routtenberg, 1985; Zweirs et al., 1980). Combined with our data on the immunological cross-reactivity of the protein from hamster, these results indicate that GAP-43 may serve the same function in the hamster as decribed for the rat, which include regulation of phosphoinositide metabolism, signal transduction in growth cones and calcium-mediated neurotransmitter release (Freeman et al., 1988; Schrama et al., 1988; Van Hooff et al, 1988).

The 67kD, pI 4.8 rapidly transported protein is similar in apparent molecular weight to the proto-oncogene pp60[src]. pp60[src] is a myristylated membrane-associated protein which exhibits tryosine kinase activity, including itself as a substrate, and is concentrated at sites of cellular adhesion and cytoskeleton/membrane interactions (Burridge and Feramisco, 1980; Cross et al., 1984; Hynes, 1982). Furthermore, neurons express high levels of this protein, and neuronal-like differentiation can be induced by the related

oncogene (Alemá et al., 1985; Brugge et al., 1985; Lynch et al., 1986; Martinez et al., 1987). The 67kD from hamster protein appears similar to a phosphoprotein associated with the plasma membrane, which is myristoylated in rat cortical neruons (Perrone-Bizzozero et al., in press, and Fig 7). In developing hamster retinal axons, the 67kD protein is present at low levels during axon elongation, increases when axons arborize in terminal areas, and then declines again, once mature terminals have differentiated (Fig 5 and Chapter 3), a time course similar to that for pp60^{src} in developing rat brain (Steedman and Landreth, 1985). When retinal axons are induced to form abnormal terminals, however, the time course of this protein is delayed, with elevated levels seen into the third postnatal week before they decline to normal levels in the adult. In order to demonstrate directly that the 67kD protein shares characteristics with pp60, experiments using [^{3}H]-myristic and conditions more appropriate for pp60 phosphorylation are currently underway.

Combined with previous studies, the results suggest mechanisms whereby specific nerve terminal membrane proteins might contribute to neuronal development. When axons are rapidly elongating, high levels of N-CAM and Ng-CAM may provide the means for axon fasciculation and guidance along non-neuronal elements. Also at this time, levels of GAP-43 are high throughout the length of axons, situating this membrane phosphoprotein to influence the growth process through the kinase C cascade and phosphoinositide metabolism (Aloyo et al., 1983; Gispen et al., 1985; Moya et al., 1988 and Chapter 4; Van Hooff et al., 1988). Terminal arbor formation coincides with declining levels of N-CAM; an associated shift to a less polysialated form would allow the junctional apposition of cell membranes and the interaction of other integral components such as integrin to facilitate the guidance and adhesion of exploratory branches in terminal regions. There is now growing evidence that the extraneuronal environment undergoes

significant changes during development (Armson et al., 1987; Silver and Sapiro, 1981; Smith et al., 1986), some of which coincide with the time when retinal axons begin to arborize in central target areas (Wu et al., 1988). The differentiation of terminal morphology and establishment of synaptic contacts coincides with the increase in the 67kD protein which then declines after mature arbors have formed. The presumed cognate protein, pp60^{src}, links the plasma membane to the cytoskeleton, concentrates at points of cell-cell attachment and is associated with neuronal differentiation. Its transient appearance during terminal arborization may be related to the phosphorylation of substrate proteins including cytoskeletal elements which may be required for cell-cell interactions and the formation of points of attachment. However, if the target is disturbed, arborization might be delayed, perhaps reflected in the delay of the 230kD basic protein and the 67kD protein after early tectal lesions (Chapter 5). An increase in the 94kD protein at this same time is consistent with the notion that interactions between integrins and the extracellular matrix components may help shape and maintain the terminal morphology through adhesive mechanisms. Thus, these data suggest specific molecular mechanisms for axonal development. Further work on the identification and functional characterization of these proteins is likely to contribute significantly to our understanding of the basic biology of neural development.

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FIGURE LEGENDS

Figure 1. Concanavilin A binding proteins identified on 2-dimensional Western blots. Labeled, rapidly transported proteins from P5 animals were separated by 2-dimensional gel electrophoresis and transferred to nitrocellulose. The fluorogram shows a number of rapidly transported proteins (A), some of which show an affinity for biotinylated concanavilin A (B).

Figure 2. Lectin affinity chromatography of labeled, transported proteins. Hamsters of different ages were labeled as described in the text, and detergent extracts of membranes were passed over a concanavilin A column. Labeled proteins extracted from membranes (A) showed a pattern identical with our previous studies (Moya et al., 1988; Chapters 3 and 5). Proteins eluted from the column with N-acetyl-glucosamine include the acidic 230kD protein as well as species of 130kD, 110kD and 100kD (B). Both 230kD proteins and the 100kD protein were eluted from the column with a-methyl-D-mannosamine (C).

Figure 3. Identification of a rapidly transported protein in developing hamster retinal axons as the neural cell adhesion molecule, N-CAM. A monospecific antibody raised against affinity purified N-CAM uniquely recognizes the acidic 230kD protein (B) labeled in P5 hamsters (A).
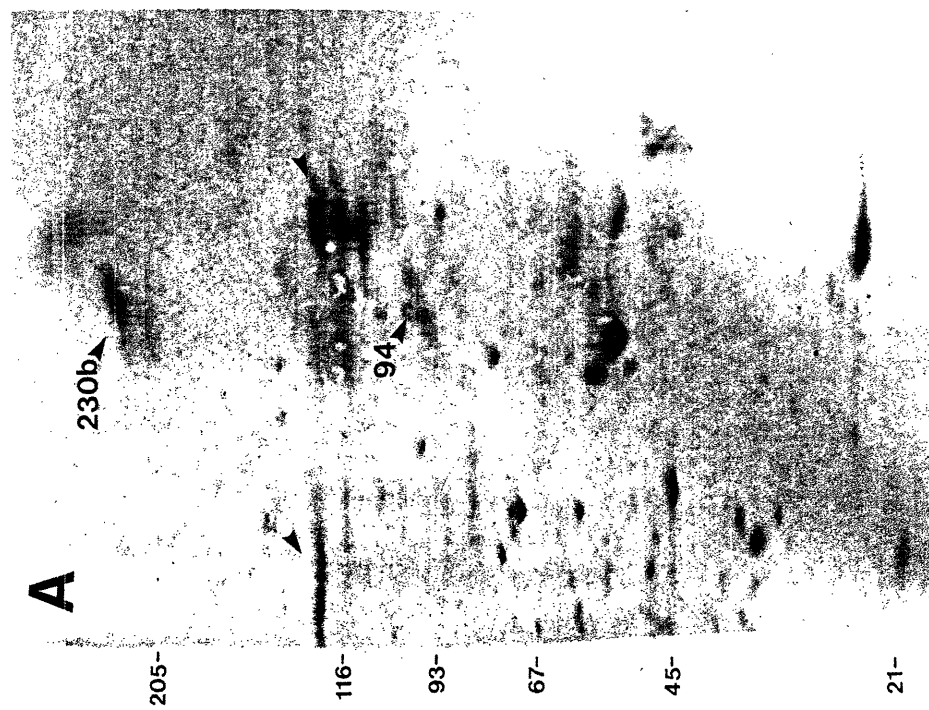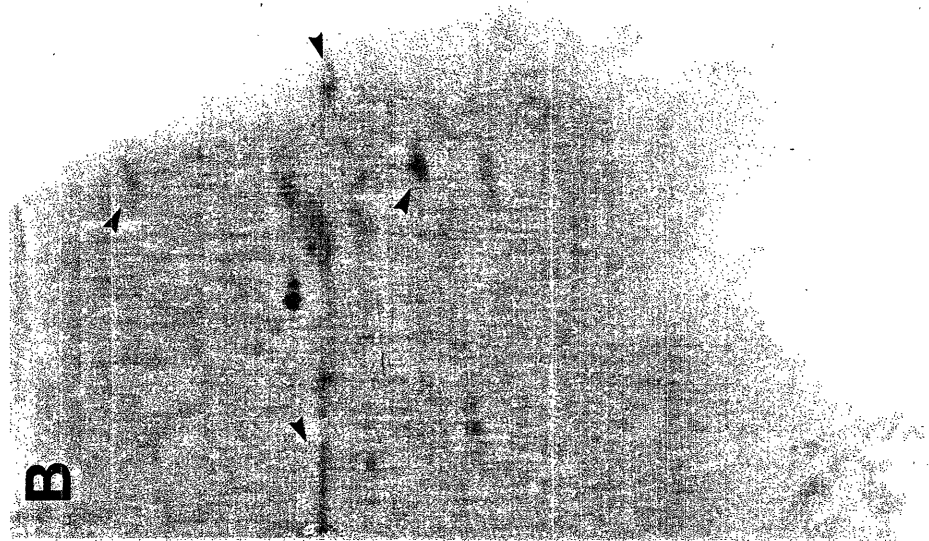
Figure 4. Proteins separated under reducing and non-reducing conditions. The pattern of silver stained proteins from neonatal hamster neocortex showed few differences under reducing (A) and non-reducing (B) conditions. Among the few changes, was an apparent decreased molecular weight in a 94kD protein under non-reducing conditions (arrow points). This shift is clearly shown in the insets (C and D, large arrow points), as well as

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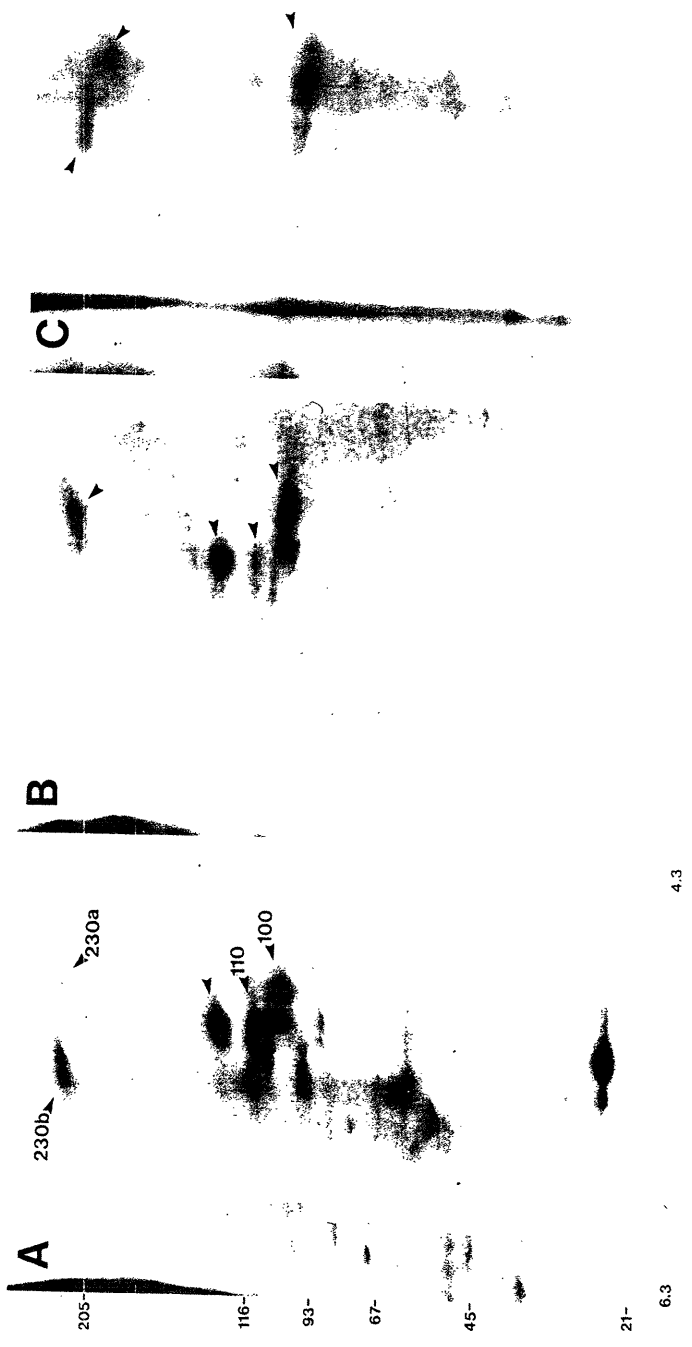several other changes indicated by small arrow points.

Figure 5. Comparison of labeled particulate and soluble proteins in the neonate hamster. The fluorogram of labeled rapidly transported proteins in the particulate fraction shows a typical pattern with the various previously identified species (A). The fluorogram of the labeled proteins in the soluble fraction (B) show actin (A) and ß-tubulin (ßT) are prominent, although these likely represent systemic labeling (see Moya et al., 1988; Chapter 3). A 100kD protein is prominent on the fluorogram of soluble proteins which corresponds in migration position to a rapidly transported protein. A 94kD protein is barely visualized in the soluble fraction (arrow point).

Figure 6. GAP-43 isolated from hamster brain is a phosphoprotein. A protein with identical 2-dimensional electrophoretic migration properties to GAP-43 from retinal axons (A) incorporates phosphate in the presence of calcium (B). GAP-43 is identical to protein B-50 from rat brain and phosphorylated under the same conditions (C).
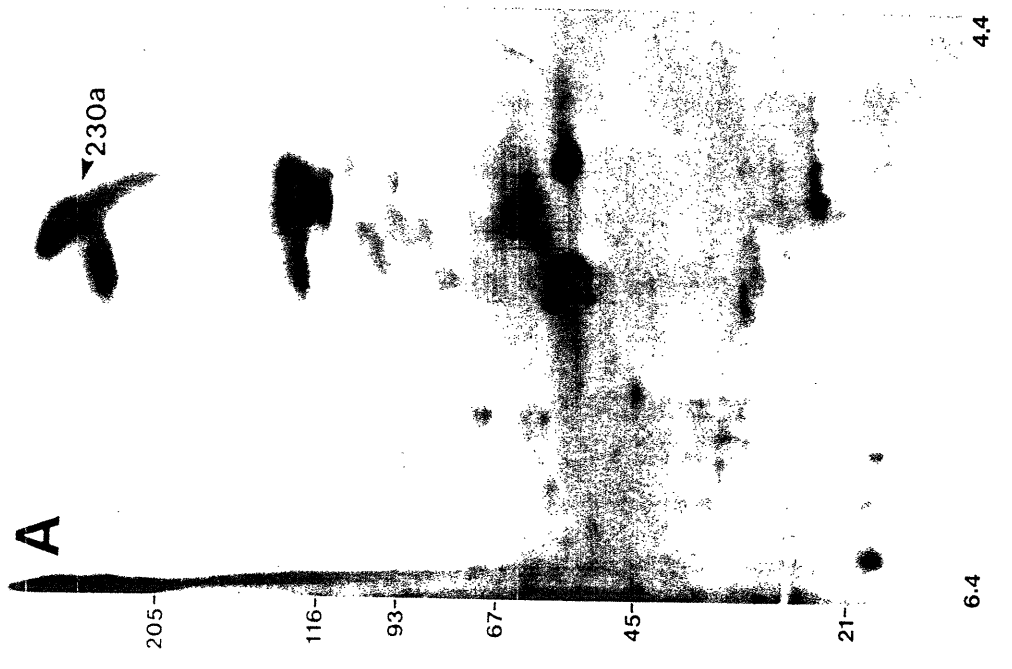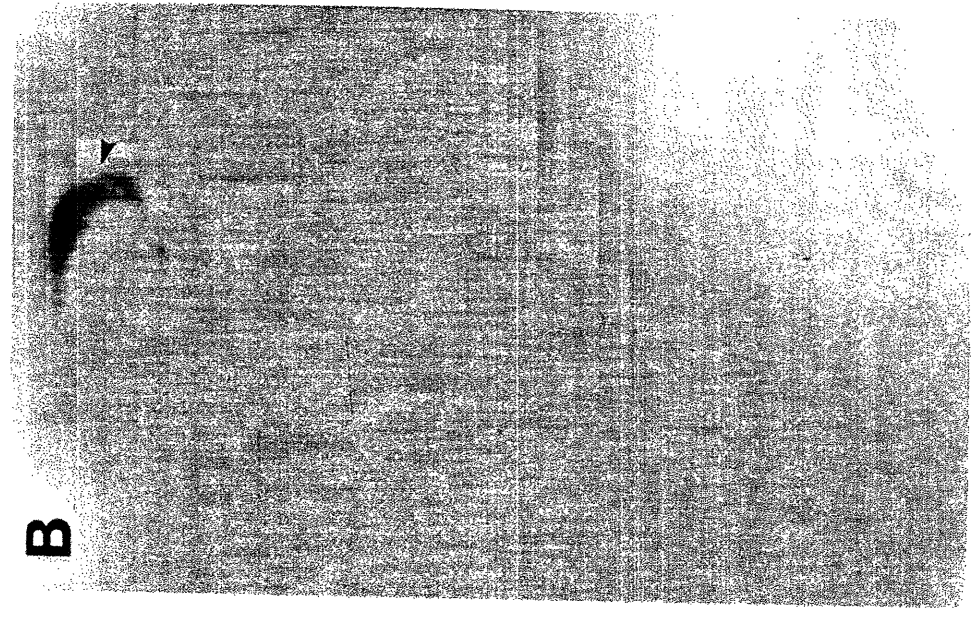
Fugure 7. The 67kD protein appears to be a myristoylated phosphoprotein. A fluorogram of proteins labeled in the eye and rapidly transported to nerve terminals in the neonate hamster show the 67kD protein and GAP-43 (A). These proteins are also pominent among proteins from P5 hamster cortex detected by silver staining (B). A protein with similar molecular weight and pI to the 67kD protein is phosphorylated in rat cortical neurons (arrow, C). Also prominently labeled with phosphate is GAP-43. The 67kD protein is also visualized in the fluorogram of proteins labeled with [^{3}H]-myristic acid (D). C and D from Perrone-Bizzozero et al., (in press) with permission.

A

B

230b

94

205—
116—
93—
67—
45—
21—

6.4

4.3

B

A

230a

205–
116–
93–
67–
45–
21–

6.4   4.4

B

A

D

C

205—
116—
93—
67—
45—
21—

4.1
6.4

A

B

230a
230b
110
100
94
64
GAP-43
27

βT
A

A

GAP

B

C

B50

A

B

C

D

67 kD

GAP-43
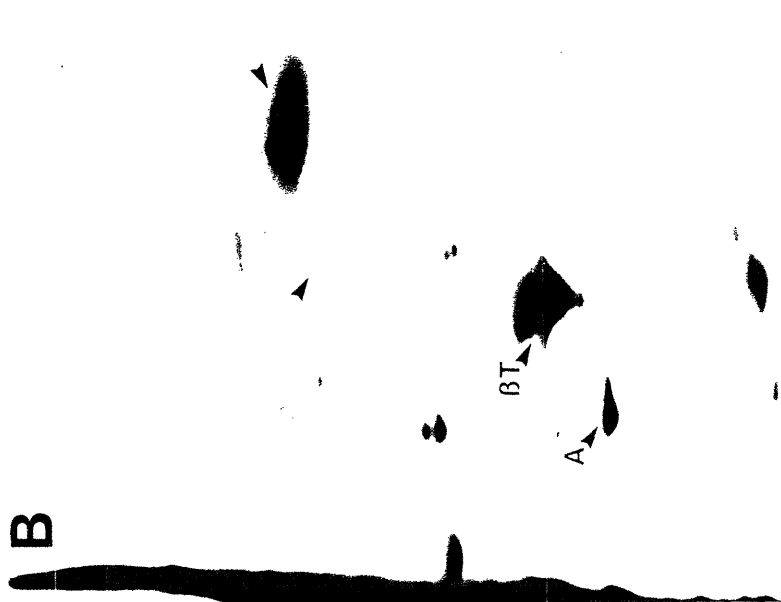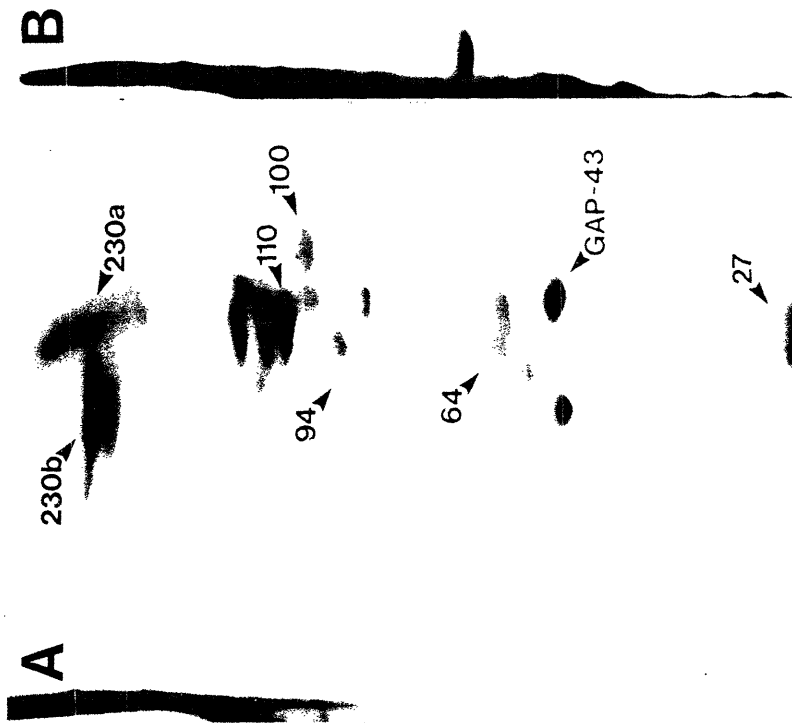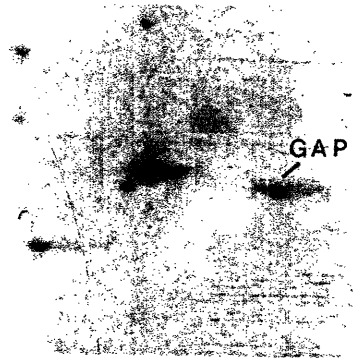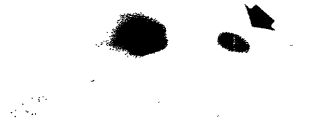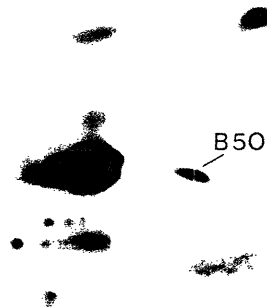
CHAPTER 8

GENERAL DISCUSSION

The studies presented here have examined molecular changes during various modes of axonal growth, in an attempt to understand some of the biological mechanisms of neural development. An underlying assumption of this work is that neural development is comprised of various and separate stages, some of which may be prerequisites for the next. For example, it is difficult to imagine the extension of neurites and formation of meaningful connections without neurons having first differentiated from precursor cells. In regard to the focus of the present studies, this assumption is supported by evidence that axonal growth consists of distinct modes, each with its own characteristic growth rate, morphology and types of interactions with other cells (see Schneider et al., 1985). Furthermore, in the experimental system used here, the plastic response to injury is quite distinct depending upon the mode of growth. When elongating retinal fibers are transected, many regenerate and functionally reinnervate appropriate targets. However, once terminal arborization is well underway, only proximal sprouting is observed. An important caveat to these observations is provided by studies which have shown the growth of some mature retinal axons over long distances when they are provided with a suitable environment (So and Aguayo, 1985).

In addition to the well characterized modes of growth, the effects of early lesions on the anatomical organization of the hamster retinofugal pathway have also been extensively studied (Crain and Hall, 1980; Schneider, 1973; So and Schneider, 1978). Lesions of the SC at about the time of birth result in an abnormal recrossing visual projection to the remaining SC and the

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formation of dense patches of retinal terminals in the LP (Chapter 5 and 6), which in normal animals receives little if any direct retinal input. Thus, an experimental manipulation such as this, in which retinal axons fail to encounter their normal complement of terminal space, provides a useful paradigm for examining the effects of interactive events on neural development.

The studies of the molecular changes in rapidly transported proteins during retinal axon development were initally hampered by the immaturity of the blood-brain barrier in neonatal hamsters, which resulted in a high background in our metabolic labeling experiments. Chapter 2 described some of the kinetics of the problem and a method for minimizing this systemic labeling. Using this method, we examined the dynamic pattern of nerve terminal proteins during development, and the results showed that specific proteins were associated with such morphogenetic events as axon elongation, terminal arborization, synaptogenesis and the maintenance of mature connections (Chapter 3). While the shift from axon elongation to arborization and the boundary of the critical period for the regeneration of transected optic fibers is well defined (Fig 1), we did not observe an abrupt change in the overall pattern of protein synthesis and transport, indicating that the distinct growth states were not accompanied by mutually exclusive patterns of nerve terminal proteins. However, significant changes between P2 and P5 were observed in several specific proteins, including N-CAM, a 67kD protein which may be identical to $pp60^{src}$, and a 100kD species (Chapters 3, 5 and 7). It is conceivable then, that the transition from elongation to arborization with the associated changes in response to injury involves a combination of changes in a subset of neruronal membrane proteins, in addition to whatever cues may be changing in the extraneuronal environment.

The results from the present studies are summarized in Table 1. One of the most prominently labeled rapidly transported proteins in neonatal hamster optic fibers was identified as the growth-associated protein, GAP-43 (Chapters 2, 3 and 7), also known as B-50, pp46 and F1 (see Benowitz and Routtenberg, 1987). GAP-43 was highest in hamster retinal axons during early stages of development, but then declined when terminal arbors were being elaborated and retinal topography had been established. The levels of this protein available in

retinal axons and terminal regions closely followed the time course of its synthesis and transport, suggesting that the turnover of this protein was relatively rapid (Chapter 4). In addition, the protein underwent a striking shift in its cellular localization. It was densely distributed throughout axons in embryonic and neonatal fiber tracts at the time of axon elongation and collateral penetration of target structures, whereas the protein had a more diffuse localization, presumably within presynaptic terminals in the neuropil of target areas when axons arborize extensively and form synapses. Whether the expression of this protein is regulated by target interactions has not been resolved; the diminished levels of this protein seen after early lesions of the SC could result from a down regulation of synthesis, or perhaps by the activation of a degradation mechanism (Chapter 5).

The developmental shift in the localization of GAP-43 from axons to the neuropil suggests that this molecule functions throughout the length of fibers early in development, while at later ages, it is confined to terminals. Consistent with this notion of multiple roles for GAP-43, recent reports have suggested that this protein may transduce extracellular signals in growth cones of developing axons, while it has been implicated in synaptic plasticity in the adult brain (Freeman et al., 1988; Lovinger et al., 1985 ; Nelson and Routtenberg, 1985; Schrama et al., 1988; Van Hooff et al., 1988). The localization of GAP-43 in the adult brain is in agreement with its putative role in synaptic plasticity since the protein is abundant in areas considered to be highly integrative and plastic in function, while it is virtually absent from primary sensory and motor areas (Benowitz et al., 1988a; Benowitz et al., in press; McGuire et al., 1988; Neve et al., 1988a). In this regard, it should be noted that in adult hamster all structures which receive direct retinal input are virtually devoid of GAP-43. However the LP, which receives secondary visual input and is a point of convergence for projections from a number of association areas, is darkly stained for this protein (Chapters 3, 4 and 6).

Experimental manipulations which result in the formation abnormal projections to the LP, alter the pattern of nerve terminal proteins in retinal axons and target structures (Chapters 5 and 6). While these experiments show that the abnormally projecting retinal axons can alter the molecular organization in the LP, earlier studies have demonstrated that these retinal terminals

are themselves induced to assume a heterotypic morphology consistent with the normal innervation of the target (Kalil and Schneider, 1975). The competitive advantage exhibited by retinal terminals as they predominate in areas of the LP and form dense patches to the exclusion of GAP-43-containing terminals, may involve the synchronous activation of post synaptic cells (Chapter 6), similar to the activity-dependent mechanisms that underlie the organization of visual input in other species (Antonian et al., 1987; Benowitz and Schmidt, 1987; Law and Constantine-Paton, 1981; Le Vay et al., 1980; Meyer, 1979; Schmidt and Edwards, 1983). We are now in the position to directly test the role that activity-dependent sorting mechanisms in the formation of the topographically organized retinal projection to the LP.

While GAP-43 exhibits striking changes during development and recent evidence suggests that this protein may be causally involved in axon outgrowth and regeneration (O'Brian et al., 1988), our data also show that a number of other nerve terminal membrane proteins undergo marked changes during development (Chapter 3). One of these (230kD, pI 5.1), is associated with early stages of axon growth and has been identified as the neural cell adhesion molecule, N-CAM (Chapter 7), which has been shown to mediate neuronal adhesion and axon fasciculation (Buskirk et al., 1980; Rutishauser et al., 1978).

A more basic 230kD protein (pI 5.3) shares properties in common with the neuron-glia cell adhesion molecule, Ng-CAM, also known as NILE and L1 (Chapter 7; Bock et al., 1985; Friedlander et al., 1986; Salton et al., 1983). Changes in the time course of this molecule may coincide with developmental changes in the glial environment (Armson et al., 1987; Silver and Shapiro, 1981; Smith et al., 1986; Chapters 3 and 5), some of which have been described in the hamster SC (Wu et al., 1988). Thus the expression of this molecule on growing axons at the time when a complementary molecule is expressed on glial cells in the nerve or near the pial surface over the optic tract, would allow for selective adhesion and could serve as a guide for axonal growth. Furthermore, levels of the 230kD protein remain elevated for about two weeks in retinal ganglion cells forming abnormal connections in the diencephalon (Chapter 5). Whether this prolongation is accompanied by a corresponding change in the glial environment in response to the injury, or a delay in the arborization of retinal terminals after early lesions is

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now being examined.

TABLE 1

Protein	Identification	Time course	Events Associated With	Altered by Target Manip.	Function
GAP-43	B-50, F1, pp46	decrease	axon elong., initial arbor. synap., plasticity	++	PKC substrate, phosphoinos. metab., Ca^{2+}-NT release
230kDa	N-CAM	decrease	axon elong.	+	adhesion, fasciculation
230kDa	?Ng-CAM?	decrease	axon elong.	++	neuron-glia adhesion, fasciculation
67kD	?pp60^{src}?	increase/ decrease	arborization only	++	tyrosine kinase, cyto-membrane linkage
100kD	?	increase/ decrease	arborization only	--	associated with cytoskeleton
110kD	?	increase/ decrease	arborization only	–	?
27kD	S1	increase	arborization, synaptogen., mature connec.	–	?
64kD	?	increase	arborization, synaptogen., mature connec.	++	?
94kD	?integrin?	increase	arborization, synaptogen., mature connec.	–	ECM adhesion

Table 1. Summary of proteins identified in the present studies, and their known or suspected identity and functions.

The potential for regeneration and functional reinnervation may require the expression of a number of molecules since axons which are capable of regeneration, significant levels of GAP-43, N-CAM and the putative Ng-CAM are high. However, after P3, the latter two

molecules decrease, while GAP-43 remains elevated for at least a few more days. Perhaps the continued expression of GAP-43 alone is permissive for the proximal sprouting observed following lesions after P3, but true regeneration may also require the expression of adhesive constituents within neurons as well as in the immediate environment in order to mediate axon fasciculation and provide a guidance mechanism for rapidly elongating fibers. This is consistent with preliminary evidence which shows that the critical period for retinal axon regeneration can be extended several days when the injured axons are exposed to a less mature non-neuronal environment (Carman et al., 1988). In regards to neuronal growth in the adult brain, sprouting has been shown to occur in regions where GAP-43 expression is high, such as the hippocampus, and is accompanied by enhanced expression of this protein (Norden et al., 1988; Benowitz et al., 1988b). Similarly, adult lesions of the LP result in the enhanced expression of GAP-43 by local interneurons and the appearance of positively stained fibers (Chapter 6). Taken together, these findings suggest that the continued expression of GAP-43 in select regions of the adult brain may be permissive for this type of plastic response to injury.

Two proteins which show marked increases during development have apparent molecular weights of 27kD (identified as S1 in Redshaw and Bisby, 1984) and 64kD. These proteins are seen at relatively low levels in the immature retinofugal pathway, increase dramatically during the formation of end-arbors, and are maintained at high levels in the adult (Chapter 3 and 5). One of these proteins, 64kD, shows a marked delay in its increase when the SC is removed in neonatal animals, although this protein eventually attains normal levels in the adult (Chapter 5). A similar 64kD protein is high in normal peripheral nerve and is diminished after nerve crush and during regeneration, but increases again at about the time when the target muscle tissue is reinnervated (See Fig 5, Redshaw and Bisby, 1984; Redshaw and Bisby, 1985). Combined with these studies, our results suggest that the 64kD protein is associated with the formation and maintenance of terminals and thus may provide a useful marker for mature connections.

A 94kD protein which also shows a general increase during development, is similar to the ß subunit of the integrin family of membrane proteins (Chapters 3, 5 and 7). Integrins, which have been shown to be involved in cellular migration and neurite outgrowth, are receptors for

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extracellular matrix components such as collagen, laminin, and fibronectin (for review see Hynes, 1987 and Ruoslathi and Piershbacher, 1987). Elevated expression of the 94kD protein at the time of terminal elaboration would enable this molecule to interact with extracellular matrix proteins in the target neuropil, providing a cell-substrate adhesion mechanism for the growth of arbors, and later this mechanism may serve to anchor the terminal structure with respect to the extracellular matrix.

The synthesis and transport of the 67kD, 100kD, and 110kD proteins precisely coincided with the elaboration of end-arbors and synaptogenesis (Chapters 3 and 5). The 67kD species was shown to be a myristoylated phosphoprotein, suggesting that this protein may be identical to pp60[src] (Chapter 7). This proto-oncogene product has been shown to be associated with neuronal differentiation and appears to be involved with membrane-cytoskelatal interactions (Martinez et al., 1987; Sefton et al., 1981). Such a molecule expressed at the time of arborization and synaptogenesis could then participate in the linkage between cytoskeletal proteins, membrane constituents and molecules on other cells or in the extracellular matrix at sites of attachment.

Figure 1 provides a model representing developmental events and changes in these proteins. During axon elongation, vigorous growth and adhesive mechanisms allow retinal fibers to elongate rapidly at a rate of 60-100 μm/hr in fascicles through the nerve and over the surface of the diencephalon to their primary targets the LGN and SC. At this time GAP-43 is densely distributed along the entire extent of axons where the protein may influence membrane addition and collateral sprouting via its role in the protein kinase C cascade and phosphoinositide metabolism, while concentrations of it in growth cones may participate in the extension of microspikes and motility, in addition to the transduction of extracellular signals. Also at high levels at this time are the adhesion molecules, N-CAM and Ng-CAM, the former mediating neuronal adhesion and axon fasciculation, while the latter could provide a mechanism for axon guidance along glial cells in the nerve and optic tract. Manipulations which alter the pattern of connections and delay the arborization of terminals prolong the expression of N-CAM and the putative Ng-CAM, while optic fibers transected at this time still

retain the molecular requirements for regeneration and functional reinnervation of appropriate target areas.

After retinal axons have reached target areas, fibers defasciculate, grow much more slowly (5-10 μm/hr) and begin to extend collaterals which penetrate into the target neuropil and form terminal arbors. At P5, considerable arborization has occurred and retinal topography has been established, perhaps mediated by activity-dependent tuning involving GAP-43, which remains high at this time. However, N-CAM and the putatively identified Ng-CAM have declined, while a number of other proteins begin to increase, including species similar to pp60^{src} and integrin. Via its tyrosine kinase acitivity for specific cytoskeletal proteins, pp60^{src} may participate in the cytoskeleton-membrane linkage at sites of attachment, thus helping to establish the internal cellular structure for the formation and maintainenance of terminal arbor morphology. Having increasing levels of integrin present when axons arborize would provide the membrane-matrix linkage to stabilize terminal structures with respect to the extracellular matrix. Also temporally correlated with this stage of axon growth are increases in several other proteins, two of which subsequently decline when the mature terminal morphology has formed, e.g., the 100kD species which is also associated with the cytoskeleton (Chapter 7). The 64kD protein and putatively identified pp60^{src} appear to be regulated by target interactions since the time course of these proteins is delayed after early lesions (Chapter 5).

By the third postnatal week, the eyes open and at least some retinal fibers are capable of conducting visual infomation as evidenced by the presence of a pupillary light reflex. Myelination of optic axons has begun and the mature terminal morphology has been established. In molecular terms, terminals would be characterized by an abundance of vesicle-associated proteins, receptors and channels. By this time, most of the proteins studied here have decreased markedly including GAP-43, N-CAM, and proteins putatively identified as Ng-CAM and pp60^{src}. The 27kD and 64kD proteins have increased however, and reached their high adult levels of synthesis and transport. As retinofugal connections mature and the adult pattern of nerve terminal proteins emerges, ganglion cells may no longer preserve the

molecular potential for further growth and synaptic modification, and so lesions of this pathway in the adult result in considerable ganglion cell loss and little or no observable plastic response to the injury.

The studies presented here have shown that specific proteins of the nerve terminal membrane are associated with specific morphogenetic events and that some of these proteins appear to be regulated by target interactions. Two rapidly transported species were positively identified as the growth- and plasticity-associated protein GAP-43, and the neuronal cell adhesion molecule, N-CAM. Definitive identification and functional characterization of proteins putatively identified here as Ng-CAM, pp60^{src} and integrin are currently in progress and would lend support to the model describing the contributions of these proteins to axonal growth. In addition, it will be important to examine developmental changes in the immunohistochemical localization of these proteins, since it is conceivable that the actual levels available in growing axons may differ somewhat from those indicated by our metabolic labeling studies, depending on turnover rates. Lesions in the adult LP indicated that local interneurons synthesize GAP-43, which may retain the capacity for neuronal sprouting, thus providing a model for further studies on this type of adult plasticity.

Early lesions of the midbrain tectum showed that the formation of abnormal retinal projections to the LP altered the molecular organization of this nucleus, and suggested that competitive activity-dependent interactions may underlie these changes. This hypothesis predicts that the blockade of activity in early SC lesioned animals would prevent the formation of the dense patches of retinal terminals in the LP and will soon be tested. These studies also showed that the expression of several proteins was regulated by target interactions, but the nature of these regulatory signals is unknown. Thus, in addition to the identity and physiological significance of the individual proteins studied here, it is of importance to identify the signals that orchestrate this pattern of changes. Some of these signals are likely to originate in the end-organ environment, while others may reflect an intrinsic program of control, suggesting that the regulatory sequences related to the genes for these proteins will also be of

great interest. These further experiments combined with the results of the studies presented here will add to our knowledge of the molecular mechanisms underlying axonal growth and contribute significantly to our understanding of the biology of neural development.

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Figure 1. Schematic representation of nerve terminal membrane proteins and their possible contribution to neural development. Upper row: Major developmental events in the hamster retinofugal pathway are presented. Age is along the abscissa in embryonic (E) or postnatal (P) days. Representation of retinal axons during elongation (second from the top), arborization (second from the bottom) or after the establishment of mature connections (bottom).

FIGURE 1