Axonally Transported Proteins Associated with Axon Growth in Rabbit Central and Peripheral Nervous Systems

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ABSTRACT   In an effort to determine whether the "growth state" and the "mature state" of a neuron are differentiated by different programs of gene expression, we have compared the rapidly transported (group I) proteins in growing and nongrowing axons in rabbits. We observed two polypeptides (GAP-23 and GAP-43) which were of particular interest because of their apparent association with axon growth. GAP-43 was rapidly transported in the central nervous system (CNS) (retinal ganglion cell) axons of neonatal animals, but its relative amount declined precipitously with subsequent development. It could not be reinduced by axotomy of the adult optic nerves, which do not regenerate; however, it was induced after axotomy of an adult peripheral nervous system nerve (the hypoglossal nerve, which does regenerate) which transported only very low levels of GAP-43 before axotomy. The second polypeptide, GAP-23 followed the same pattern of growth-associated transport, except that it was transported at significant levels in uninjured adult hypoglossal nerves and not further induced by axotomy. These observations are consistent with the "GAP hypothesis" that the neuronal growth state can be defined as an altered program of gene expression exemplified in part by the expression of GAP genes whose products are involved in critical growth-specific functions. When interpreted in terms of the GAP hypothesis, they lead to the following conclusions: (a) the growth state can be subdivided into a "synaptogenic state" characterized by the transport of GAP-23 but not GAP-43, and an "axon elongation state" requiring both GAPs; (b) with respect to the expression of GAP genes, regeneration involves a recapitulation of a neonatal state of the neuron; and (c) the failure of mammalian CNS neurons to express the GAP genes may underly the failure of CNS axons to regenerate after axon injury.

Certain rapidly transported proteins (which we have designated "growth-associated polypeptides" [GAPs]) are specifically induced during the regeneration of amphibian retinal ganglion cell axons (17). In the preceding paper we proposed that the GAPs are proteins involved in key functions related to axon growth and that their induction is a critical event in axon regeneration. The mammalian nervous system provides a particularly interesting and important system in which to further evaluate this hypothesis, because it is possible to distinguish between metabolic changes resulting from axon injury itself and those directly related to axon growth: mature neurons of the mammalian peripheral nervous system (PNS) (e.g., the hypoglossal and vagus nerves) retain the ability to regenerate their axons after injury, while those of the central nervous system (CNS) (e.g., the optic nerve and spinal cord) generally

do not. The failure of CNS regeneration is an important clinical problem and in the past has been explained in terms of models emphasizing either extrinsic or intrinsic factors. A particularly durable extrinsic model has been that regenerating CNS axons are physically blocked by the formation of an astrocytic scar at the lesion site (3, 13). On the other hand, mature CNS neurons might fail to mount a regenerative response to axon injury (4, 5). For example, if axon growth in mammals in fact requires GAPs, a failure to induce these proteins after axon injury would result in a failure to regenerate. Here we consider, first, whether any proteins are specifically associated with periods of axon growth in the mammalian CNS (i.e., neonatal optic nerves) and PNS (regenerating hypoglossal nerve), and, if so, whether they bear any resemblance to the toad GAPs. Second, we consider whether neurons of the mature mammalian CNS

fail to induce growth-associated proteins when their axons are injured. Because GAPs are components of the most rapidly transported groups of proteins (groups I and II) in amphibian optic nerves, and because toads and mammals show considerable homology in their organization and composition of axonally transported proteins (18), we have concentrated here on the most rapidly transported proteins (group I, reference 22) of the rabbit nervous system, in our search for mammalian GAPs.

MATERIALS AND METHODS

Labeling and Analysis of Transported Polypeptides

We labeled proteins of rabbit retinal ganglion cells—CNS neurons whose axons pass through the optic nerve—by intraocular injection of [35S]methionine (typically 0.3 mCi at ~1,000 Ci/mmol) and recovered the rapidly transported proteins by removing the optic nerve 3–4 h after injection (24). Axonally transported proteins of the hypoglossal or vagus nerves were similarly labeled by exposing the fourth ventricle in a rabbit anesthetized with ketamine and chloral hydrate and applying [35S]methionine (~1,000 Ci/mmol) either by introducing a cotton swab soaked in an aqueous solution of the isotope (0.75–1.0 mCi in 20–50 μl) into the fourth ventricle (12) or by injecting 0.5 μl of an aqueous solution (1 mCi/μl) of the precursor directly into the hypoglossal nucleus, using the stereotaxic coordinates of Wallach and Loewy (22). Hypoglossal and/or vagus nerves (segments ~1 cm long, 7–8 cm from the brainstem) were removed and frozen on dry ice, 10 h after injection. The nerve segments were homogenized in 4 ml of H Buffer (18) and divided into particulate and soluble fractions (pellet and supernate, respectively, of a 6×10^6 g_{avg}·min centrifugation), and the labeled polypeptides were analyzed by one- and two-dimensional electrophoresis/fluorography as described previously (17, 18).

Surgery for Crushing Nerves

Rabbits were anesthetized by an intramuscular injection of ketamine hydrochloride (50–100 mg/kg) followed by an intravenous injection of chloral hydrate (15% aqueous solution, ~1 ml/kg, adjusted to achieve surgical depth). Hypoglossal nerves were exposed by a skin incision and blunt dissection through muscle layers medial to the common carotid artery. The hypoglossal nerve was crushed with jewelers' forceps at the level of the hypoglossus muscle. To expose the optic nerve near the optic chiasm, we exposed the dura through a skull hole bordered laterally by the rim of the orbit, and posteriorly by a line beginning at Bregma and extending anterolaterally to the rim of the orbit (5–6 mm anterior to the transverse suture). The anterior/posterior width of the skull hole was typically 7–8 mm, and the hole extended medially ~1–2 mm past the midline. A flap of dura was removed, and a smooth metal spatula (width, 6 mm) was inserted slowly between the lateral aspect of the hemisphere and the dura. The brain was slowly compressed medially to expose the optic nerve on the floor of the skull. The optic nerve was then crushed by pinching it with extra-fine-tip jewelers' forceps.

RESULTS

Developmental Changes in the Composition of CNS Rapidly Transported Proteins

Extensive axon growth in the mammalian CNS occurs only during development. To compare the rapidly transported proteins in the retinal ganglion cells of neonatal and adult rabbits, we labeled retinal proteins with [35S]methionine in rabbits ranging from 2 d of age to adults and analyzed the composition of radioactive axonally transported proteins in the ipsilateral optic nerve and contralateral superior colliculus by means of one- and two-dimensional electrophoresis followed by fluorography of the gels. (In albino rabbits, the vast majority of retinal ganglion cell axons pass through the ipsilateral optic nerve [~17 mm long], contralateral optic tract [~10 mm], and make synaptic connections in the contralateral lateral geniculate nucleus and superior colliculus.) Although the composition of rapidly transported proteins was qualitatively similar in 2-d and adult rabbits (Fig. 1), three age-dependent alterations were reproducibly apparent.

First, the labeling (relative to the total rapidly transported radioactivity) of a 43,000 polypeptide declined from its maximum value (4–7 d after birth) to one-half this value by 12 d, and to less than one-twentieth this value in juvenile (4–5 lb) and adult rabbits (Fig. 1). The 43,000 polypeptide could be resolved by both one- and two-dimensional electrophoresis (its pI was 4.5), in both the optic nerve and superior colliculus. In the superior colliculus (but not the optic nerve) the labeled 43,000 polypeptide coelectrophoresed in two dimensions with a Coomassie Blue-stained polypeptide that was one of the more intensely stained spots on two-dimensional gels (system D) of 2- to 10-d rabbits. This spot decreased in its relative staining intensity as the animals became older; the time-course was similar to that of the labeled 43,000 polypeptide. The 43,000 polypeptide thus appears to be a significant component of both the axonally transported proteins and the total superior colliculus proteins, but only during the neonatal period. The 43,000 polypeptide resembled toad GAP-43 in molecular weight and pI.

A second transported polypeptide, of 23,000 mol wt, also decreased with age (Fig. 2). Its labeling was maximal in the optic nerve 7–12 d after birth, declined to one-half that value by 21 d, and, when it could be detected at all in juvenile and adult optic nerve, was about ninefold less than its 12-d level. The 23,000 polypeptide was resolved by one-dimensional polyacrylamide gel electrophoresis (PAGE) (system B, reference 17) and by two-dimensional analysis when isoelectric focusing (IEF) (but not NEPHGE) was the first dimension; its isoelectric point was 5.6. It was not present in sufficient abundance to be stained by Coomassie Blue, and it was not labeled above the background level in the superior colliculus after 3 h of transport.

A third rapidly transported polypeptide, of 18,000 mol wt, appeared to undergo an alteration resulting in a decrease in its mobility on NEPHGE gels between 12 d and 3 wk after birth (Fig. 1). Such a transition could be explained by a post-translational modification affecting the charge (and, therefore, the rate of migration in the NEPHGE gels) of the 18,000 polypeptide. This modification appears to correspond to a shift in pI from ~6.2 (neonatal) to 5.5 (adult) (not shown).

The labeling of the polypeptides described here presumably reflects their transport from the retina (as opposed to their local synthesis in the optic nerve) because local synthesis appears to be negligible in these experiments. This conclusion is based upon the observation that the composition of proteins locally synthesized from precursor that is topically applied to the optic nerve is complex and distinct from the composition of group I transported proteins (unpublished experiments from this laboratory); the composition observed here corresponds to group I proteins and not locally synthesized proteins.

Rapidly Transported Proteins in Axotomized Adult PNS and CNS Neurons

A comparison of the low molecular weight, rapidly transported proteins in the adult hypoglossal and vagus (PNS) nerves with those in the optic nerve (CNS) of adult rabbits showed that the composition is similar in the CNS and PNS. One exception was a 23,000 polypeptide that was labeled in both peripheral nerves but not in the adult optic nerves (Fig. 3). A second difference was that an 18,000 PNS polypeptide migrated more rapidly on NEPHGE gels (similarly to the neonatal optic nerve 18,000 polypeptide) than a polypeptide of
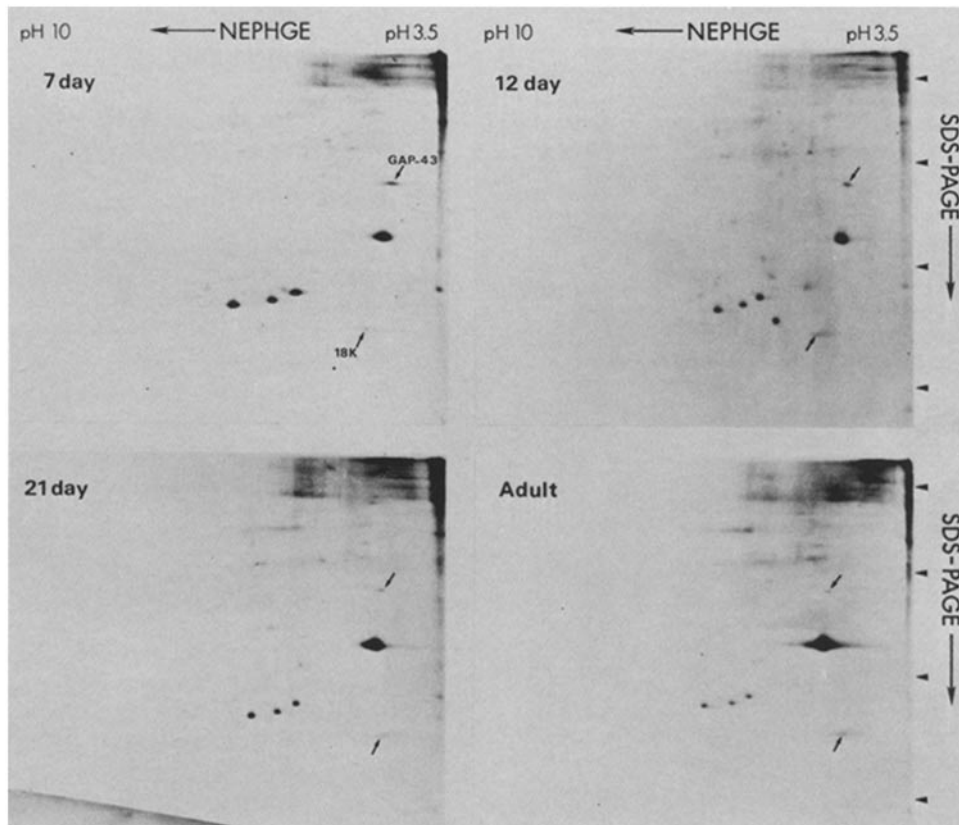
FIGURE 1 Changes in rapidly transported polypeptides during development of rabbit optic nerves. At the indicated times after birth, albino rabbits were injected intraocularly with [^{35}S]methionine; 3 h later, labeled rapidly transported proteins were recovered in the optic nerves. Particulate fractions of the labeled nerves were subjected to two-dimensional electrophoresis (system D) and fluorography. The first dimension is NEPHGE, with the origin at right; the second dimension is SDS PAGE (12% polyacrylamide, 8 M urea, system B, reference 17). The arrowheads at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.

the same molecular weight from the adult optic nerve (compare Figs. 4 and 1).

When the hypoglossal nerve was crushed either 10 or 13 d before the axonally transported polypeptides were labeled, a 43,000 polypeptide was labeled in the regenerating hypoglossal axons to a significantly greater degree than in the contralateral (uncrushed) hypoglossal nerve (Fig. 4) or in nerves from unoperated rabbits. This 43,000 polypeptide resembles both the 43,000 polypeptide of developing optic nerve axons and GAP-43 in regenerating toad optic nerves in its two-dimensional electrophoresis. Densitometry of the corresponding regions of fluorographs derived from control (contralateral or unoperated) and regenerating hypoglossal nerves showed that the relative labeling of the 43,000 polypeptide was at least nine times the control level by 13 d after the nerve was crushed.

In contrast, when adult optic nerve was crushed near the optic chiasm at various times (4, 5, 9, 10, and 14 d) before the rapidly transported polypeptides were labeled and analyzed by electrophoresis (Figs. 5–7), the only reproducible changes that were apparent were a minor increase in a 22,000 polypeptide and small decreases in 21,000 and 20,000 polypeptides (Fig. 7). The analytical systems (B and D) that resolved GAPs 24 and 43 in regenerating toad retinal ganglion cells, and similar polypeptides in the neonatal rabbit retinal ganglion cells showed that neither of these polypeptides is selectively increased after axotomy of the adult rabbit optic nerve (Figs. 6 and 7). Furthermore, the migration on NEPHGE gels of the 18,000 polypeptide that appeared to have a different form in neonates and adults (Fig. 1) was not affected when the adult

optic nerve was crushed (Fig. 7). Thus, none of the compositional changes in polypeptides most specifically associated with axon growth occurred after axotomy of the adult mammalian optic nerve.

Relationship of the Rabbit 23,000 Polypeptide to Toad GAP-24

Because a 23,000 polypeptide is rapidly transported in two adult peripheral nerves (which can regenerate), but only during development of a central nerve (which does not regenerate), we considered the possible relationship of this polypeptide to toad GAP-24, whose rapid axonal transport is specifically induced during regeneration. An unusual feature of the toad GAP-24 is the sensitivity of its migration on SDS PAGE to urea at high pH (19). When toad GAP-24 is electrophoresed in a two-dimensional SDS PAGE system in which the second dimension differs from the first only in its inclusion of 8 M urea at pH 9.25 (compared to pH 8.9 in the first dimension), GAP-24 is one of the few polypeptides that migrate substantially above the diagonal line formed by the polypeptides whose relative mobilities are not differentially altered by urea and high pH (19). Fig. 8 shows that a 23,000 urea-sensitive polypeptide can be detected among the axonally transported proteins of adult rabbit peripheral nerves and a neonatal central nerve, but not in the adult central nerves, regardless of whether they have been crushed. The degree of urea sensitivity of the mammalian 23,000 protein is less than in the case of toad GAP-24 (Fig. 8); however, this characteristic is sufficiently
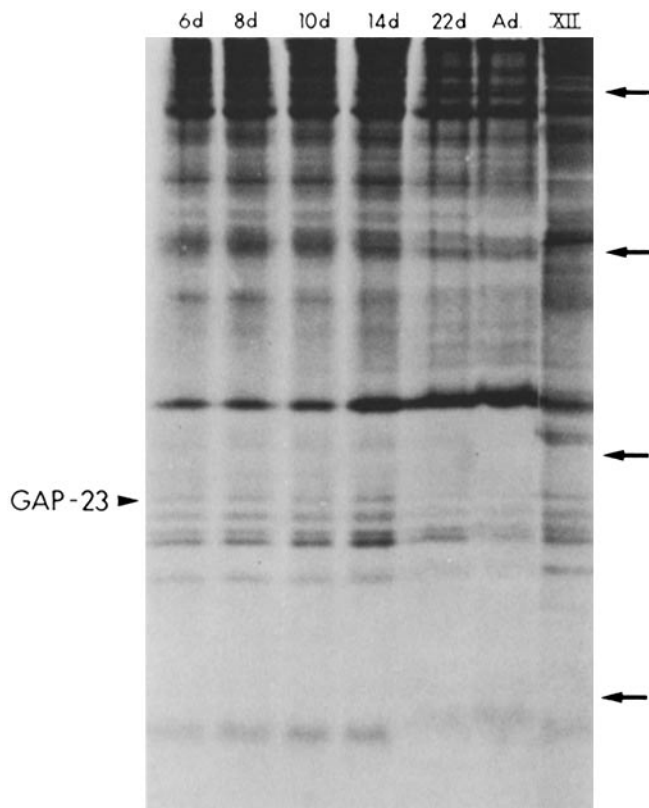
FIGURE 2 Decline in relative labeling of GAP-23 during development of rabbit optic nerves. At the indicated times after birth, [35S] methionine was injected into the posterior eye chambers of albino rabbits; 3 h later, labeled rapidly transported proteins were recovered in the optic nerves. Particulate fractions of the optic nerves were subjected to one-dimensional electrophoresis (12% polyacrylamide, 8 M urea, system B of reference 17) and fluorography. The samples contained equal amounts of radioactivity. The rapidly transported polypeptides in an undamaged hypoglossal nerve of an adult rabbit (XII) are shown for comparison. The arrows at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.

unique that its manifestation in the rabbit 23,000 polypeptide strongly indicates that the two polypeptides share some unusual structural homology.

DISCUSSION

We previously identified three rapidly transported proteins that are specifically induced during the regeneration of toad retinal ganglion cell axons and suggested that these polypeptides might be essential for axon growth. To further evaluate this hypothesis, we have asked whether any rapidly transported polypeptides are specifically associated with growing neurons in the mammalian nervous system, and if so, whether they share properties with the toad GAPs. Three transported polypeptides of the rabbit CNS and PNS are candidates for roles in mammalian growth-specific processes.

The specific induction of GAP-43 transport was completely correlated with periods of axon growth; it was induced in regenerating hypoglossal nerves (but not in axotomized adult optic nerves, which do not regenerate), and in neonatal (but not adult) optic nerves. The period during which GAP-43 was expressed at elevated levels in neonatal rabbit retinal ganglion cells (i.e., the first several weeks after birth) corresponds to a period in which both the number of synapses per neuron and
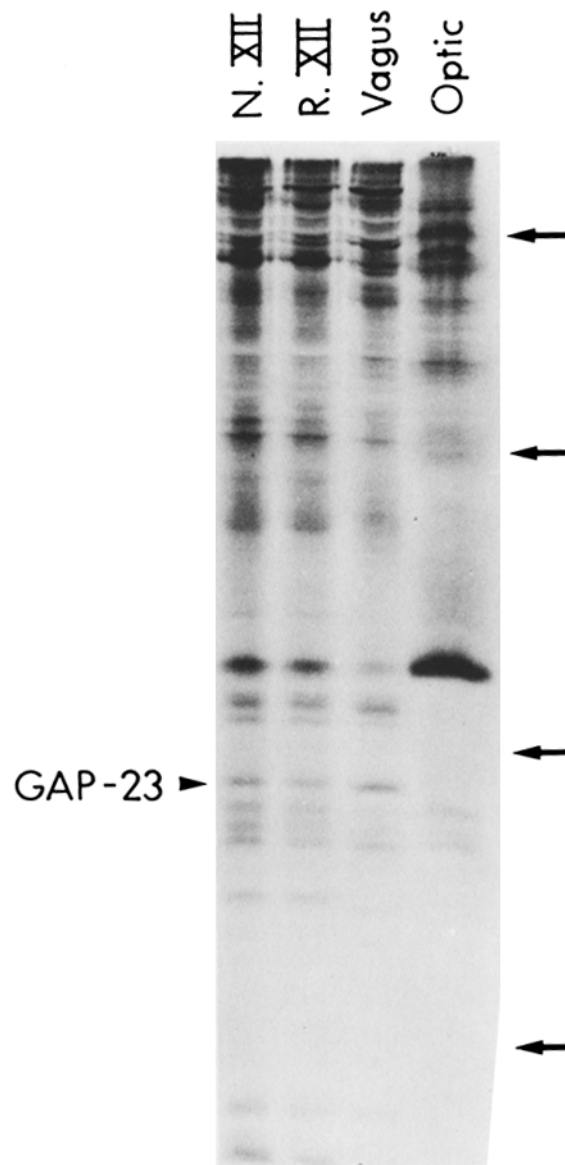

FIGURE 3 Comparison of rapidly transported polypeptides in peripheral and central nerves of adult rabbits. The left hypoglossal nerve of an adult rabbit was crushed (see Materials and Methods). 12 d after surgery, [35S]methionine was applied to the surface of the dorsal medulla; 10 h after isotope application, labeled rapidly transported proteins were recovered in the regenerating hypoglossal nerves (R. XII). Rapidly transported proteins in the hypoglossal (N. XII) and vagus nerves of an unoperated rabbit were labeled similarly. Group I proteins in optic nerves were labeled by intraocular injection of an unoperated adult rabbit and recovered in the optic nerve 3 h after isotope injection. Particulate fractions of the labeled nerves were subjected to electrophoresis (12% polyacrylamide, 8 M urea, system B, reference 17) and fluorography. The samples contained equal amounts of radioactivity. The arrows at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.

the number of multiple synapses (taken to be of retinal origin) in the superior colliculus increase sharply, reaching 85–90% of their adult level by 21 d after birth (11). Furthermore, the total protein-associated radioactivity rapidly transported into the superior colliculus increases sharply at ~21 d after birth, indicating that growth of new axons into the superior colliculus or maturation of axons already in the superior colliculus continues through the period that GAP-43 is transported at elevated

pH 10 ←— NEPHGE   pH 3.5

SDS-PAGE →

NORMAL OPTIC

pH 3.5 ←— IEF   pH 10

SDS-PAGE →

REGEN. HYPOGLOSSAL

pH 10 ←— NEPHGE   pH 3.5

GAP-43

SDS-PAGE →

CRUSHED OPTIC

pH 3.5 ←— IEF   pH 10
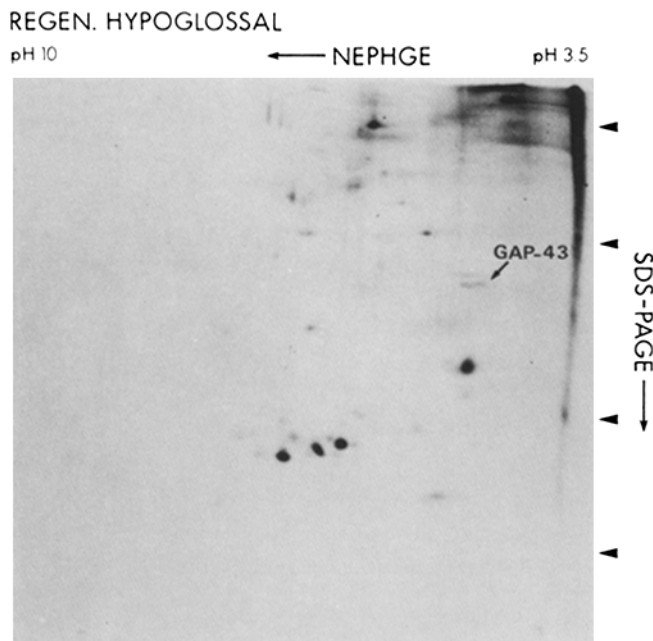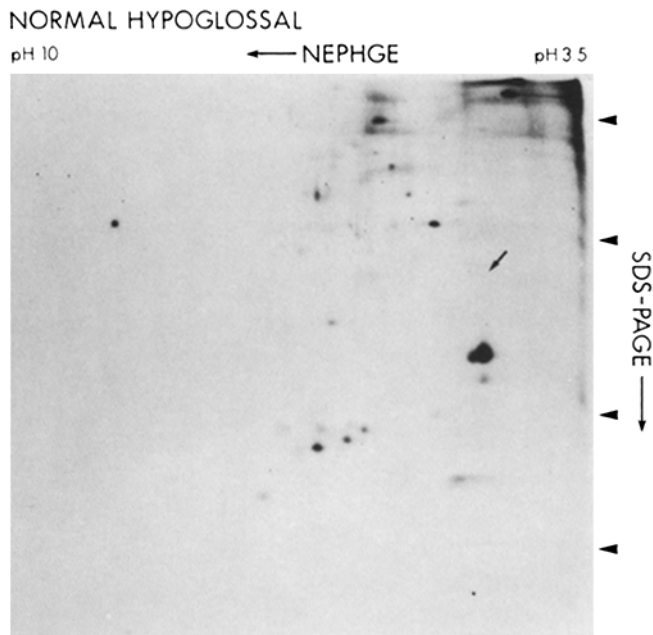
SDS-PAGE →

FIGURE 4   Induction of GAP-43 during hypoglossal nerve regeneration in rabbits. The left hypoglossal nerve of an adult rabbit was crushed as described in Materials and Methods; 13 d later, [^{35}S]-methionine was injected bilaterally into the hypoglossal nuclei (see Materials and Methods), and labeled rapidly transported proteins were recovered in the hypoglossal nerves 10 h later. Particulate fractions of the nerves were subjected to two-dimensional electrophoresis (system D) and fluorography. Equivalent amounts of total radioactivity were loaded onto the two gels. "Normal" = contralateral to crush. The first dimension is NEPHGE, with the origin at right; the second dimension is SDS PAGE (12% polyacrylamide, 8 M urea, system B). The arrowheads at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.
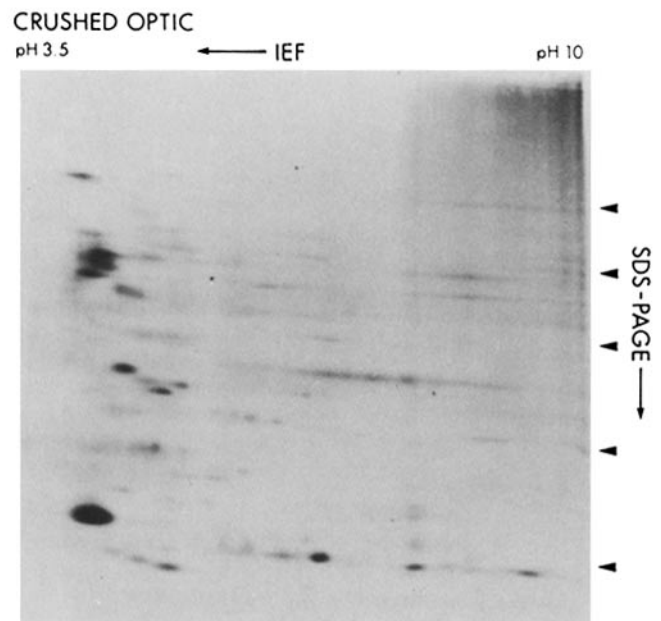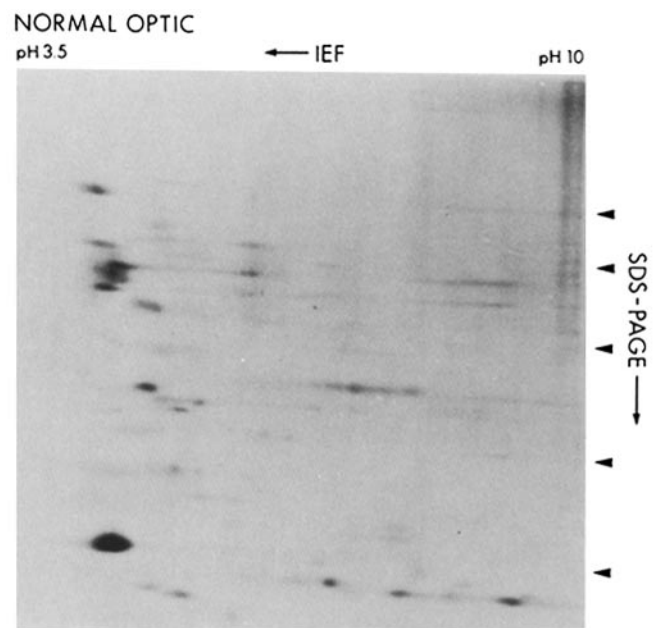
FIGURE 5   Lack of changes in group I polypeptides after the optic nerves of adult rabbits were crushed. The optic nerve of an adult rabbit was crushed near the optic chiasm (see Materials and Methods). 10 d after surgery, [^{35}S]methionine was injected intraocularly in the operated rabbit and in an unoperated control rabbit, and labeled group I proteins were recovered in the optic nerves 3 h after isotope injection. Particulate fractions of the labeled nerves were subjected to two-dimensional electrophoresis (system C, reference 17) and fluorography; equal amounts of radioactivity were loaded onto the two gels. The first dimension is IEF (pH 3.5-10), with the origin at right; the second dimension is SDS PAGE (4-12% polyacrylamide; 4-8 M urea, system A). Small changes in the relative labeling of individual polypeptides are detectable in these gels but are not reproducible. The arrowheads at right indicate molecular weights of 200,000; 150,000; 100,000; 50,000; and 25,000.

levels (7). Endogenous neurons of the superior colliculus also undergo maturation during the first three postnatal weeks (10); therefore, the disappearance from the superior colliculus of the stained polypeptide corresponding to GAP-43 during this period is also correlated with neuronal maturation regardless of

whether this stained polypeptide originates primarily by transport from the retinal ganglion cells, or by synthesis in neurons of the superior colliculus.

GAP-43 has the same molecular weight (43,000) and pI in

TOAD | **RABBIT**
Norm | Crush | Norm | Norm | Crush | Sham

GAP-50
GAP-43
GAP-24
22K

NORMAL OPTIC
pH 10 ←— NEPHGE pH 3.5
GAP-43
22K
SDS-PAGE →

CRUSHED OPTIC
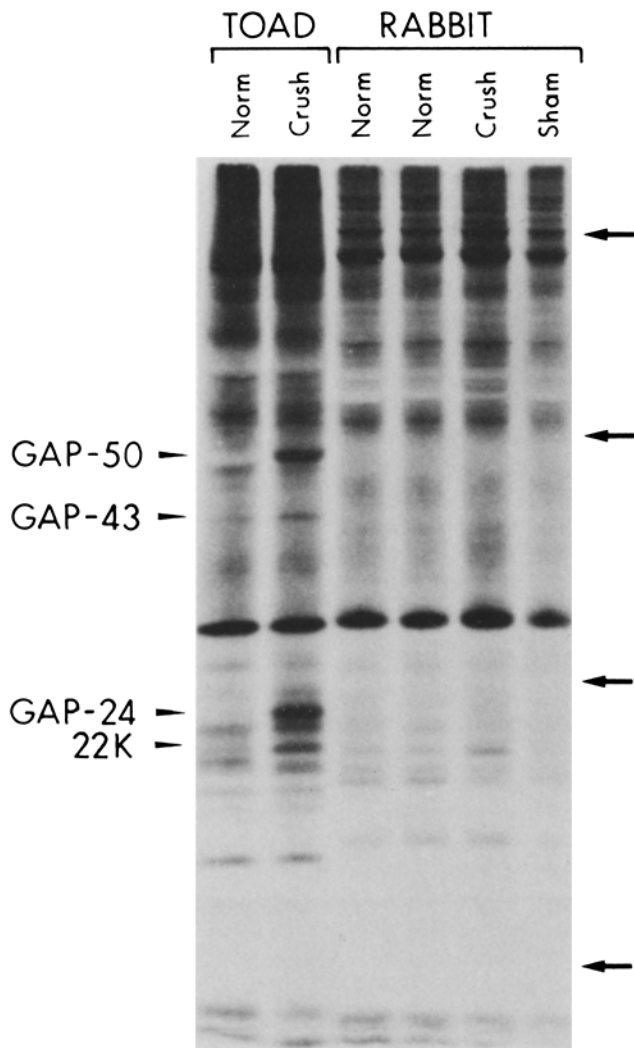pH 10 ←— NEPHGE pH 3.5
SDS-PAGE →

FIGURE 6 Failure to induce GAP-24-like polypeptide after optic nerve crush in adult rabbits. The left optic nerve of an adult rabbit was crushed near the optic chiasm. 5 d after surgery, [^{35}S]methionine was injected intraocularly in the operated rabbit, two unoperated control rabbits, and a sham-operated rabbit (optic nerve exposed, but not crushed), and labeled group I proteins were recovered in the optic nerves 3 h after isotope injection. Particulate fractions of the nerves were subjected to electrophoresis (12% polyacrylamide, 8 M urea, system B, reference 17) and fluorography. The samples contained equal amounts of radioactivity. Group I polypeptides from normal and regenerating (5 d after axotomy) toad optic nerves are shown for comparison. The arrows at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.

toad optic nerves, rabbit optic nerves, and rabbit peripheral nerves. It, therefore, seems very likely that the rabbit 43,000 polypeptide is the equivalent, both structurally and functionally, of toad GAP-43. Because elevated transport of GAP-43 in all of these systems is more closely correlated with axon growth than with age, species, neuronal type, or axonal injury (Table I), GAP-43 is very likely to mediate some function prerequisite for axon growth.

The elevated axonal transport of the 23,000 polypeptide is also correlated with the period of axon growth and maturation in rabbit retinal ganglion cells; it is considerably more intensely labeled in the neonatal rabbits than the adults and, as with GAP-43, it is not induced by axotomy of adult optic nerves. Unlike GAP-43, the 23,000 polypeptide was normally trans-
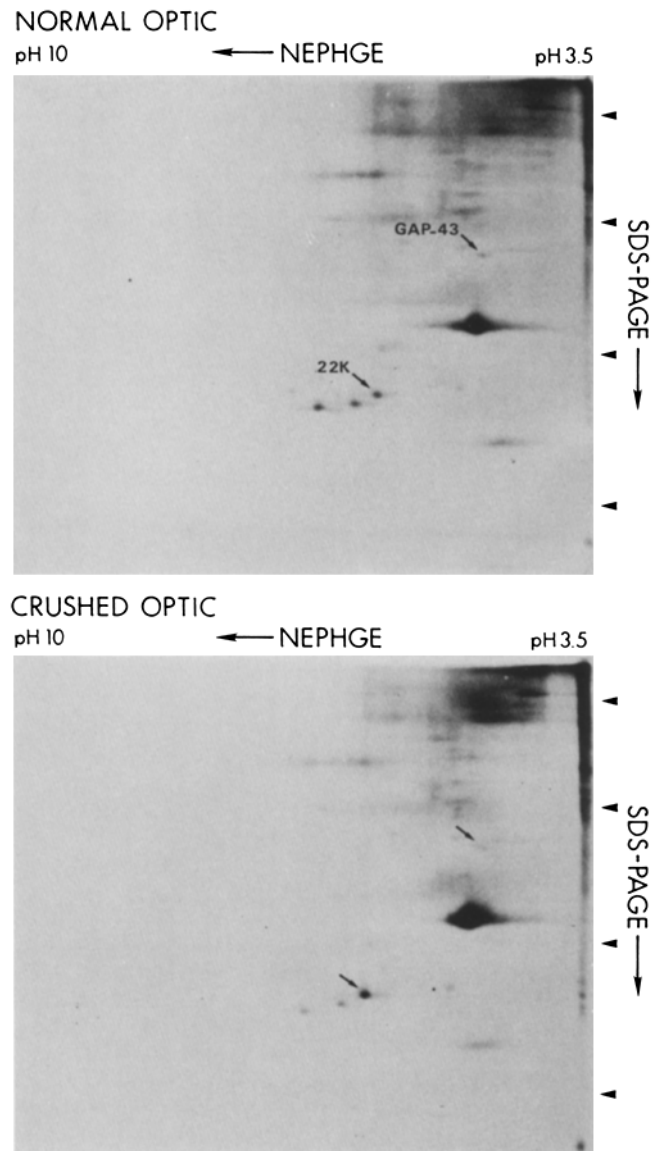
FIGURE 7 Failure to induce GAP-43 after adult rabbit optic nerves are crushed. An adult rabbit's optic nerve was crushed near the optic chiasm. 9 d after surgery, [^{35}S]methionine was injected intraocularly in the operated rabbit and in an unoperated control, and labeled group I proteins were recovered in the optic nerves 3 h after isotope injection. Particulate fractions of the labeled nerves were subjected to two-dimensional electrophoresis (system D) and fluorography. Equal amounts of radioactivity were loaded onto the two gels. The first dimension is NEPHGE, with the origin at right; the second dimension in SDS PAGE (12% polyacrylamide, 8 M urea, system B of ref. 17). The fluorographs were exposed long enough to detect the low level of GAP-43 labeling that occurs in adult rabbit retinal ganglion cells. The arrowheads at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000.

ported at significant levels in peripheral nerves (vagus and hypoglossal nerve), and its relative labeling in these neurons was not appreciably affected by axotomy. Therefore, we cannot dismiss the possibility that the PNS 23,000 polypeptide is not involved in growth-specific function. However, the similarities (discussed below) between the PNS 23,000 polypeptide and both the CNS 23,000 polypeptide and toad GAP-24 suggest the alternative interpretation that the labeling of the 23,000 polypeptide in undamaged hypoglossal and vagal neurons is related to spontaneous axon sprouting and synapse formation.

Several reports (1, 19, 21) have shown that peripheral motor neurons in both mammals and amphibians continue to elaborate axonal sprouts over short distances to form new neuromuscular synapses throughout adult life. It therefore seems reasonable to consider that peripheral neurons (at least motor neurons) may exist in an intermediate physiological state between the developing or regenerating neurons, characterized
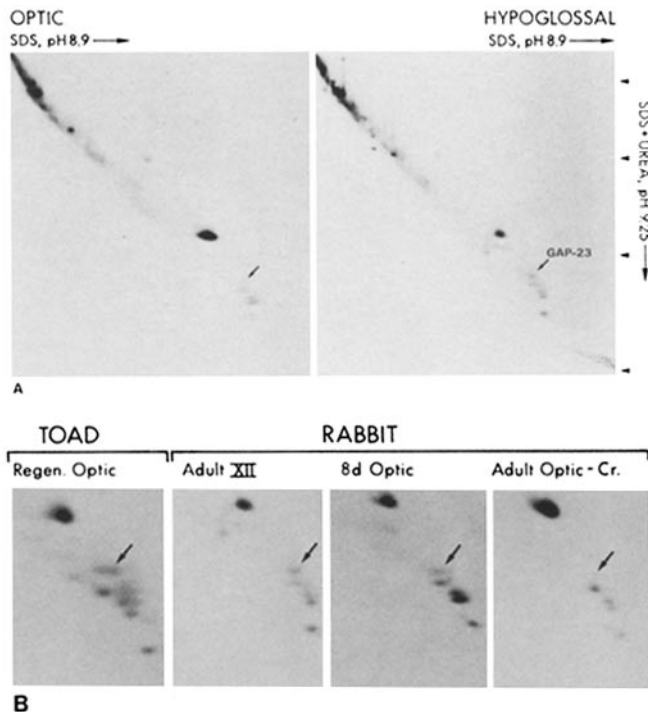

FIGURE 8 Similar effect of urea and high pH on the electrophoretic mobility of toad GAP-24 and rabbit GAP-23. Rapidly transported proteins were labeled in a toad optic nerve 8 d after axotomy (regenerating optic); in an undamaged adult rabbit hypoglossal nerve (Adult XII); in the optic nerve of an 8-d-old rabbit (8d Optic); and in adult rabbit optic nerves either unoperated (Optic) or crushed 5 d previously (Adult Optic—Cr.). The proteins were labeled by injecting [³⁵S]methionine into the posterior eye chambers (optic) or dorsal medulla (XII); the labeled rapidly transported proteins were recovered in the respective nerves 4 h (toad optic), 10 h (rabbit hypoglossal), or 3 h (rabbit optic) after isotope injection. The labeled nerves were subjected to electrophoresis in the absence of urea (horizontal dimension, origin at left) and then in the presence of urea at a higher pH (19). (A) Fluorographs of whole gels of unoperated adult rabbit optic nerve and adult rabbit hypoglossal nerve. Arrowheads at right indicate molecular weights of 100,000; 50,000; 25,000; and 15,000. (B) Higher magnification of fluorographs showing gel regions containing labeled GAP-24 (toad) and GAP-23 (neonatal rabbit), indicated by arrows. In this series, the adult rabbit optic nerve was crushed 10 d before isotope injection.

by extensive axon elongation and transport of both the 43,000 and 23,000 polypeptides, and the mature state of central neurons, characterized by very little axon growth and minimum levels of the two polypeptides described here. This interpretation suggests that the 43,000 and 23,000 polypeptides are involved in separate aspects of axon growth, since they are expressed under different conditions.

The 23,000 polypeptide of rabbit peripheral nerve and neonatal optic nerves are electrophoretically indistinguishable and resemble toad GAP-24 in the following respects: both toad and rabbit polypeptides are rapidly transported, are associated with rapidly sedimenting material, have similar apparent molecular weights (23,000 vs. 24,000), and show an unusual sensitivity of their electrophoretic mobility to urea; this latter property is sufficiently rare to suggest rather strongly that the polypeptides from the two animals share some unusual structural feature. On the other hand, rabbit GAP-23 and toad GAP-24 are not identical proteins as they differ slightly in molecular weight, degree of urea sensitivity, and more significantly in pI (toad $\simeq$ 6.7 vs. rabbit $\simeq$ 5.6) and mobility in NEPHGE. Although these differences are sufficient that the relationship between the toad and rabbit polypeptide is not conclusive, a minor change in protein structure could probably account for all of these differences. For example, if rabbit GAP-23 lacked only a few basic amino acids of toad GAP-24, it would have a lower pI, slower migration on NEPHGE gels, and might well have an electrophoretic mobility that would be less retarded by urea (16), leading to a lower apparent molecular weight when measured by electrophoresis in the presence of urea. Thus, the differences would seem to be well within the range of those that might be expected for functionally related proteins in species as widely separated in evolution as toads and rabbits.

A third polypeptide with a molecular weight of 18,000 showed an alteration in mobility on NEPHGE gels that was correlated with periods of axon growth in a pattern similar to the induction of the 23,000 polypeptide; a slower migrating (NEPHGE) form was associated with adult optic nerves and was not affected by axotomy, while a faster migrating form was associated with both normal and regenerating hypoglossal nerves, as well as neonatal optic nerves. This protein probably corresponds to a toad 18,000 polypeptide whose NEPHGE mobility appeared to change after axotomy of the optic nerve (manuscript in preparation), and also to a low molecular weight polypeptide that has been reported to have different pI's in the optic nerve and superior colliculus of adult rabbits (9). The 18,000 polypeptide differs from the GAPs in that its alteration is most easily explained in terms of a post-translational modification (e.g., phosphorylation, methylation, glycosylation) of a normally transported protein, rather than by an alteration in its synthesis. The number of distinct forms of the 18,000 polypeptide is not yet clear, as we have observed its behavior

TABLE I

Correlation of GAP Transport with Aspects of Axon Growth

	Toad optic		Rabbit optic			Rabbit hypoglossal	
	Normal	Crushed	Neonatal	Adult	Crushed adult	Normal*	Crushed
Sprouting	−	+	+	−	−	+	+
Directed elongation	−	+	+	−	−	−	+
Synaptogenesis	−	+	+	−	−	+	+
GAP-23/24	−	++	+	−	−	+	+
GAP-43	±	++	++	±	±	−	+

* Assuming that undamaged rabbit hypoglossal neurons resemble other mammalian motor neurons (1, 19) in their growth characteristics.

predominantly in the nonequilibrium, two-dimensional system; we are currently evaluating (by means of IEF) the possibility that its modifications, coupled with retrograde transport, could serve as a growth-regulating signal to the cell body. We did not detect a homologue of toad GAP-50, a fucosylated glycoprotein (19), in the rabbit CNS or PSN.

In previous analyses of proteins of the mammalian neurons during periods of axon growth (e.g., references 2, 6, and 20), compositional alterations have sometimes been reported, but because of the differences in analytical techniques employed, it is difficult to compare meaningfully these changes with the GAPs described here. It is interesting, however, that the largest axotomy-induced increase in labeling of rat sciatic nerve polypeptides reported by Theiler and McClure (20) was in an area of a one-dimensional gel corresponding to a molecular weight of 42,000, suggesting the possibility that this neuronal system also induces GAP-43 in order to regenerate.

Inability of Mammalian CNS Neurons to Regenerate

If rabbit GAP-23 and/or GAP-43 are involved in functions essential for full axon growth, the failure of axotomized CNS neurons to induce these proteins would be sufficient to explain the failure of these neurons to regenerate. It is consistent with this possibility that we observed no large reproducible change in the relative labeling of GAP-23, GAP-43, or other group I axonally transported proteins after axotomy of adult rabbit retinal ganglion cells during a period when they typically exhibit limited abortive axon sprouting (14, 15), chromatolysis, and degeneration (8). Because we examined the group I proteins with several electrophoretic systems including those that had resolved GAPs in these cells during the neonatal period, it is unlikely that a GAP induction would have been undetected, had it occurred. We therefore suggest that mature mammalian CNS neurons do not regenerate because some step (e.g., the generation of an appropriate signal, or the response to that signal) leading to the induction of GAPs fails to be accomplished in the mammalian CNS. This hypothesis is contrary to the alternative, that injured mammalian CNS axons become competent to regenerate, but are prevented from doing so only by extrinsic agents that physically block or misrout the fibers. The competency of CNS axons to regenerate has been inferred from the abortive sprouting of axotomized retinal ganglion cells (14, 15). However, similar sprouting occurs from the distal stumps of severed optic nerves (14); abortive sprouting is thus most likely a locally initiated reaction independent of the cell body and not necessarily an indication that a neuron is competent to undergo directed axon elongation.

GAP Hypothesis for Regulation of Axon Growth

In the preceding paper, we proposed that the transition of neurons between mature states and growth states requires an alteration in the program of gene expression, including the expression of GAP genes, whose products perform critical functions in axon growth. When interpreted in terms of the GAP hypothesis, the observations reported here (which themselves lend support to the hypothesis) lead to the following additional conclusions: (a) In addition to mature states (characterized by no GAP transport) and full axon growth states (characterized by transport of both GAPs -23 and -43), there are intermediate growth states (possibly including a "synaptogenic state") characterized here by the transport of GAP-23,

but not GAP-43; furthermore, some aspects of axon growth that do not themselves lead to successful regeneration (e.g., limited sprouting), are mediated by local reactions independent of GAP transport. (b) Failure to accomplish some step leading to the re-expression of GAP genes repressed during development leads to the failure of many mammalian CNS tracts to regenerate after injury.

These considerations suggest that further studies of the GAPs could be important in two ways. First, understanding the reactions mediated by each of these polypeptides may clarify the molecular mechanism of axon growth. Second, even in the absence of a detailed knowledge of the function of the GAPs, their induction could be a useful assay for tracing the steps leading from axotomy to regeneration, with the hope of determining the molecular event at which axon regeneration fails in the mammalian CNS.

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